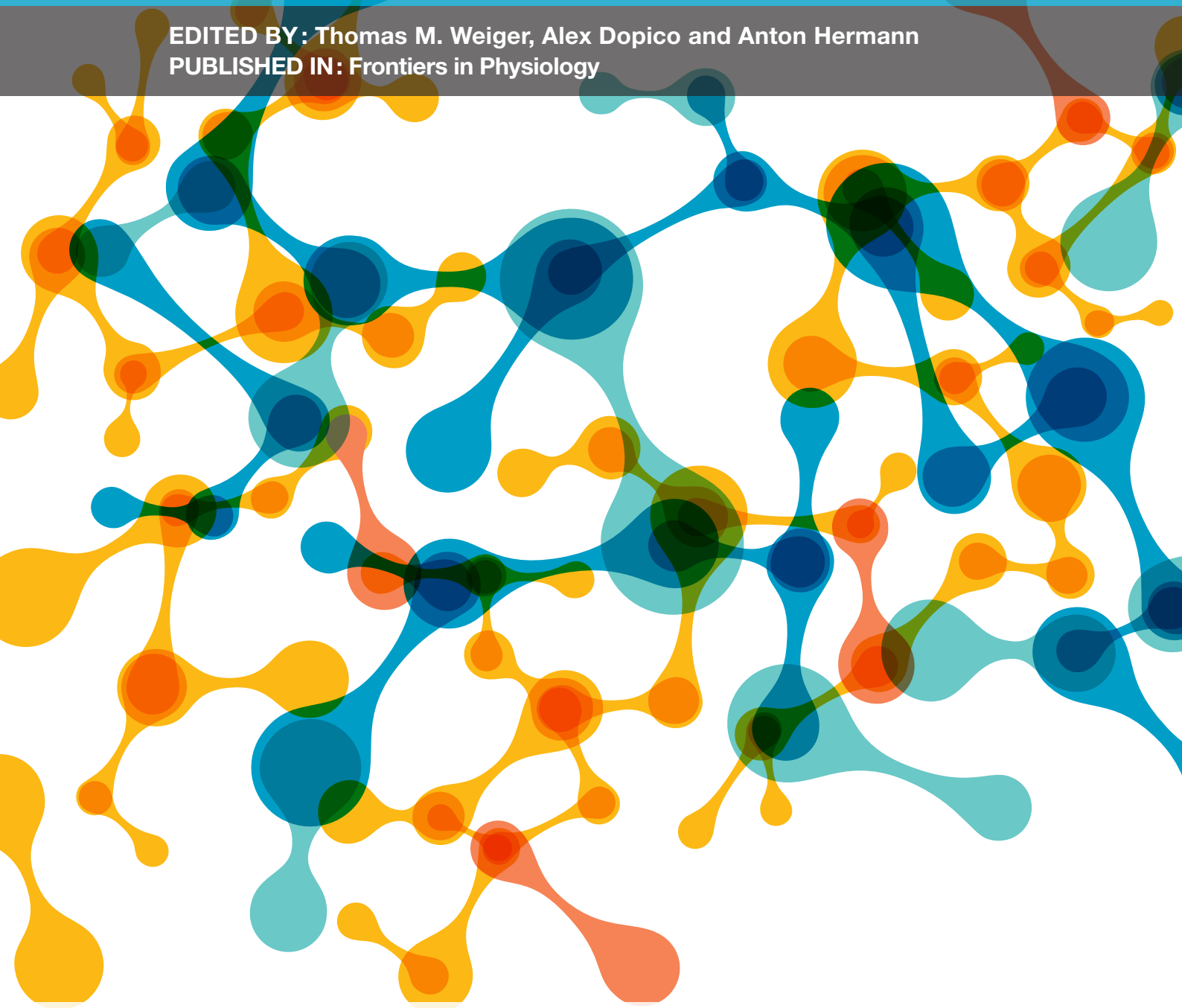
BK CHANNELS: INTEGRATORS OF CELLULAR SIGNALS IN HEALTH AND DISEASE

EDITED BY: Thomas M. Weiger, Alex Dopico and Anton Hermann
PUBLISHED IN: Frontiers in Physiology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-291-0
DOI 10.3389/978-2-88945-291-0

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

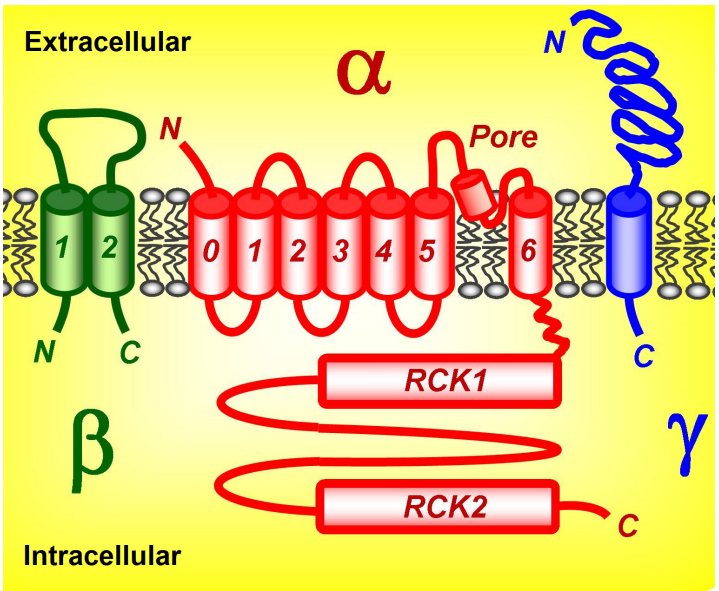
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

BK CHANNELS: INTEGRATORS OF CELLULAR SIGNALS IN HEALTH AND DISEASE

Topic Editors:
Thomas M. Weiger, University of Salzburg, Austria
Alex Dopico, The University of Tennessee, United States
Anton Hermann, University of Salzburg, Austria



Cartoon showing a BK channel-forming slo1 protein (BK alpha subunit, in red) accompanied by modulatory beta (in green) and gamma (in blue) subunits. The BK alpha subunit contains seven transmembrane segments (0-6), resulting in an extracellular amino (N) end. They include the pore and voltage-gating domains, which are conserved to those of purely voltage-gated K+ channels of the TM6 superfamily. These seven segments are connected via a short linker (zig-zag line) to a large cytosolic tail domain which contains two Regulator of Conductance for K+ structures (RCKs 1 and 2), which include the sensing sites for physiological calcium and magnesium. The assembly of four BK alpha subunits around a central pore results in the formation of the gating ring with eight RCKs and thus, a fully functional BK channel. The complex may include beta (types 1-4) or gamma subunits (LRRC proteins). The stoichiometry of native BK complexes with different beta and gamma subunits is not fully resolved, although recombinant channels made of slo1+beta2+gamma1 with a novel current phenotype have been reported (Gonzalez-Perez et al., 2015).

"Gonzalez-Perez, V., Xia, X.M., Lingle, C.J. (2015). Two classes of regulatory subunits coassemble in the same BK channel and independently regulate gating. *Nat Commun.* 6:8341. doi: 10.1038/ncomms9341. PMID: 26388335"

Maxi calcium-activated potassium channels (BK) are an amazing category of ion channels which are found in cellular plasma membranes as well as in membranes of intracellular organelles. The function of these channels is to repolarize any excited membrane by passing a potassium outward current, in response to depolarization and/or increase in local calcium levels. Thus, voltage and calcium ions are involved in gating the channel under physiological conditions. This dual activation makes them perfect sensors for many cellular events that require integration between intracellular calcium levels and electrical signals.

A plethora of physiological and pathophysiological functions, such as membrane hyperpolarization, modulation of synaptic transmission, hormone secretion or mental deficiencies, vaso-regulation, epilepsies, heart diseases, myotonic dystrophies, hypertension etc, in almost all cells and tissues were reported for these channels. BK channels are main targets for important ligands like alcohol and gaseous neurotransmitters, such as NO, CO or H_2S, to name a few.

In the last years, the molecular entities and mechanisms involved in modulation of BK channels have gained tremendous attention, as the key role of these channels in cellular processes became increasingly recognized. Indeed, accessory proteins such as slowb, beta and gamma subunits, all serve to modulate the channel gating characteristics. Moreover, channel subunit expression and function is further tuned by phosphorylation/ dephosphorylation processes, redox mechanisms and the lipid microenvironment of the BK channel protein complex.

This e-book contains structural and functional aspects of BK channels, channel modulation by a variety of agents and cellular components, as well as the channel's relevance in health and disease.

Citation: Weiger, T. M., Dopico, A., Hermann, A., eds. (2017). BK Channels: Integrators of Cellular Signals in Health and Disease. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-291-0

Table of Contents

Chapter: BK channels primary targets of ethanol and hydrogen sulfide

Chapter: BK channels in health and disease

frontiers in PHYSIOLOGY

REVIEW ARTICLE
published: 06 February 2015
doi: 10.3389/fphys.2015.00029

BK channels: multiple sensors, one activation gate

Huanghe Yang[1,2], Guohui Zhang[3] and Jianmin Cui[3,4,5]*

[1] *Ion Channel Research Unit, Duke University Medical Center, Durham, NC, USA*
[2] *Department of Biochemistry, Duke University Medical Center, Durham, NC, USA*
[3] *Department of Biomedical Engineering, Washington University in Saint Louis, St. Louis, MO, USA*
[4] *Cardiac Bioelectricity and Arrhythmia Center, Washington University in Saint Louis, St. Louis, MO, USA*
[5] *Center for The Investigation of Membrane Excitability Disorders, Washington University in Saint Louis, St. Louis, MO, USA*

Edited by:
Alex M. Dopico, The University of Tennessee Health Science Center, USA

Reviewed by:
Brad S. Rothberg, Temple University School of Medicine, USA
Karl L. Magleby, University of Miami, USA

****Correspondence:***
Jianmin Cui, Department of Biomedical Engineering, Washington University, 1 Brookings Drive, Whitaker Hall, Room 290C, St. Louis, MO 63130, USA
e-mail: jcui@wustl.edu

Ion transport across cell membranes is essential to cell communication and signaling. Passive ion transport is mediated by ion channels, membrane proteins that create ion conducting pores across cell membrane to allow ion flux down electrochemical gradient. Under physiological conditions, majority of ion channel pores are not constitutively open. Instead, structural region(s) within these pores breaks the continuity of the aqueous ion pathway, thereby serves as activation gate(s) to control ions flow in and out. To achieve spatially and temporally regulated ion flux in cells, many ion channels have evolved sensors to detect various environmental stimuli or the metabolic states of the cell and trigger global conformational changes, thereby dynamically operate the opening and closing of their activation gate. The sensors of ion channels can be broadly categorized as chemical sensors and physical sensors to respond to chemical (such as neural transmitters, nucleotides and ions) and physical (such as voltage, mechanical force and temperature) signals, respectively. With the rapidly growing structural and functional information of different types of ion channels, it is now critical to understand how ion channel sensors dynamically control their gates at molecular and atomic level. The voltage and Ca^{2+} activated BK channels, a K^+ channel with an electrical sensor and multiple chemical sensors, provide a unique model system for us to understand how physical and chemical energy synergistically operate its activation gate.

Keywords: BK channels, allosteric gating, calcium binding proteins, modular organization, ion permeation, voltage sensor domain, magnesium binding, ion channel gating

INTRODUCTION

BK channels, also known as MaxiK, Slo1 or $K_{Ca}1.1$ channels, are one type of calcium-activated potassium channels that have large single channel conductance of 100–300 pS (Marty, 1981; Pallotta et al., 1981; Latorre et al., 1982). As a member of the six transmembrane (TM) voltage-gated potassium (K_V) channel superfamily, the basic functional unit of BK channels is a tetramer of the pore-forming α-subunits encoded by *Slo1* or *KCNMA1* gene in human (**Figure 1A**). The *slo1* gene was first identified by studying a mutation of the *Drosophila Slowpoke* locus that specifically abolished a Ca^{2+}-activated K^+ current in fly muscles and neurons (Atkinson et al., 1991; Adelman et al., 1992). BK channel activation can be regulated by membrane voltage and various intracellular chemical ligands such as Ca^{2+} (Marty, 1981; Pallotta et al., 1981; Adams et al., 1982; Barrett et al., 1982; Latorre et al., 1982; Methfessel and Boheim, 1982; Moczydlowski and Latorre, 1983), Mg^{2+} (Squire and Petersen, 1987; Zamoyski et al., 1989; Ferguson, 1991; McLarnon and Sawyer, 1993; Zhang et al., 1995, 2001; Morales et al., 1996; Wachter and Turnheim, 1996; Bringmann et al., 1997; Shi and Cui, 2001; Shi et al., 2002; Xia et al., 2002), protons (Schubert et al., 2001; Avdonin et al., 2003; Brelidze and Magleby, 2004; Hou et al., 2009), heme (Tang et al., 2003; Horrigan et al., 2005), carbon monoxide (Williams et al., 2004, 2008; Hou et al., 2008a), ethanol (Jakab et al., 1997; Dopico et al., 1998; Davies et al., 2003; Liu et al., 2008c; Bukiya et al., 2014; Davis et al., 2014), and lipid molecules (Braun, 2008; Vaithianathan et al., 2008; Yuan et al., 2011; Bukiya et al., 2011b; Dopico et al., 2012; Latorre and Contreras, 2013; Hoshi et al., 2013b,c,d; Tang et al., 2014) (**Figures 1A,B**, **3** and **Table 1**). The properties of BK channels can be further diversified through various splicing variants (Tseng-Crank et al., 1994; Navaratnam et al., 1997; Rosenblatt et al., 1997; Fury et al., 2002), post-translational modifications (Schubert and Nelson, 2001; Li et al., 2010), and association with the tissue-specific auxiliary β (Tseng-Crank et al., 1996; Wallner et al., 1996; Behrens et al., 2000; Orio et al., 2002) and γ subunits (Yan and Aldrich, 2010, 2012). Owing to their big conductance, the opening of BK channels allows rapid efflux of potassium ions, which effectively hyperpolarizes membrane potential, regulates membrane excitability, intracellular ion homeostasis, calcium signaling and cell volume. Therefore, BK channels are important in controlling various physiological processes, including smooth muscle contraction (Brayden and Nelson, 1992; Nelson et al., 1995; Tanaka et al., 1998; Perez et al., 1999; Pluger et al., 2000; Wellman and Nelson, 2003), hormone secretion (Petersen and Maruyama, 1984; Wang et al., 1994; Ghatta et al., 2006; Braun et al., 2008), neural excitation (Adams et al., 1982; Lancaster and Nicoll, 1987; Storm, 1987; Roberts et al., 1990; Robitaille and Charlton, 1992; Robitaille

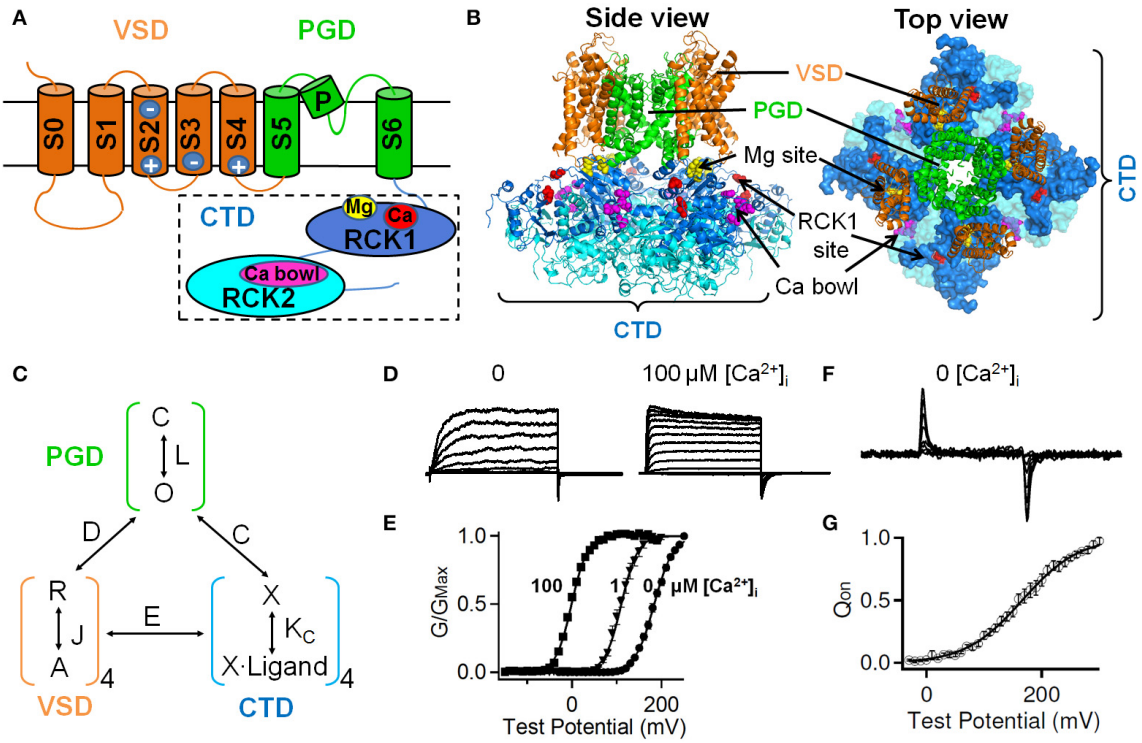


FIGURE 1 | Structural domains of the large-conductance, Ca^{2+}- and voltage-activated BK channels and their allosteric interactions during channel gating. (A) A BK channel can be divided into three structure domains: the pore-gate domain (PGD), the voltage sensor domain (VSD) and the cytosolic tail domain (CTD). Major elements for voltage and ion sensing (see text) are illustrated. **(B)** A homology model of BK channels based on the CTD structure of the zebra fish BK channel (PDB ID: 3U6N) and the membrane spanning domain of the Kv1.2–Kv2.1 chimera channel structure (PDB ID: 2R9R) by superimposing to the corresponding conserved regions of the MthK channel structure (PDB ID: 1LNQ) using UCSF Chimera software. Different structural domains are shown in colors as in A. **(C)** A general allosteric gating mechanism including allosteric interactions among three structure domains. *C* and *O*: closed and open conformations of PGD, respectively; *L*: the equilibrium constant for the *C–O* transition in the absence of voltage sensor activation and Ca^{2+} binding; R and A: resting and activated states of VSD; *J*: the equilibrium constant for VSD activation; K_C: equilibrium constant for ligand binding to closed channels; *D, C, and E*: allosteric factors describing the interaction between PGD-VSD, PGD-CTD, and VSD-CTD, respectively. **(D)** Macroscopic ionic current of BK channels in the absence and presence of 100 μM $[Ca^{2+}]_i$. **(E)** Increasing $[Ca^{2+}]_i$ shifts the conductance-voltage (*G–V*) relation to more negative voltages. **(F,G)** In the absence of Ca^{2+}, VSD can move in response to membrane voltage changes. **(F)** Gating current traces. Gating currents are generated due to the movement of the voltage sensor in the electric field across the membrane. **(G)** The voltage dependence of gating charge movement, the *Q–V* relation.

Table 1 | Molecular nature of chemical sensors in BK channels*.

Ligands	Effect	Binding site(s)	References
Ca^{2+}	Activation	D367, R514, and E535 (in RCK1) and Ca-bowl: D889, D892, D895, and D897 (in RCK2)	Schreiber and Salkoff, 1997; Shi et al., 2002; Xia et al., 2002; Zhang et al., 2010a
Mg^{2+}	Activation	D99 and N172 (in VSD) + E374 and E399 (in RCK1)	Shi et al., 2002; Xia et al., 2002; Yang et al., 2008
H^+	Activation	H365 and H394 (in RCK1)	Hou et al., 2008b
Carbon monoxide	Activation	Potentially through H365, D367, and H394 (in RCK1)	Hou et al., 2008a
Ethanol	Activation	K361 and R514 (in RCK1)	Bukiya et al., 2014
Heme	Inhibition	$C_{612}KAC_{615}H_{616}$ (between RCK1 and RCK2)	Tang et al., 2003
PIP_2	Stabilization?	K392, R393 (in RCK1)	Tang et al., 2014
Omega-3	Activation	Y318 (in S6)	Hoshi et al., 2013d

**The residue numbers are according to the sequence of the mbr5 clone.*

et al., 1993), hearing (Hudspeth and Lewis, 1988b,a; Wu et al., 1995; Rosenblatt et al., 1997; Fettiplace and Fuchs, 1999), circadian rhythms (Meredith et al., 2006), and gene expression (Marty, 1981; Li et al., 2014a). Consistent with their important physiological roles, BK channels have been discovered involving in pathogenesis of various diseases such as epilepsy (Du et al., 2005; N'Gouemo, 2011), cerebellar ataxia (Sausbier et al., 2004), autism and mental retardation (Laumonnier et al., 2006; Deng

et al., 2013), stroke (Gribkoff et al., 2001), hypertension (Brenner et al., 2000), asthma (Seibold et al., 2008), tumor progression (Weaver et al., 2004; Sontheimer, 2008), obesity (Jiao et al., 2011), hypoxia and ischemia (Kumar, 2007; Tano and Gollasch, 2014). With the collective efforts of the BK channel field, the understanding of molecular mechanisms of BK channel function has been greatly advanced over the past three decades. This review summarizes the recent structure-function understanding of the sensors and the activation gate of BK channels, their allosteric coupling, and implications of their assembly in 3-dimension. The readers may refer to other excellent reviews with regard to BK channel structure-function, physiology and regulations (Toro et al., 1998; Magleby, 2003; Cox, 2006; Latorre and Brauchi, 2006; Salkoff et al., 2006; Cui et al., 2009; Latorre et al., 2010; Lee and Cui, 2010; Horrigan, 2012; Rothberg, 2012; Singh et al., 2012b; Hoshi et al., 2013a; Yang and Cui, 2015) and reviews in this special topics series.

BK CHANNEL STRUCTURE FOLLOWS A MODULAR DESIGN

A functional BK channel is comprised of four Slo1 subunits. Each Slo1 subunit has three main structural domains with distinct functions (**Figure 1A**). The pore-gate domain (PGD) opens and closes to control ion selectivity and K^+ permeation; the voltage sensor domain (VSD) senses membrane potential changes; and the large cytosolic tail domain (CTD) that occupies two third of Slo1 sequence forms a gating-ring and serves as the chemical sensor to detect intracellular Ca^{2+} ions and various other ligands (**Figures 1B**, **3** and **Table 1**). Two sensory domains, the VSD and the CTD, covalently attach to the N- and C-terminus of the PGD, respectively. The basic function of these sensory domains is to transduce electric or chemical energy to mechanical forces on the PGD to toggle its conformation between closed and open states to control K^+ flux (**Figure 1C**).

The three distinct structural domains in BK channels can work as functionally independent modules and their homologs are widely expressed in various organisms. The PGD of BK channel is homologous to the PGDs of numerous prokaryotic and eukaryotic 2-TM and 6-TM K^+ channels, while the BK channel VSD follows similar design as the VSDs of voltage-gated cation channels (Long et al., 2005b,a, 2007; Payandeh et al., 2011), proton channels (Ramsey et al., 2006; Sasaki et al., 2006; Takeshita et al., 2014) and voltage sensitive phosphatase (VSPs) (Murata et al., 2005; Li et al., 2014b). Homologs of BK CTD have been found in the cytosolic domains of the bacterial K^+ channel complex (Cao et al., 2013), prokaryotic ligand-gated K^+ channels (Jiang et al., 2002a,b), as well as of the CTDs of Na^+- and Cl^-- activated Slo2 and pH-regulated Slo3 K^+ channels (Schreiber et al., 1998; Yuan et al., 2003, 2010; Salkoff et al., 2006). Based on the sequence homology, it seems plausible that multiple lateral gene transfer and gene fusion events might have occurred during the evolution of BK channels to link all three individual modules to form a multi-functional ion channel. Consistent with this possibility, a recent study elegantly demonstrated that a truncated BK channel without the entire CTD specifically eliminates its capability to sense intracellular ligands; but the voltage sensing and K^+ permeation are largely intact (Budelli et al., 2013). On the other hand, a prokaryotic MthK channel that is comprised of a PGD and a similar cytosolic gating-ring structure but lacks VSD is activated by intracellular Ca^{2+} (Jiang et al., 2002a,b).

The three distinct structural domains interact with one other and dynamically regulate channel gating, making BK channels an exemplar model system to study principles of sensor-gate coupling in ion channel function. Under physiological conditions, Ca^{2+} and depolarization work on the CTD and the VSD, respectively. The free energy derived from these two separate sensory modules activates the PGD of BK channels. The structure-function relationships of each individual module and the current understanding of their couplings are described below.

THE VOLTAGE SENSOR DOMAIN SERVES AS THE ELECTRIC SENSOR OF BK CHANNELS

Membrane depolarization alone is sufficient to activate BK channels as evidenced by the voltage-dependent macroscopic ionic current and the fast gating current that proceeds the ionic current in the absence of Ca^{2+} (**Figures 1D–G**). The voltage-dependence is mainly derived from voltage sensing residues in their intrinsic voltage sensor domain (VSD), which transverse membrane electrical field resulting in the measurable gating current (**Figures 1F,G**). The VSD of BK channels resembles a similar design to the VSDs of other voltage sensitive transmembrane proteins that include four transmembrane helices S1–S4. Unique to BK channel VSD, an additional transmembrane helix S0 (Meera et al., 1997) had been evolutionarily fused to its N-terminus through a long (~70 amino acids) intracellular loop (the S0–S1 linker), rendering the N-terminus of Slo1 peptide to the extracellular side (**Figure 1A**). Biochemical and electrophysiological evidence suggests that the extracellular end of S0 is located in close proximity of S3 and S4 (Liu et al., 2008a,b; Wang and Sigworth, 2009) and contributes to the folding and function of BK channel VSD (Meera et al., 1997; Koval et al., 2007; Pantazis et al., 2010b).

The VSD of BK channels exhibits three major functional differences from the VSDs of other Kv channels. First, BK channel VSD carries much less voltage-sensing charges (gating charge) than Kv channel VSDs (Stefani et al., 1997; Horrigan and Aldrich, 1999; Ma et al., 2006). The canonical Shaker K^+ channel has ~12–13*e* effective gating charges (Zagotta et al., 1994; Aggarwal and MacKinnon, 1996; Seoh et al., 1996), whereas each VSD of a BK channel only carries 0.6*e* gating charge or 2.4*e* charges per channel. The smaller number of gating charges indicates that more membrane depolarization is needed to move the VSD of BK channels into the fully activated state, as evidenced by the shallower slope of the gating charge-voltage (Q–V) relationship in gating current measurement (**Figure 1G**) and the conductance-voltage (G–V) relationship in ionic current measurement (**Figure 1E**). This weaker voltage sensitivity is critical to the physiological role of BK channels because it enables BK channels to operate in a wide range of membrane potentials to fine-tune channel activation, and in turn the membrane voltage. Second, only one out of three Arginine residues in BK channel S4 contributes to gating charge. Mutations of the other two Arginine residues, R207 or R210, do not affect the total gating charge, while voltage-sensing R213 merely contributes 0.3*e* to each VSD (Ma et al., 2006). This is drastically different from the S4 of Kv channels, in which each

of the first four Arginine (R1-R4) residues accounts for about 1*e* gating charge (Aggarwal and MacKinnon, 1996; Gandhi and Isacoff, 2002; Bezanilla, 2008). Third, the voltage sensing residues in BK channels are not restricted to S4. It has been well established that the R1-R4 residues in S4 serve as the primary voltage sensor of Kv channels and account for nearly all their gating charge (Aggarwal and MacKinnon, 1996). Nevertheless, BK channel S4 only contributes about half of total gating charge (Ma et al., 2006). In addition, E219, an acidic residue at the C-terminus of S4, was suggested to sense voltage (Zhang et al., 2014), bringing the contribution of S4 to total gating charge even lower. The other voltage sensing residues, D153 and R167 in S2 and D186 in S3 (**Figure 1A**), collectively contribute at least 50% of gating charge of BK channels. Interestingly, the corresponding residues in the Shaker K^+ channel have minimal contribution to its gating charge (Seoh et al., 1996). Instead, the acidic residues corresponding to D153 and D186 in Kv channels have been shown to form a network of electrostatic interaction with arginine residues in S4 at either the resting or active state, thereby controlling the conformational stability of the VSD (Seoh et al., 1996; Tiwari-Woodruff et al., 1997; Silverman et al., 2003; Long et al., 2005a). On the other hand, E293, the only major voltage sensing residue in Shaker S2, corresponds to an uncharged residue in the BK channel (Y163) (Seoh et al., 1996). Even replacing Y163 with a glutamate residue did not enhance the voltage sensing of BK channels (Ma et al., 2006). The decentralized distribution of gating charges and small contribution of each voltage sensing residue to BK channel activation thus suggest that the VSD movement in BK channels during channel gating may differ from that in Kv channels (Ma et al., 2006). Consistent with this scenario, recent voltage clamp fluorometry studies demonstrated that the transmembrane helices in BK channel VSD undergo complex relative motions during voltage-dependent activation. Upon depolarization, S2 approaches S1, while S4 diverges from S0, S1, and S2 (Pantazis et al., 2010b; Pantazis and Olcese, 2012). The relative movements of the voltage sensing S2 and S4 segments in the membrane electrical field result in reciprocal and cooperative interactions between these two transmembrane segments as evidenced by the fact that the neutralization of voltage-sensing residues in one segment impairs the voltage-dependent motions of the other (Pantazis et al., 2010a). This cooperativity between S2 and S4 may derive from mechanical coupling between the two segments. Alternatively or additionally, this cooperativity may be mediated by the rearrangements of the aqueous crevices within the VSD, which can change the dynamic focusing of the membrane electric field.

THE CYTOSOLIC TAIL DOMAIN SERVES AS THE CHEMICAL SENSOR OF BK CHANNELS

STRUCTURES OF THE CYTOSOLIC TAIL DOMAIN (CTD)

The CTD of BK channel contains multiple ligand binding sites (**Figure 3**), serving as the primary chemical sensor to respond to changes of Ca^{2+} and other intracellular ligands. The main structural components of a CTD are two regulators of K^+ conductance (RCK) domains (RCK1 and RCK2) that are connected by a ∼100-amino acid linker (**Figure 1A**). Evolutionarily conserved in the CTDs of some eukaryotic and many prokaryotic ligand-gated K^+ channels, as well as many prokaryotic K^+ transport systems, RCK domains regulate K^+ transport in response to intracellular ligand binding (Jiang et al., 2001, 2002a; Kuo et al., 2005; Loukin et al., 2005; Albright et al., 2006; Fodor and Aldrich, 2006). Recently, three crystal structures of eukaryotic BK CTD (PDB ID: 3MT5, 3U6N and 3NAF) were solved (Wu et al., 2010; Yuan et al., 2010). These structures provide the molecular basis for further understanding of how BK channel CTD regulate channel gating upon ligand binding.

The overall architecture and some key structural features are conserved between BK channel CTD and its prokaryotic counterparts. Four CTDs or eight RCK domains from a tetrameric BK channel stack together and form a large gating ring structure that covalently connects to the C-terminus of the PGD through four ∼20-amino acid C-linker (**Figure 1A**). Nevertheless, BK channel gating ring structures exhibit unique features. Different from the prokaryotic MthK channel whose CTD contains two separate identical RCK domains (Jiang et al., 2002a), the BK CTD contains the tandem non-identical RCK1-RCK2 domains and assembles into a more expanded gating ring structure. Each RCK domain can be further divided into three subdomains: the Rossmann-fold subdomain (βA–βF) forms central core of the gating ring; the intermediate helix-crossover (αF-turn-αG) interlocks RCK1 and RCK2 domains within the same subunit; and the C-terminal subdomain (αH–C-terminus) stays in the periphery and helps to hold the integrity of the gating ring structure. Extensive inter-RCK interactions at the helix crossover and C-terminal subdomain result in a more extensive "flexible interface" within the same subunit; while the "assembly interface" is mainly restricted to the Rossmann-fold subdomains between neighboring subunits. Under physiological conditions, Ca^{2+} is the major BK channel regulator that binds to the CTD. CTD also interacts with VSD via intracellular Mg^{2+} to activate the channel. Other ligands such as protons, heme, phosphatidylinositol 4,5-bisphosphate (PIP_2) and ethanol also bind to the CTD and regulate BK channel activation. Based on the functional and structural information of the CTD, here we review the current molecular understanding of Ca^{2+} and Mg^{2+}-dependent activation and also briefly summarize the action of other physiological ligands.

CA^{2+} SENSORS AND THEIR ACTION

Intracellular Ca^{2+} binds to the CTD of BK channels to increase channel opening, typically in the range of 100 nM to 300 μM (**Figures 1D,E**). Electrophysiological and mutagenesis experiments have identified two Ca^{2+} high affinity binding sites for each Slo1 subunits (**Figures 2A,B**): one is located in the C-terminus of RCK2 domain, containing a string of Asp residues known as the "Ca^{2+} bowl" (Schreiber and Salkoff, 1997), and the other is located in RCK1 domain presumably including the side-chain carboxylates of D367 and E535, as well as the main-chain carbonyl of R514 (Shi et al., 2002; Xia et al., 2002; Zhang et al., 2010b) (**Figures 1A,B**).

In the recent crystal structures of the human and zebra fish BK channel CTD domains (Wu et al., 2010; Yuan et al., 2010), the Ca^{2+} bowl binding site was mapped to a short consecutive peptide containing an EF-hand-like motif at the "assembly

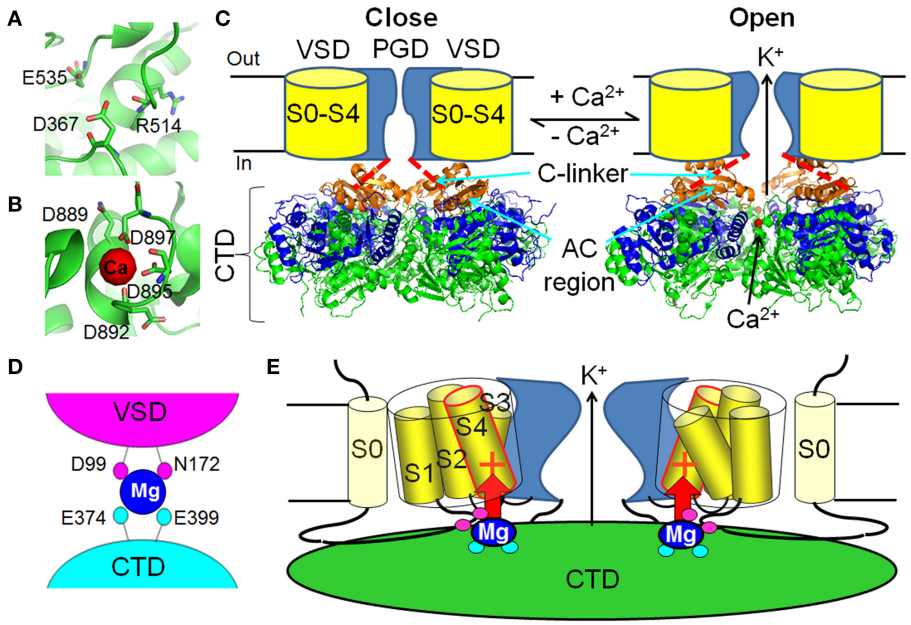


FIGURE 2 | Ca^{2+} and Mg^{2+}-dependent activation of BK channels. (A) The putative Ca^{2+} binding pocket in the RCK1 site (PDB ID: 3NAF). **(B)** The Ca^{2+} binding pocket in the Ca^{2+} bowl site (PDB ID: 3U6N). **(C)** Ca^{2+} binding changes the conformation of the cytosolic tail domain (CTD), which pulls the C-linker to open the activation gate of BK channels. The Ca^{2+}-free (3NAF) and Ca^{2+} bound to the Ca^{2+} bowl (3U6N) CTD structures are shown in the left and right panels, respectively. One of the most dramatic Ca^{2+}-induced conformational changes happens in the AC region (βA–αC, orange). The rest of the RCK1 domain is shown in blue and the RCK2 domain is shown in green. The bound Ca^{2+} in the Ca^{2+} bowl is shown as red dot in the right panel. **(D)** The low affinity Mg^{2+} binding site is composed of D99 and N172 in the voltage sensory domain (VSD) and E374 and E399 in the CTD. Magenta and cyan of these residues illustrate that D99/N172 and E374/E399 are from neighboring Slo1 subunit. **(E)** Mg^{2+} binds to the interface of the VSD and the CTD to activate BK channels through electrostatic interaction with the voltage sensor. The red + sign in S4 represents the major voltage sensing residue R213.

interface" between two neighboring subunits. Within the Ca^{2+} bowl, the side chain carboxylate groups of D895 and D897 and the main chain carbonyl groups of D889 and D892 provide direct coordinates to the bound Ca^{2+} ion (**Figure 2B**), consistent with previous mutagenesis and biochemistry experiments (Bian et al., 2001; Braun and Sy, 2001; Bao et al., 2002, 2004). The side chain of D894 does not directly contact with Ca^{2+}. Instead, it forms salt bridges with R1018 and K1030, presumably helping to stabilize the conformation of the Ca^{2+} bowl. D896, on the other hand, does not contact with other parts of the protein or Ca^{2+}, and is thereby not important for Ca^{2+} binding.

Compared to the Ca^{2+} bowl site, the high affinity Ca^{2+} binding site in RCK1 domain was less well defined by the recent BK channel CTD structures. No diffraction of Ca^{2+} ion was resolved within the putative high affinity Ca^{2+} binding pocket in the RCK1 domain even when 10 or 50 mM Ca^{2+} was present during the crystallization procedures (Yuan et al., 2010). Nevertheless, the key residues (D367, E535, and R514) that have been shown to be important for Ca^{2+} sensing by functional studies (Shi et al., 2002; Xia et al., 2002; Zhang et al., 2010b) stay in close proximity in these structures, giving insights on the molecular details of this putative high affinity Ca^{2+} binding site (**Figure 2A**). Other residues implicated by mutational studies as being important for Ca^{2+} dependent activation, such as M513 (Bao et al., 2002) and D362 (Xia et al., 2002) do not seem to be part of the putative Ca^{2+} binding pocket as they are either chemically unfavorable (M513) or spatially far away from the binding site (D362) (Zhang et al., 2010a). It is likely that these resides indirectly contribute to Ca^{2+} binding by stabilizing the RCK1 Ca^{2+} site. Does the absence of Ca^{2+} at the RCK1 site in these structures represent artifacts during crystallization or result from the potential distortions of the Ca^{2+} site due to the absence of the entire transmembrane spanning domain that intimately interacts with the RCK1 domain (see discussion in later sections)? Further structural endeavors are thus needed to address this intriguing question.

Structural and functional studies suggest that the RCK1 Ca^{2+} site and Ca^{2+} bowl are not identical in terms of ion binding and the subsequent allosteric activation mechanism. These two sites are located, ~25Å apart, near the periphery of the gating ring (**Figure 1B**) with the Ca^{2+} bowl at the assembly interface between neighboring subunits and the RCK1 site in the N-lobe of the RCK1 domain (Wu et al., 2010; Yuan et al., 2010). Although both sites face toward the plasma membrane, the RCK1 site stays closer, and thus may exert more influence on the transmembrane domain including the VSD of BK channels (**Figure 1B**). Consistent with this scenario, voltage dependence was only observed in the binding of Ca^{2+} to the RCK1 site but not to the Ca^{2+} bowl (Sweet and Cox, 2008). The same study also discovered that the Ca^{2+} sensors exhibit different apparent Ca^{2+} binding affinity with the Ca^{2+} bowl showing higher Ca^{2+} affinity than the RCK1 site at −80 mV. Indeed, these two sites may adopt different cation coordination chemistry as evidence by their different selectivity toward various divalent cations (Zeng

et al., 2005). The Ca^{2+} bowl specifically binds Ba^{2+}, while the RCK1 site only senses Cd^{2+}, though both sites can bind Ca^{2+} and Sr^{2+}. Moreover, the two Ca^{2+} sites also exert different effects on channel kinetics (Zeng et al., 2005). The Ca^{2+} bowl mainly accelerates activation kinetics at low Ca^{2+} concentrations, while the RCK1 site influences both activation and deactivation kinetics. Considering the fact that these two Ca^{2+} sensors contribute about equally and independently to Ca^{2+} activation (Bao et al., 2002; Xia et al., 2002) with small cooperativity in activating the channel (Qian et al., 2006; Sweet and Cox, 2008), it is reasonable to assume that they operate the activation gate through different allosteric pathways. Interestingly, D369G, the human hereditary mutation associated with generalized epilepsy and paroxysmal dyskinesia (GEPD) (Du et al., 2005), enhances BK channel Ca^{2+} sensitivity specifically through the RCK1 site but not the Ca^{2+} bowl (Yang et al., 2010). The enhancing effect of the mutation was lost when the RCK1 site was destroyed, but still remained intact when the Ca^{2+} bowl site was mutated (Yang et al., 2010). Although only two amino acids away from D367 in the RCK1 site, D369G seems not directly affect Ca^{2+} binding to the RCK1 site. Instead, this mutation increases Ca^{2+} sensitivity at low Ca^{2+} concentrations by enhancing the rigidity of the N-terminal AC region (βA–αC) of the RCK1 domain (**Figure 2C**), a critical regulatory gating region (Krishnamoorthy et al., 2005) that couples the CTD, VSD and PGD of BK channels and exhibits most dramatic conformational changes when comparing the Ca^{2+}-free and Ca^{2+}-bound CTD structures (Wu et al., 2010; Yang et al., 2010; Yuan et al., 2010). With the new CTD structures, it is promising to unveil the molecular mechanism of these Ca^{2+}-induced activation pathways and their potential interactions in the near future.

MG^{2+} SENSOR AND ITS ACTION

Under physiological conditions, millimolar intracellular Mg^{2+} can activate BK channels by shifting activation voltage to more negative ranges (Golowasch et al., 1986; Oberhauser et al., 1988). The low affinity (in millimolar range) Mg^{2+}-dependent activation is independent from the high affinity (in micromolar range) Ca^{2+}-dependent activation as the Mg^{2+} sensitivity remains unaltered at both zero and saturating Ca^{2+} concentrations (100 μM) (Shi and Cui, 2001). Indeed, Mg^{2+} activates the channel by binding to a low affinity divalent cation binding site distinct from the high affinity Ca^{2+} bowl and RCK1 binding sites. Electrophysiological characterization of mutations in the N-terminus of the RCK1 domain identified two acidic residues, E374 and E399 (**Figure 1B**), which are critical to Mg^{2+} sensing and likely to be part of the putative Mg^{2+} binding site (Shi et al., 2002; Xia et al., 2002). A comprehensive screening of all the potential oxygen-containing residues in the membrane spanning domain pinpointed D99 and N172 as the other two putative Mg^{2+} coordinates, which are located in the C-terminus of the long S0–S1 loop and the S2–S3 loop, respectively (Yang et al., 2007, 2008) (**Figure 2D**). Functional evidence suggests that D99 and N172 in the VSD are spatially close to E374/E399 in the RCK1 domain, thereby forming an inter-domain Mg^{2+} binding site at the interface between the VSD and the CTD (Yang et al., 2008, 2013). In the recent BK channel CTD structures (Wu et al., 2010; Yuan et al., 2010), E374 and E399 are located at the top plateau of the CTD with their carboxylate containing side chains pointing to the membrane, providing further support to the functional findings (**Figure 1B**).

Distinct from the Ca^{2+}-dependent activation that is largely independent of the BK VSD (Horrigan and Aldrich, 2002), Mg^{2+} actually activates the channel through an electrostatic interaction with the VSD (Yang et al., 2007; Horrigan and Ma, 2008) (**Figure 2E**). Two lines of evidence indicate the involvement of the VSD in Mg^{2+}-dependent activation. First, millimolar Mg^{2+} has no measurable effect on channel activation at negative voltages when voltage sensors are in the resting state (Yang et al., 2007; Horrigan and Ma, 2008); in contrast, 70 μM $[Ca^{2+}]_i$ can increase the open probability >2000-fold under similar voltages (Horrigan and Aldrich, 2002; Yang et al., 2010). This suggests that Mg^{2+} activates the channel only when the VSD stays in the activated state (Chen et al., 2011). Second, neutralization of R213, the most important voltage-sensing residue in S4, specifically eliminated Mg^{2+} sensitivity, but had no effect on Ca^{2+} sensing (Hu et al., 2003). A study further demonstrated that an electrostatic repulsion between R213 and the bound Mg^{2+} at the interface of the VSD and CTD is responsible for the activation effect of Mg^{2+} (Yang et al., 2007). This electrostatic interaction can stabilize the VSD in the activated state and alter the VSD-pore coupling (Horrigan and Ma, 2008), thereby facilitating BK channel opening.

OTHER LIGAND SENSORS AND THEIR ACTIONS

In addition to Ca^{2+} and Mg^{2+}, other intracellular ligands can also bind to the CTD domain and regulate BK channel activation (**Figure 3** and **Table 1**). In the absence of Ca^{2+}, intracellular protons have been found to be able to activate BK channels presumably by protonating the side chains of H365 and H394 (Avdonin et al., 2003; Hou et al., 2008b). As H365 is located near the RCK1 Ca^{2+} binding site, it is likely that its protonated imidazole side chains electrostatically interact with the nearby putative Ca^{2+} sensor D367 to facilitate Ca^{2+} binding. On the other hand, H394, a residue that stays further away from the RCK1 Ca^{2+} site, may indirectly affect Ca^{2+} binding and thus plays a less important role on proton sensing (Hou et al., 2008b). Interestingly, carbon monoxide (CO) also stimulates BK channels using the same sensors (Hou et al., 2008a). Mutations of H365, H394, or D367, also eliminate the CO sensitivity. These studies thus suggest that both CO and H^+ enhance channel activation by mimicking the action of Ca^{2+} on its RCK1 sensor.

Ethanol can directly activate BK channels in isolated inside-out membrane patches (Dopico et al., 1998) in the presence of Ca^{2+} and potentiate *Caenorhabditis elegans* BK channels *in vivo* to produce alcohol intoxication by reducing excitatory neurotransmitter release (Davies et al., 2003). Further study (Liu et al., 2008c) shows that in the presence of Ca^{2+}, mutations in the Ca^{2+} bowl (5D5N) or the Mg^{2+} binding site (E374A/E399A) fail to eliminate ethanol effect while mutations in the RCK1 high-affinity site (D362A/D367A) abolish ethanol inhibition of current. Based on crystallographic structure, computational modeling, mutagenesis and electrophysiology, a recent study discovered a putative ethanol recognition site in the CTD including key residues K361 and R514 (Bukiya et al., 2014). When Ca^{2+} binds to the CTD,

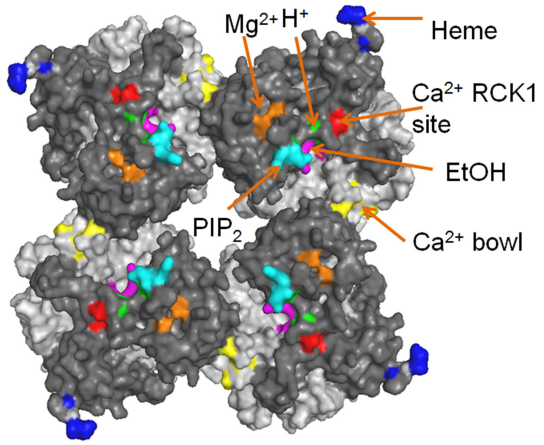


FIGURE 3 | The cytosolic tail domain (CTD) serves as chemical sensors for BK channels. The putative binding sites for different ligands are labeled with colors. RCK1 domain and RCK2 domain are shown in dark gray and light gray, respectively. PDB ID: 3U6N.

K361 (in the middle of αA helix) likely forms hydrogen bond with the hydroxyl group of ethanol, while R514 (in the linker between αG and αH) may help to stabilize the ethanol binding pocket. It is known that ethanol cannot active BK channels in the absence of Ca^{2+}. This new study gave structural explanation to this phenomenon. In the absence of Ca^{2+}, the hydrogen bonding between K361 and ethanol is blocked by a nearby residue M909, and R514 swings away from the binding pocket. Both factors likely make the putative binding site inaccessible to ethanol. Consistent with this scenario, adding bulk side chains to the nearby residues E354, S357, and N358 also abolishes ethanol action, presumably by blocking ethanol accessibility. Interestingly, R514 is also involved in Ca^{2+} sensing (Zhang et al., 2010b). It is therefore plausible that Ca^{2+} binding opens up the putative ethanol binding pocket and the binding of ethanol, in turn, further facilitates Ca^{2+}-dependent activation by increasing Ca^{2+} binding and/or by enhancing the allosteric coupling between Ca^{2+} binding at the RCK1 site and channel opening. By screening *C. elegans* strains with different predicted missense mutations in the *Slo1* channel from the Million Mutation Project (Thompson et al., 2013), another mutation, T352I, was found to reduce ethanol-induced activation (Davis et al., 2014). It was concluded that the T352I mutation may alter a binding site for ethanol and/or interfere with ethanol-induced conformational changes that are critical for behavioral responses to ethanol.

Lipid molecules such as PIP_2, cholesterol and omega-3 fatty acids can also modulate BK channel activities (Braun, 2008; Vaithianathan et al., 2008; Yuan et al., 2011; Bukiya et al., 2011b; Dopico et al., 2012; Latorre and Contreras, 2013; Hoshi et al., 2013b,c,d; Tang et al., 2014). PIP_2, a ubiquitous lipid modulator of numerous ion channels and transporters, enhances Ca^{2+}-dependent gating of BK channels. Neutralizing the positively charged residue K392 and R393 in αB greatly reduced the apparent sensitivity to PIP_2, suggesting that these two resides might be part of the putative PIP_2 binding site (Tang et al., 2014). The localization of these residues on the top surface of RCK1 domain is consistent with this hypothesis, which potentially allows electrostatic interactions between the positively charged residues and the negatively charged PIP_2 head group in the inner leaflet of the plasma membrane (**Figure 3**). Interestingly, the PIP_2 effect was only apparent when potent PIP_2 depleting reagents were applied, suggesting that PIP_2 might tightly bind to BK channels and/or the intimate interactions between the CTD and the membrane spanning domain of BK channels create a physical barrier to limit the free diffusion of this highly charged lipid species. Different from PIP_2, Omega-3 fatty acids were recently discovered to act on BK channels through Y318 at the C-terminus of S6 segment (Hoshi et al., 2013d). These lipids potentiate BK current in the presence of auxiliary β1 subunit and lower blood pressure in mice (Hoshi et al., 2013c), thereby providing a molecular mechanism to explain potential health benefits of omega-3 fatty acids on regulating blood pressure. In contrast, cholesterol inhibits BK channel activity (Bolotina et al., 1989; Bregestovski and Bolotina, 1989; Dopico et al., 2012). Cholesterol either works directly on BK channel complexes and/or alters BK channel activity indirectly by modulating membrane lipids or lipid-channel interfaces (Bukiya et al., 2011a,b; Dopico et al., 2012; Singh et al., 2012a).

Heme inhibits BK channel activity by binding to the CTD with high affinity (IC_{50} =~70 nM) (Tang et al., 2003; Horrigan et al., 2005). Bioinformatics predication and the subsequent functional characterization identified the sequence "CKACH" in the N-terminus of the RCK1-RCK2 linker to be responsible for heme binding (Tang et al., 2003). A comprehensive analysis of heme effects using the HA allosteric model (Horrigan and Aldrich, 2002) suggested that heme exerts its apparent inhibitory effect by increasing open probability (Po) at negative voltages and reducing Po at more positive voltages (Horrigan et al., 2005). The binding of heme to the RCK1-RCK2 linker segment that is located at the periphery of the CTD domain (**Figure 3**) may impede the gating ring conformational changes and the CTD-VSD interaction that normally accompanies the activation of BK channels.

It is worth noting that, except for the Ca^{2+} bowl in the RCK2 domain, all the known chemical sensors are located on or close to the top surface of the RCK1 domain that faces the membrane or the membrane-spanning domain of the channel (**Figure 3**). Since these sites are sensors of cytoplasmic ligands, they are exposed to aqueous solution. It is interesting that all the "activating" sensors in the RCK1 domain are clustered at the center of the gating ring, while the "inhibiting" heme sensor is located at the periphery. Does this design reflect a coincidence or an evolutionary advantage in regulating BK channel activation? How do these sensors interact with each other? Do they have any cooperativity? Answers to these questions will further our understanding of the mechanisms of BK channel activation.

THE PORE-GATE DOMAIN CONTROLS K^+ PERMEATION OF BK CHANNELS

Free energies provided by membrane voltage and intracellular ligand binding ultimately alters the PGD to open to K^+ flux across the membrane. The PGD domain, comprising S5–S6 segments, forms the center of a BK channel, where the ion selection and permeation occur. Like most of other K^+ channels, a short peptide including the signature "GYG" sequence from four Slo1 subunits

form the ion selectivity filter of a BK channel, which separates the external and internal aqueous solution and selectively permits K^+ ions to go through (Doyle et al., 1998). Four S6 helices (equivalent to the inner helix in 2-TM K^+ channels) from each of Slo1 subunit form the central ion pathway. Despite these similarities, BK channels exhibit a number of functional and structural features that distinguish them from other Kv channels.

First, BK channels have the largest unitary conductance of all K^+ channels. Its large conductance is partly derived from two clusters of acidic residues that are located at the intracellular and extracellular entrances of the K^+ permeation pathway. D261 in the extracellular entrance contribute to ∼18% of BK channel unitary conductance for the inward K^+ current (Carvacho et al., 2008), while E321 and E324, which are located in the cytosolic end of the S6 segment, form a ring of 8 negative charges and contribute up to 50% of BK unitary conductance for the outward K^+ current (Brelidze et al., 2003; Nimigean et al., 2003). These clusters of negative charges thus serve as electrostatic traps to attract and concentrate local K^+ concentration to enhance BK unitary conductance (Brelidze et al., 2003; Nimigean et al., 2003; Carvacho et al., 2008). Nevertheless, these negative charges at the extracellular and intracellular entrances of K^+ permeation pathway only account for part of the large single channel conductance of BK channels. Other structural features specific to BK channels, including the larger negative electrostatic potential inside the pore and the wider entrance to the inner vestibule, may also contribute to BK channels' large conductance (discussed below) (Nimigean et al., 2003; Li and Aldrich, 2004; Brelidze and Magleby, 2005; Carvacho et al., 2008; Geng et al., 2011).

Second, BK channels have a much larger inner vestibule with a wide cytosolic entrance compared to most of K^+ channels. Chemicals with various sizes and properties were used to probe the size of the central cavity and its cytosolic entrance (Li and Aldrich, 2004; Brelidze and Magleby, 2005; Wilkens and Aldrich, 2006). Smaller size quaternary ammoniums (QA) such as tetrabutylammonium (TBA) can have relatively free access to the inner vestibule independent of the states the activation gate. These QAs show much faster blocking and unblocking kinetics in BK channels than in other Kv channels, indicating BK channels have an enlarged inner vestibule and broader cytosolic entrance (Li and Aldrich, 2004; Wilkens and Aldrich, 2006). Based on the changes of the K^+ diffusion rate from bulk intracellular solution to the central cavity due to interference by sucrose, the cytosolic mouth of BK channel pore when open was estimated to be twice (∼16–20Å) as large as that of the Shaker K^+ channel (Brelidze and Magleby, 2005). Consistent with this estimation, recent cysteine substitution and modification studies of S6 with different MTS reagents showed that modification can occur even when the channel is in closed states (Geng et al., 2011; Zhou et al., 2011). The cytosolic opening of the central cavity at the level of the C-terminus of S6 (around E321 and E324) is at least 13–18 Å in diameter, which allows MTS reagents to go through and modify the cysteine residues inside the central cavity (Zhou et al., 2011) or the cysteine residues at the cytosolic entrance to alter outward single channel conductance (Geng et al., 2011). All these results suggest that BK channel S6 lacks the cytosolic activation gate around the "bundle crossing" in canonical K^+ channels, where the hydrophobic residues at the C-terminus of the four inner helices form a tight seal to restrict K^+ ion flux when these channels are closed (Hille et al., 1999). Instead, the activation gate of BK channels is likely near or within the selectivity filter, a design that is also observed in other ligand-gated ion channels such as CNG (cyclic nucleotide-gated) channels (Flynn and Zagotta, 2001) and SK (small conductance, Ca^{2+}-activated K^+) channels (Bruening-Wright et al., 2007). The interaction between permeating thallium ion (Tl^+) and the selectivity filter altering BK channel activation further supports this scenario (Piskorowski and Aldrich, 2006).

Third, the orientation of the pore-lining residues in BK channels is different from those of Kv channels. Cysteine substitution and modification studies of the BK S6 demonstrated that A313, A316, and S317 are facing to the inner pore, while the corresponding residues in Shaker K^+ channels tend to face away from the aqueous environment (Zhou et al., 2011). Therefore, a relative rotation of the S6 has to occur to account for this experimental observation. One possible cause of this rotation may derive from the two consecutive glycine residues (G310 and G311) in BK channel S6 (**Figure 4A**). An additional glycine residue (G310) may make S6 more flexible around the highly conserved "Glycine hinge" region in Kv channels, thereby rearranging the orientations of the residues downstream of this di-glycine hinge.

The forth unique feature of the BK PGD is that the movement of the pore-lining S6 helix of BK channels is different from that of canonical K^+ channels. Although BK channel pore has an enlarged intracellular entrance, state-dependent blockade by a synthetic Shaker ball peptide (ShBP) suggests that the S6 segment corresponding to this entrance indeed moves during gating to restrict the entry of bulky ShBP but not smaller K^+ and QA ions (Li and Aldrich, 2006). Structural and functional studies of K^+ channels suggest that the highly conserved glycine hinge in the middle (Jiang et al., 2002b) and/or the Pro-Val-Pro (PVP motif) at the C-terminus of the pore-lining S6 helix (Webster et al., 2004) are two critical pivot points for the movement of the activation gate. Ala mutations of the double glycine resides (Magidovich and Yifrach, 2004) significantly hinders channel activation, suggesting that the flexibility around the di-Gly hinge is critical for BK channel gating. Indeed, the recent mutagenesis, cysteine modification, histidine protonation and pharmacological studies of BK channels show that multiple pore residues (L312, A313, M314, and A316) downstream of the di-glycine hinge reorient their side chains during channel gating (Chen et al., 2014). Remarkably, charged or polar side-chain substitutions at each of the sites resulted in constitutively opened mutant channels that largely or completely loss voltage and Ca^{2+} dependence, presumably by exposing the hydrophilic side chains to the aqueous environment of the pore to reduce their side-chain solvation energy. Based on the fact that multiple pore residues in BK displayed side-chain hydrophilicity-dependent constitutive openness, it is proposed that BK channel opening involves structural rearrangement of the deep-pore region.

ASSEMBLY OF THE BK CHANNEL MODULAR DOMAINS

Increasing structural and functional information is available on the individual PGD, VSD, and CTD and their intimate

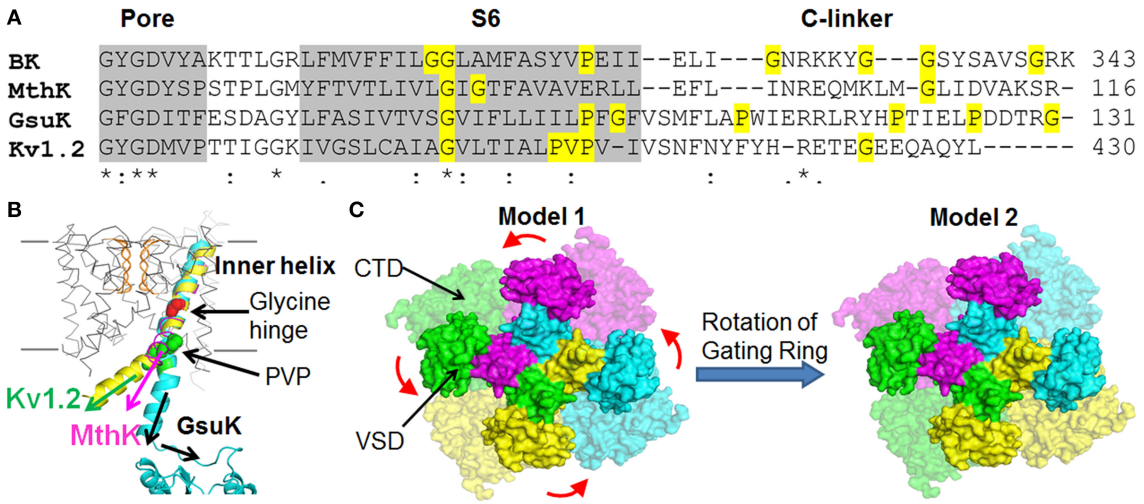


FIGURE 4 | Assembly of the BK channel structural domains. (A) Sequence alignment of the inner helices and the C-linkers of different K^+ channels. Residues that can increase peptide flexibility and alter α helix orientation (i.e., Glycine and Proline) are highlighted in yellow. **(B)** The C-termini of inner helix and the C-linkers of different K^+ channels may point to different directions. The PGDs of MthK (PDB ID: 1LNQ), Kv1.2 (PDB ID: 2R9R) and GsuK (PDB ID: 4GX5) channels are superimposed at the selectivity filter by using UCSF Chimera. **(C)** The relative assembly of the VSD and the CTD in BK channels might be different from the homology model shown in **Figure 1B** with a relative ∼90° angular rotation about the central axis between the PGD and CTD.

interactions. However, how these domains spatially assemble to form a quaternary structure of a functional tetrameric BK channel is still unclear largely owing to the lack of a full-length atomic structure. Thus, the most widely used method to address this problem is to construct a BK channel structure through homology modeling (**Figure 1B**). With the crystal structures of Kv1.2–Kv2.1 chimera channel (Long et al., 2007) and the BK CTD (Wu et al., 2010; Yuan et al., 2010), a homology model of BK channels can be built using the MthK structure, which contains a homologous PGD and CTD, as a template (Jiang et al., 2002a,b). A VSD structure was included in the BK channel model by superimposing the PGDs of the Kv1.2–Kv2.1 chimera channel and the MthK channel; while the gating ring of MthK was replaced by the BK channel CTD structure. Despite the fact that the VSD lacks S0 and the long S0–S1 linker, the overall structure of this homology model is consistent with most of functional findings in BK channels. According to this model, the membrane spanning domain and the CTD gating ring are closely packed to each other with the VSD residing on the top surface of RCK1 domain along the four-fold (central) axis. The close proximity of the CTD to the membrane spanning domain is consistent with the following evidence. First, the CTD and the membrane spanning domain were tightly packed to each other in a cryo-electron microscopy structure of the full-length BK channel (Wang and Sigworth, 2009). Second, the top plateau of the RCK1 domain, especially αB helix might move toward the membrane-spanning domain along the central axis when compared the Ca^{2+}-bound CTD structure (3U6N) with the Ca^{2+}-free CTD structure (3NAF) (Wu et al., 2010; Yuan et al., 2012) (**Figure 2C**). Third, four residues from the VSD and the CTD are in close proximity and form an inter-domain Mg^{2+} binding site (Yang et al., 2008) (**Figure 2D**). Fourth, Mg^{2+} or the charges around the Mg^{2+} binding site in the CTD can electrostatically interact with R213 in S4 of VSD (Hu et al., 2003) (**Figure 2E**). Fifth, introducing charges to N172 in the VSD and E399 in the CTD creates electrostatic interactions that affect the voltage- and Ca^{2+}-dependent activation of BK channels (Yang et al., 2013). Last but not least, K392 and R393 on the top surface of the CTD can electrostatically interact with negatively charged PIP_2 molecules on the inner leaflet of the plasma membrane (Tang et al., 2014) (**Figure 3**).

While the relative longitudinal packing of the VSD and the CTD along the four-fold central axis in the homology model is consistent with experimental results, the relative angular positions about the central axis between the PGD and CTD of the same subunit are not defined in the BK channel models (**Figure 4C**). First of all, the 17-amino acid peptide C-linker between the PGD and CTD was not resolved in the template MthK structure (Jiang et al., 2002a,b), leaving some uncertainties in the assignment of the PGD and the CTD to the same subunit. Secondly, although both MthK and BK channels contain two glycine residues at the "Glycine hinge" in S6 of the PGD the localization of the Glycine residues differs between the two channels (**Figure 4A**). The C-terminus of S6 in the MthK channel also lacks a Proline, the helix-breaking residue, compared to BK channels. In addition, the C-linker between S6 and the CTD of the BK channel differs from that of the MthK channel in both length and amino acid sequences. This series of differences suggest that the CTD angular position relative to the PGD can differ between MthK and BK channels. Interestingly, recently published crystal structures of a full-length GsuK channel, a RCK-containing, multi-ligand gated K^+ channel from bacteria *Geobacter sulfurreducens*, show a ∼50 degree of counterclockwise rotation of the CTD relative to the PGD as compared to the MthK structure (Kong et al., 2012). The "glycine hinge" residues and the C-terminal Proline residue in S6 are not conserved between the GsuK and MthK channels (**Figure 4A**). In addition, the C-linker

of the GsuK channel is six amino acids longer than that of the MthK channel. Containing three proline residues and one glycine residue, the GsuK C-linker is also flexible as evident in the crystal structure (**Figure 4B**). These properties might contribute to this rotation.

Based on the study of the Mg^{2+} binding site (Yang et al., 2008), which is composed of D99/N172 from the VSD and E374/E399 from the CTD (**Figure 2D**), it was proposed that the VSD of each subunit is aligned with the CTD from a neighboring subunit. This alignment requires relative angular positions between the CTD and PGD differing from the homology model based on MthK structure (**Figure 4C**). While individual mutations D99R, N172R, E374R, and E399C abolished Mg^{2+} sensitivity of the homotetrameric BK channels, the heterotetrameric channels resulting from the co-expression of the BK channel subunits containing D99R in the VSD and E374R in the CTD, respectively, still retained partial Mg^{2+} sensitivity. The retention of Mg^{2+} sensitivity can only be explained by the BK homology model 2 (**Figure 4C**), in which D99/N172 in the VSD of one subunit and E374/E399 in the CTD from the neighboring subunit form an intersubunit Mg^{2+} binding site. In this way, one or two out of four Mg^{2+} binding sites could remain intact in some of the heterotetrameric channels. The experimental results could be nicely fitted with a binomial distribution of the mixed mutant channels. On the other hand, the BK homology model based on the MthK structure predicts that all four Mg^{2+} binding residues are from the same Slo1 subunit to form an intrasubunit Mg^{2+} site. Since one single mutation is sufficient to eliminate Mg^{2+} binding, none of the heterotetrameric channels could retain intact Mg^{2+} binding site based on this model. Consistent with this alignment, it is recently reported (Zhang et al., 2014) that mutation E219R in S4 and E321/E324 in the cytosolic side of S6 of a neighboring subunit engage in electrostatic interactions to alter voltage and Ca^{2+} dependent activation. These results suggest that the BK channel S6 may bend at the inner vestibule as compared to the structure of Kv1.2/Kv2.1 (**Figure 4B**), directing the downstream CTD to an angular position shown in the homology model 2 (**Figure 4C**). This arrangement may also explain why BK channels have large openings at the inner vestibule even when the channel is closed.

ALLOSTERIC COUPLING BETWEEN THE SENSORS AND THE PORE-GATE

The VSD and CTD in BK channels sense voltage and intracellular signaling molecules and open the channel gate in the PGD by electromechanical and chemomechanical couplings between the PGD and the VSD and CTD, respectively (**Figure 1C**). Voltage and Ca^{2+} activate BK channels mainly by destabilizing the closed state with small effects on stabilizing the activated state of the PGD (Geng and Magleby, 2014). Various allosteric models have been developed to describe Ca^{2+}- and voltage-dependent BK channel gating based on the analysis of single channel kinetics and macroscopic current (McManus and Magleby, 1991; Cox et al., 1997; Horrigan et al., 1999; Rothberg and Magleby, 1999, 2000; Cui and Aldrich, 2000; Horrigan and Aldrich, 2002; Shelley et al., 2010). In a model that integrates both Ca^{2+} and voltage dependent activation (**Figure 1C**) (Horrigan and Aldrich, 2002), three structural domains, the PGD, VSD and CTD, undergo separate conformational changes but also allosterically coupled to each other, reflecting the modular design of BK channels. The BK channel activation gate can open in the absence of voltage sensor activation and Ca^{2+} binding with an intrinsic open probability of $\sim 10^{-7}$ (Horrigan et al., 1999; Cui and Aldrich, 2000). On the other hand, voltage sensor activation and Ca^{2+} binding can enhance channel opening in a relatively independent fashion. In the absence of Ca^{2+} binding, extreme depolarization ($> +110$ mV) enhances channel open probability when the voltage sensor moves from resting state to activated state (**Figures 1D–G**) (Cui et al., 1997). Similarly, saturating Ca^{2+} increases BK channel open probability by four orders of magnitude from $\sim 10^{-7}$–10^{-3} when the voltage sensors are at the resting state (Horrigan and Aldrich, 2002; Yang et al., 2010), indicating a strong interaction between Ca^{2+} binding and channel opening. A weak interaction between voltage sensor activation and Ca^{2+} binding also exists (Horrigan and Aldrich, 2002; Sweet and Cox, 2008), though the mechanism of this interaction is less clear.

The allosteric coupling between the CTD and the PGD of BK channels is mainly mediated by the C-linker that covalently connects these two domains (**Figure 2C**). A comparison between the CTD crystal structures with and without Ca^{2+}-bound to the Ca^{2+} bowl suggests that the N-terminal lobe of RCK1 domain undergoes most dramatic conformational changes upon Ca^{2+} binding to the Ca^{2+} Bowl compared to other regions in the CTD (Wu et al., 2010; Yuan et al., 2010). The conformational changes are distinct from the conformation changes of the MthK channel gating ring, which reduces its height and expands its diameter upon Ca^{2+} binding (Jiang et al., 2002a,b). In BK channels, the N-terminal lobe of RCK1 resides on the top layer of the gating ring directly facing the membrane and covalently connecting to the PGD through the C-linker. Upon Ca^{2+} binding, this lobe rotates relative to RCK2 domains as a rigid body, resulting in an expansion of the top layer of the gating ring (from a diameter of 81–93 Å measured at Cα atoms of the N-terminal residues of RCK1, K343). Opening like the petals of a flower, this mechanical force will directly pull the C-linker to open the PGD. This mechanical model is consistent with an early functional study by the Magleby group, who discovered that the length of the C-linker is critical to channel activation. Shortening the C-linker enhances channel activity and lengthening the linkers decreases channel activity, both in the presence and absence of intracellular Ca^{2+} (Niu et al., 2004). Therefore, the C-linker might serve as a passive spring to control BK channel gating. Interestingly, a recent functional study demonstrated that BK channel openers, such as Cym04 and NS1619, activate BK channels by functionally interacting with the C-linker, thereby mimicking site-specific shortening of the C-linker (Gessner et al., 2012).

As discussed above, the free energy of Ca^{2+} binding to the Ca^{2+} bowl and the RCK1 site may propagate via different pathways to open the activation gate. As the C-linker provides the only covalent linkage between the CTD and the PGD, it is conceivable that these two separate Ca^{2+}-activation pathways may converge at the C-linker to operate the gate. Nevertheless, the non-covalent domain-domain interactions among the CTD, VSD and the PGD may provide additional pathways to differentially mediate Ca^{2+}

dependent activation originated from the Ca^{2+} bowl and the RCK1 site. It is unclear whether the possible modes of the quaternary assembly of BK channels (**Figure 4C**) have any impact on the coupling of the Ca^{2+} sensors to the activation gate. Further experiments are needed to address this question.

The molecular mechanism of electromechanical coupling between the VSD and the PGD in BK channels is less well understood compared to other Kv channels. In Kv channels, the S4–S5 linker directly contact with the C-terminus of S6 to transduce the energy of the VSD movement to gate opening (Lu et al., 2001). In a recent study, the Arginine mutation of E219 in the lower S4 segment was shown to have an electrostatic interaction with E321 and E324 at the C-terminus of S6 (Zhang et al., 2014), suggesting that BK channels may use the similar mechanism to couple the VSD to its PGD as Kv channels. Nevertheless, this electrostatic interaction is rather long range compared to the short-range hydrophobic interactions observed in Kv channels. Given that the conformation of BK S6 might be different from that of Kv channels, it is therefore very likely other coupling sites and mechanism also exist to couple the VSD to the PGD.

Although the coupling between the VSD and CTD is relatively weak (Horrigan and Aldrich, 2002), the interaction between these two domains does exist and is important in controlling BK channel activation. A well-understood example is the interactions among the residues around the Mg^{2+} binding resides in both the CTD and VSD that can affect VSD activation and the intrinsic open probability of the activation gate (Yang et al., 2006, 2007, 2013). Considering the clusters of the ligand binding sites that are located at the interface between the CTD and VSD (**Figure 3**), it is conceivable that more direct interactions between these two sensory modules may exist and mediate their synergy in activating the PGD.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing, revising, and approval of the manuscript.

ACKNOWLEDGMENTS

This work was supported by NIH Grants R01-HL70393 (to Jianmin Cui) and K99NS086916 (to Huanghe Yang).

REFERENCES

Adams, P. R., Constanti, A., Brown, D. A., and Clark, R. B. (1982). Intracellular Ca^{2+} activates a fast voltage-sensitive K^+ current in vertebrate sympathetic neurones. *Nature* 296, 746–749. doi: 10.1038/296746a0

Adelman, J. P., Shen, K. Z., Kavanaugh, M. P., Warren, R. A., Wu, Y. N., Lagrutta, A., et al. (1992). Calcium-activated potassium channels expressed from cloned complementary DNAs. *Neuron* 9, 209–216. doi: 10.1016/0896-6273(92)90160-F

Aggarwal, S. K., and MacKinnon, R. (1996). Contribution of the S4 segment to gating charge in the Shaker K^+ channel. *Neuron* 16, 1169–1177. doi: 10.1016/S0896-6273(00)80143-9

Albright, R. A., Ibar, J. L., Kim, C. U., Gruner, S. M., and Morais-Cabral, J. H. (2006). The RCK domain of the KtrAB K+ transporter: multiple conformations of an octameric ring. *Cell* 126, 1147–1159. doi: 10.1016/j.cell.2006.08.028

Atkinson, N. S., Robertson, G. A., and Ganetzky, B. (1991). A component of calcium-activated potassium channels encoded by the Drosophila slo locus. *Science* 253, 551–555. doi: 10.1126/science.1857984

Avdonin, V., Tang, X. D., and Hoshi, T. (2003). Stimulatory action of internal protons on Slo1 BK channels. *Biophys. J.* 84, 2969–2980. doi: 10.1016/S0006-3495(03)70023-X

Bao, L., Kaldany, C., Holmstrand, E. C., and Cox, D. H. (2004). Mapping the BKCa channel's "Ca^{2+} bowl": side-chains essential for Ca^{2+} sensing. *J. Gen. Physiol.* 123, 475–489. doi: 10.1085/jgp.200409052

Bao, L., Rapin, A. M., Holmstrand, E. C., and Cox, D. H. (2002). Elimination of the BK(Ca) channel's high-affinity Ca(2+) sensitivity. *J. Gen. Physiol.* 120, 173–189. doi: 10.1085/jgp.20028627

Barrett, J. N., Magleby, K. L., and Pallotta, B. S. (1982). Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol.* 331, 211–230. doi: 10.1113/jphysiol.1982.sp014370

Behrens, R., Nolting, A., Reimann, F., Schwarz, M., Waldschutz, R., and Pongs, O. (2000). hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family [In Process Citation]. *FEBS Lett.* 474, 99–106. doi: 10.1016/S0014-5793(00)01584-2

Bezanilla, F. (2008). Ion channels: from conductance to structure. *Neuron* 60, 456–468. doi: 10.1016/j.neuron.2008.10.035

Bian, S., Favre, I., and Moczydlowski, E. (2001). Ca^{2+}-binding activity of a COOH-terminal fragment of the drosophila BK channel involved in Ca^{2+}-dependent activation. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4776–4781. doi: 10.1073/pnas.081072398

Bolotina, V., Omelyanenko, V., Heyes, B., Ryan, U., and Bregestovski, P. (1989). Variations of membrane cholesterol alter the kinetics of Ca2(+)-dependent K+ channels and membrane fluidity in vascular smooth muscle cells. *Pflugers Arch.* 415, 262–268. doi: 10.1007/BF00370875

Braun, A. P. (2008). BK channels and the expanding role for PIP2-mediated regulation. *Channels (Austin)* 2, 229. doi: 10.4161/chan.2.4.6873

Braun, A., and Sy, L. (2001). Contribution of potential EF hand motifs to the calcium-dependent gating of a mouse brain large conductance, calcium-sensitive K(+) channel. *J. Physiol.* 533, 681–695. doi: 10.1111/j.1469-7793.2001.00681.x

Braun, M., Ramracheya, R., Bengtsson, M., Zhang, Q., Karanauskaite, J., Partridge, C., et al. (2008). Voltage-gated ion channels in human pancreatic beta-cells: electrophysiological characterization and role in insulin secretion. *Diabetes* 57, 1618–1628. doi: 10.2337/db07-0991

Brayden, J. E., and Nelson, M. T. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* 256, 532–535. doi: 10.1126/science.1373909

Bregestovski, P. D., and Bolotina, V. N. (1989). Membrane fluidity and kinetics of Ca2+-dependent potassium channels. *Biomed. Biochim. Acta* 48, S382–S387.

Brelidze, T. I., and Magleby, K. L. (2004). Protons block BK channels by competitive inhibition with K+ and contribute to the limits of unitary currents at high voltages. *J. Gen. Physiol.* 123, 305–319. doi: 10.1085/jgp.200308951

Brelidze, T. I., and Magleby, K. L. (2005). Probing the geometry of the inner vestibule of BK channels with sugars. *J. Gen. Physiol.* 126, 105–121. doi: 10.1085/jgp.200509286

Brelidze, T. I., Niu, X., and Magleby, K. L. (2003). A ring of eight conserved negatively charged amino acids doubles the conductance of BK channels and prevents inward rectification. *Proc. Natl. Acad. Sci. U.S.A.* 100, 9017–9022. doi: 10.1073/pnas.1532257100

Brenner, R., Perez, G. J., Bonev, A. D., Eckman, D. M., Kosek, J. C., Wiler, S. W., et al. (2000). Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature* 407, 870–876. doi: 10.1038/35038011

Bringmann, A., Faude, F., and Reichenbach, A. (1997). Mammalian retinal glial (Muller) cells express large-conductance Ca^{2+}- activated K^+ channels that are modulated by Mg^{2+} and pH and activated by protein kinase A. *Glia* 19, 311–323.

Bruening-Wright, A., Lee, W. S., Adelman, J. P., and Maylie, J. (2007). Evidence for a deep pore activation gate in small conductance Ca2+-activated K+ channels. *J. Gen. Physiol.* 130, 601–610. doi: 10.1085/jgp.200709828

Budelli, G., Geng, Y., Butler, A., Magleby, K. L., and Salkoff, L. (2013). Properties of Slo1 K+ channels with and without the gating ring. *Proc. Natl. Acad. Sci. U.S.A.* 110, 16657–16662. doi: 10.1073/pnas.1313433110

Bukiya, A. N., Kuntamallappanavar, G., Edwards, J., Singh, A. K., Shivakumar, B., and Dopico, A. M. (2014). An alcohol-sensing site in the calcium- and voltage-gated, large conductance potassium (BK) channel. *Proc. Natl. Acad. Sci. U.S.A.* 111, 9313–9318. doi: 10.1073/pnas.1317363111

Bukiya, A. N., Singh, A. K., Parrill, A. L., and Dopico, A. M. (2011a). The steroid interaction site in transmembrane domain 2 of the large conductance, voltage-

and calcium-gated potassium (BK) channel accessory beta1 subunit. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20207–20212. doi: 10.1073/pnas.1112901108

Bukiya, A. N., Vaithianathan, T., Kuntamallappanavar, G., Asuncion-Chin, M., and Dopico, A. M. (2011b). Smooth muscle cholesterol enables BK beta1 subunit-mediated channel inhibition and subsequent vasoconstriction evoked by alcohol. *Arterioscler. Thromb. Vasc. Biol.* 31, 2410–2423. doi: 10.1161/ATVBAHA.111.233965

Cao, Y., Pan, Y., Huang, H., Jin, X., Levin, E. J., Kloss, B., et al. (2013). Gating of the TrkH ion channel by its associated RCK protein TrkA. *Nature* 496, 317–322. doi: 10.1038/nature12056

Carvacho, I., Gonzalez, W., Torres, Y. P., Brauchi, S., Alvarez, O., Gonzalez-Nilo, F. D., et al. (2008). Intrinsic electrostatic potential in the BK channel pore: role in determining single channel conductance and block. *J. Gen. Physiol.* 131, 147–161. doi: 10.1085/jgp.200709862

Chen, R. S., Geng, Y., and Magleby, K. L. (2011). Mg(2+) binding to open and closed states can activate BK channels provided that the voltage sensors are elevated. *J. Gen. Physiol.* 138, 593–607. doi: 10.1085/jgp.201110707

Chen, X., Yan, J., and Aldrich, R. W. (2014). BK channel opening involves side-chain reorientation of multiple deep-pore residues. *Proc. Natl. Acad. Sci. U.S.A.* 111, E79–E88. doi: 10.1073/pnas.1321697111

Cox, D. H. (2006). "BKCa-channel structure and function," in *Biological Membrane Ion Channels*, eds S. H. Chung, O. S. Anderson, and V. V. Krishnamurthy, (Berlin: Springer) 171–218.

Cox, D. H., Cui, J., and Aldrich, R. W. (1997). Allosteric gating of a large conductance Ca-activated K^+ channel. *J. Gen. Physiol.* 110, 257–281. doi: 10.1085/jgp.110.3.257

Cui, J., and Aldrich, R. W. (2000). Allosteric linkage between voltage and Ca^{2+}-dependent activation of BK-type mslo1 K^+ channels. *Biochemistry* 39, 15612–15619. doi: 10.1021/bi001509+

Cui, J., Cox, D. H., and Aldrich, R. W. (1997). Intrinsic voltage dependence and Ca^{2+} regulation of mslo large conductance Ca-activated K^+ channels. *J. Gen. Physiol.* 109, 647–673. doi: 10.1085/jgp.109.5.647

Cui, J., Yang, H., and Lee, U. (2009). Molecular mechanisms of BK channel activation. *Cell. Mol. Life Sci.* 66, 852–875. doi: 10.1007/s00018-008-8609-x

Davies, A. G., Pierce-Shimomura, J. T., Kim, H., VanHoven, M. K., Thiele, T. R., Bonci, A., et al. (2003). A central role of the BK potassium channel in behavioral responses to ethanol in C. elegans. *Cell* 115, 655–666. doi: 10.1016/S0092-8674(03)00979-6

Davis, S. J., Scott, L. L., Hu, K., and Pierce-Shimomura, J. T. (2014). Conserved single residue in the BK potassium channel required for activation by alcohol and intoxication in C. elegans. *J. Neurosci.* 34, 9562–9573. doi: 10.1523/JNEUROSCI.0838-14.2014

Deng, P. Y., Rotman, Z., Blundon, J. A., Cho, Y., Cui, J., Cavalli, V., et al. (2013). FMRP regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels. *Neuron* 77, 696–711. doi: 10.1016/j.neuron.2012.12.018

Dopico, A. M., Anantharam, V., and Treistman, S. N. (1998). Ethanol increases the activity of Ca(++)-dependent K+ (mslo) channels: functional interaction with cytosolic Ca++. *J. Pharmacol. Exp. Ther.* 284, 258–268.

Dopico, A. M., Bukiya, A. N., and Singh, A. K. (2012). Large conductance, calcium- and voltage-gated potassium (BK) channels: regulation by cholesterol. *Pharmacol. Ther.* 135, 133–150. doi: 10.1016/j.pharmthera.2012.05.002

Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., et al. (1998). The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* 280, 69–77. doi: 10.1126/science.280.5360.69

Du, W., Bautista, J. F., Yang, H., Diez-Sampedro, A., You, S. A., Wang, L., et al. (2005). Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat. Genet.* 37, 733–738. doi: 10.1038/ng1585

Ferguson, W. B. (1991). Competitive Mg^{2+} block of a large-conductance, Ca^{2+}-activated K^+ channel in rat skeletal muscle. Ca^{2+}, Sr^{2+}, and Ni^{2+} also block. *J. Gen. Physiol.* 98, 163–181. doi: 10.1085/jgp.98.1.163

Fettiplace, R., and Fuchs, P. A. (1999). Mechanisms of hair cell tuning. *Annu. Rev. Physiol.* 61, 809–834. doi: 10.1146/annurev.physiol.61.1.809

Flynn, G. E., and Zagotta, W. N. (2001). Conformational changes in S6 coupled to the opening of cyclic nucleotide-gated channels. *Neuron* 30, 689–698. doi: 10.1016/S0896-6273(01)00324-5

Fodor, A. A., and Aldrich, R. W. (2006). Statistical limits to the identification of ion channel domains by sequence similarity. *J. Gen. Physiol.* 127, 755–766. doi: 10.1085/jgp.200509419

Fury, M., Marx, S. O., and Marks, A. R. (2002). Molecular BKology: the study of splicing and dicing. *Sci. STKE.* 2002:PE12. doi: 10.1126/stke.2002.123.pe12

Gandhi, C. S., and Isacoff, E. Y. (2002). Molecular models of voltage sensing. *J. Gen. Physiol.* 120, 455–463. doi: 10.1085/jgp.20028678

Geng, Y., and Magleby, K. L. (2014). Single-channel kinetics of BK (Slo1) channels. *Front. Physiol.* 5:532. doi: 10.3389/fphys.2014.00532

Geng, Y., Niu, X., and Magleby, K. L. (2011). Low resistance, large dimension entrance to the inner cavity of BK channels determined by changing side-chain volume. *J. Gen. Physiol.* 137, 533–548. doi: 10.1085/jgp.201110616

Gessner, G., Cui, Y. M., Otani, Y., Ohwada, T., Soom, M., Hoshi, T., et al. (2012). Molecular mechanism of pharmacological activation of BK channels. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3552–3557. doi: 10.1073/pnas.1114321109

Ghatta, S., Nimmagadda, D., Xu, X., and O'Rourke, S. T. (2006). Large-conductance, calcium-activated potassium channels: structural and functional implications. *Pharmacol. Ther.* 110, 103–116. doi: 10.1016/j.pharmthera.2005.10.007

Golowasch, J., Kirkwood, A., and Miller, C. (1986). Allosteric effects of Mg^{2+} on the gating of Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *J. Exp. Biol.* 124, 5–13.

Gribkoff, V. K., Starrett, J. E. Jr., Dworetzky, S. I., Hewawasam, P., Boissard, C. G., Cook, D. A., et al. (2001). Targeting acute ischemic stroke with a calcium-sensitive opener of maxi-K potassium channels. *Nat. Med.* 7, 471–477. doi: 10.1038/86546

Hille, B., Armstrong, C. M., and MacKinnon, R. (1999). Ion channels: from idea to reality. *Nat. Med.* 5, 1105–1109. doi: 10.1038/13415

Horrigan, F. T. (2012). Perspectives on: conformational coupling in ion channels: conformational coupling in BK potassium channels. *J. Gen. Physiol.* 140, 625–634. doi: 10.1085/jgp.201210849

Horrigan, F. T., and Aldrich, R. W. (1999). Allosteric voltage gating of potassium channels II. Mslo channel gating charge movement in the absence of Ca^{2+}. *J. Gen. Physiol.* 114, 305–336. doi: 10.1085/jgp.114.2.305

Horrigan, F. T., and Aldrich, R. W. (2002). Coupling between voltage sensor activation, Ca^{2+} binding and channel opening in large conductance (BK) potassium channels. *J. Gen. Physiol.* 120, 267–305. doi: 10.1085/jgp.20028605

Horrigan, F. T., Cui, J., and Aldrich, R. W. (1999). Allosteric voltage gating of potassium channels I. Mslo ionic currents in the absence of Ca^{2+}. *J. Gen. Physiol.* 114, 277–304. doi: 10.1085/jgp.114.2.277

Horrigan, F. T., Heinemann, S. H., and Hoshi, T. (2005). Heme regulates allosteric activation of the Slo1 BK Channel. *J. Gen. Physiol.* 126, 7–21. doi: 10.1085/jgp.200509262

Horrigan, F. T., and Ma, Z. (2008). Mg^{2+} enhances voltage sensor/gate coupling in BK channels. *J. Gen. Physiol.* 131, 13–32. doi: 10.1085/jgp.200709877

Hoshi, T., Pantazis, A., and Olcese, R. (2013a). Transduction of voltage and Ca2+ signals by Slo1 BK channels. *Physiology (Bethesda)* 28, 172–189. doi: 10.1152/physiol.00055.2012

Hoshi, T., Tian, Y., Xu, R., Heinemann, S. H., and Hou, S. (2013b). Mechanism of the modulation of BK potassium channel complexes with different auxiliary subunit compositions by the omega-3 fatty acid DHA. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4822–4827. doi: 10.1073/pnas.1222003110

Hoshi, T., Wissuwa, B., Tian, Y., Tajima, N., Xu, R., Bauer, M., et al. (2013c). Omega-3 fatty acids lower blood pressure by directly activating large-conductance Ca(2)(+)-dependent K(+) channels. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4816–4821. doi: 10.1073/pnas.1221997110

Hoshi, T., Xu, R., Hou, S., Heinemann, S. H., and Tian, Y. (2013d). A point mutation in the human Slo1 channel that impairs its sensitivity to omega-3 docosahexaenoic acid. *J. Gen. Physiol.* 142, 507–522. doi: 10.1085/jgp.201311061

Hou, S., Horrigan, F. T., Xu, R., Heinemann, S. H., and Hoshi, T. (2009). Comparative effects of H+ and Ca2+ on large-conductance Ca2+- and voltage-gated Slo1 K+ channels. *Channels (Austin)* 3, 249–258. doi: 10.4161/chan.3.4.9253

Hou, S., Xu, R., Heinemann, S. H., and Hoshi, T. (2008a). The RCK1 high-affinity Ca^{2+} sensor confers carbon monoxide sensitivity to Slo1 BK channels. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4039–4043. doi: 10.1073/pnas.0800304105

Hou, S., Xu, R., Heinemann, S. H., and Hoshi, T. (2008b). Reciprocal regulation of the Ca^{2+} and H^+ sensitivity in the SLO1 BK channel conferred by the RCK1 domain. *Nat. Struct. Mol. Biol.* 15, 403–410. doi: 10.1038/nsmb.1398

Hu, L., Shi, J., Ma, Z., Krishnamoorthy, G., Sieling, F., Zhang, G., et al. (2003). Participation of the S4 voltage sensor in the Mg^{2+}-dependent activation of large

conductance (BK) K^+ channels. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10488–10493. doi: 10.1073/pnas.1834300100

Hudspeth, A. J., and Lewis, R. S. (1988a). Kinetic analysis of voltage- and ion-dependent conductances in saccular hair cells of the bull-frog, Rana catesbeiana. *J. Physiol.* 400, 237–274. doi: 10.1113/jphysiol.1988.sp017119

Hudspeth, A. J., and Lewis, R. S. (1988b). A model for electrical resonance and frequency tuning in saccular hair cells of the bull-frog, Rana catesbeiana. *J. Physiol.* 400, 275–297. doi: 10.1113/jphysiol.1988.sp017120

Jakab, M., Weiger, T. M., and Hermann, A. (1997). Ethanol activates maxi Ca2+-activated K+ channels of clonal pituitary (GH3) cells. *J. Membr. Biol.* 157, 237–245. doi: 10.1007/PL00005895

Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T., and MacKinnon, R. (2002a). Crystal structure and mechanism of a calcium-gated potassium channel. *Nature* 417, 515–522. doi: 10.1038/417515a

Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T., and MacKinnon, R. (2002b). The open pore conformation of potassium channels. *Nature* 417, 523–526. doi: 10.1038/417523a

Jiang, Y., Pico, A., Cadene, M., Chait, B. T., and MacKinnon, R. (2001). Structure of the RCK domain from the *E. coli* K^+ channel and demonstration of its presence in the human BK channel. *Neuron* 29, 593–601. doi: 10.1016/S0896-6273(01)00236-7

Jiao, H., Arner, P., Hoffstedt, J., Brodin, D., Dubern, B., Czernichow, S., et al. (2011). Genome wide association study identifies KCNMA1 contributing to human obesity. *BMC Med. Genomics* 4:51. doi: 10.1186/1755-8794-4-51

Kong, C., Zeng, W., Ye, S., Chen, L., Sauer, D. B., Lam, Y., et al. (2012). Distinct gating mechanisms revealed by the structures of a multi-ligand gated K(+) channel. *Elife* 1:e00184. doi: 10.7554/eLife.00184

Koval, O. M., Fan, Y., and Rothberg, B. S. (2007). A role for the S0 transmembrane segment in voltage-dependent gating of BK channels. *J. Gen. Physiol.* 129, 209–220. doi: 10.1085/jgp.200609662

Krishnamoorthy, G., Shi, J., Sept, D., and Cui, J. (2005). The NH2 terminus of RCK1 domain regulates Ca^{2+}-dependent BK(Ca) channel gating. *J. Gen. Physiol.* 126, 227–241. doi: 10.1085/jgp.200509321

Kumar, P. (2007). Sensing hypoxia in the carotid body: from stimulus to response. *Essays Biochem.* 43, 43–60. doi: 10.1042/BSE0430043

Kuo, M. M., Haynes, W. J., Loukin, S. H., Kung, C., and Saimi, Y. (2005). Prokaryotic K(+) channels: from crystal structures to diversity. *FEMS Microbiol. Rev.* 29, 961–985. doi: 10.1016/j.femsre.2005.03.003

Lancaster, B., and Nicoll, R. A. (1987). Properties of two calcium-activated hyperpolarizations in rat hippocampal neurones. *J. Physiol. (Lond.)* 389, 187–203. doi: 10.1113/jphysiol.1987.sp016653

Latorre, R., and Brauchi, S. (2006). Large conductance Ca^{2+}-activated K^+ (BK) channel: activation by Ca^{2+} and voltage. *Biol. Res.* 39, 385–401. doi: 10.4067/S0716-97602006000300003

Latorre, R., and Contreras, G. (2013). Keeping you healthy: BK channel activation by omega-3 fatty acids. *J. Gen. Physiol.* 142, 487–491. doi: 10.1085/jgp.201311100

Latorre, R., Morera, F. J., and Zaelzer, C. (2010). Allosteric interactions and the modular nature of the voltage- and Ca^{2+}-activated (BK) channel. *J. Physiol.* 588, 3141–3148. doi: 10.1113/jphysiol.2010.191999

Latorre, R., Vergara, C., and Hidalgo, C. (1982). Reconstitution in planar lipid bilayers of a Ca^{2+}-dependent K^+ channel from transverse tubule membranes isolated from rabbit skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.* 79, 805–809. doi: 10.1073/pnas.79.3.805

Laumonnier, F., Roger, S., Guerin, P., Molinari, F., M'Rad, R., Cahard, D., et al. (2006). Association of a functional deficit of the BKCa channel, a synaptic regulator of neuronal excitability, with autism and mental retardation. *Am. J. Psychiatry.* 163, 1622–1629. doi: 10.1176/appi.ajp.163.9.1622

Lee, U. S., and Cui, J. (2010). BK channel activation: structural and functional insights. *Trends Neurosci.* 33, 415–423. doi: 10.1016/j.tins.2010.06.004

Li, B., Jie, W., Huang, L., Wei, P., Li, S., Luo, Z., et al. (2014a). Nuclear BK channels regulate gene expression via the control of nuclear calcium signaling. *Nat. Neurosci.* 17, 1055–1063. doi: 10.1038/nn.3744

Li, M., Tanaka, Y., Alioua, A., Wu, Y., Lu, R., Kundu, P., et al. (2010). Thromboxane A2 receptor and MaxiK-channel intimate interaction supports channel trans-inhibition independent of G-protein activation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 19096–19101. doi: 10.1073/pnas.1002685107

Li, Q., Wanderling, S., Paduch, M., Medovoy, D., Singharoy, A., McGreevy, R., et al. (2014b). Structural mechanism of voltage-dependent gating in an isolated voltage-sensing domain. *Nat. Struct. Mol. Biol.* 21, 244–252. doi: 10.1038/nsmb.2768

Li, W., and Aldrich, R. W. (2004). Unique inner pore properties of BK channels revealed by quaternary ammonium block. *J. Gen. Physiol.* 124, 43–57. doi: 10.1085/jgp.200409067

Li, W., and Aldrich, R. W. (2006). State-dependent Block of BK channels by synthesized shaker ball peptides. *J. Gen. Physiol.* 128, 423–441. doi: 10.1085/jgp.200609521

Liu, G., Zakharov, S. I., Yang, L., Deng, S. X., Landry, D. W., Karlin, A., et al. (2008a). Position and role of the BK channel alpha subunit S0 helix inferred from disulfide crosslinking. *J. Gen. Physiol.* 131, 537–548. doi: 10.1085/jgp.200809968

Liu, G., Zakharov, S. I., Yang, L., Wu, R. S., Deng, S. X., Landry, D. W., et al. (2008b). Locations of the beta1 transmembrane helices in the BK potassium channel. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10727–10732. doi: 10.1073/pnas.0805212105

Liu, J., Vaithianathan, T., Manivannan, K., Parrill, A., and Dopico, A. M. (2008c). Ethanol modulates BKCa channels by acting as an adjuvant of calcium. *Mol. Pharmacol.* 74, 628–640. doi: 10.1124/mol.108.048694

Long, S. B., Campbell, E. B., and Mackinnon, R. (2005a). Crystal structure of a mammalian voltage-dependent Shaker family K^+ channel. *Science* 309, 897–903. doi: 10.1126/science.1116269

Long, S. B., Campbell, E. B., and Mackinnon, R. (2005b). Voltage sensor of Kv1.2: structural basis of electromechanical coupling. *Science* 309, 903–908. doi: 10.1126/science.1116270

Long, S. B., Tao, X., Campbell, E. B., and MacKinnon, R. (2007). Atomic structure of a voltage-dependent K+ channel in a lipid membrane-like environment. *Nature* 450, 376–382. doi: 10.1038/nature06265

Loukin, S. H., Kuo, M. M., Zhou, X. L., Haynes, W. J., Kung, C., and Saimi, Y. (2005). Microbial K+ channels. *J. Gen. Physiol.* 125, 521–527. doi: 10.1085/jgp.200509261

Lu, Z., Klem, A. M., and Ramu, Y. (2001). Ion conduction pore is conserved among potassium channels. *Nature* 413, 809–813. doi: 10.1038/35101535

Ma, Z., Lou, X. J., and Horrigan, F. T. (2006). Role of charged residues in the S1-S4 Voltage sensor of BK channels. *J. Gen. Physiol.* 127, 309–328. doi: 10.1085/jgp.200509421

Magidovich, E., and Yifrach, O. (2004). Conserved gating hinge in ligand- and voltage-dependent K^+ channels. *Biochemistry* 43, 13242–13247. doi: 10.1021/bi048377v

Magleby, K. L. (2003). Gating mechanism of BK (Slo1) channels: so near, yet so far. *J. Gen. Physiol.* 121, 81–96. doi: 10.1085/jgp.20028721

Marty, A. (1981). Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature* 291, 497–500. doi: 10.1038/291497a0

McLarnon, J. G., and Sawyer, D. (1993). Effects of divalent cations on the activation of a calcium-dependent potassium channel in hippocampal neurons. *Pflugers Arch.* 424, 1–8. doi: 10.1007/BF00375095

McManus, O. B., and Magleby, K. L. (1991). Accounting for the Ca^{2+}-dependent kinetics of single large-conductance Ca^{2+}-activated K^+ channels in rat skeletal muscle. *J. Physiol.* 443, 739–777. doi: 10.1113/jphysiol.1991.sp018861

Meera, P., Wallner, M., Song, M., and Toro, L. (1997). Large conductance voltage- and calcium-dependent K^+ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0-S6), an extracellular N terminus, and an intracellular (S9-S10) C terminus. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14066–14071. doi: 10.1073/pnas.94.25.14066

Meredith, A. L., Wiler, S. W., Miller, B. H., Takahashi, J. S., Fodor, A. A., Ruby, N. F., et al. (2006). BK calcium-activated potassium channels regulate circadian behavioral rhythms and pacemaker output. *Nat. Neurosci.* 9, 1041–1049. doi: 10.1038/nn1740

Methfessel, C., and Boheim, G. (1982). The gating of single calcium-dependent potassium channels is described by an activation/blockade mechanism. *Biophys. Struct. Mech.* 9, 35–60. doi: 10.1007/BF00536014

Moczydlowski, E., and Latorre, R. (1983). Gating kinetics of Ca^{2+}-activated K^+ channels from rat muscle incorporated into planar lipid bilayers. Evidence for two voltage-dependent Ca^{2+} binding reactions. *J. Gen. Physiol.* 82, 511–542. doi: 10.1085/jgp.82.4.511

Morales, E., Cole, W. C., Remillard, C. V., and Leblane, N. (1996). Block of large conductance Ca^{2+}-activated K^+ channels in rabbit vascular myocytes by internal Mg^{2+} and Na^+. *J. Physiol.* 495, 701–716. doi: 10.1113/jphysiol.1996.sp021627

Murata, Y., Iwasaki, H., Sasaki, M., Inaba, K., and Okamura, Y. (2005). Phosphoinositide phosphatase activity coupled to an intrinsic voltage sensor. *Nature* 435, 1239–1243. doi: 10.1038/nature03650

Navaratnam, D. S., Bell, T. J., Tu, T. D., Cohen, E. L., and Oberholtzer, J. C. (1997). Differential distribution of Ca^{2+}-activated K^+ channel splice variants among hair cells along the tonotopic axis of the chick cochlea. *Neuron* 19, 1077–1085. doi: 10.1016/S0896-6273(00)80398-0

Nelson, M. T., Cheng, H., Rubart, M., Santana, L. F., Bonev, A. D., Knot, H. J., et al. (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science* 270, 633–637. doi: 10.1126/science.270.5236.633

N'Gouemo, P. (2011). Targeting BK (big potassium) channels in epilepsy. *Expert. Opin. Ther. Targets* 15, 1283–1295. doi: 10.1517/14728222.2011.620607

Nimigean, C. M., Chappie, J. S., and Miller, C. (2003). Electrostatic tuning of ion conductance in potassium channels. *Biochemistry* 42, 9263–9268. doi: 10.1021/bi0348720

Niu, X., Qian, X., and Magleby, K. L. (2004). Linker-gating ring complex as passive spring and Ca^{2+}-dependent machine for a voltage- and Ca^{2+}-activated potassium channel. *Neuron* 42, 745–756. doi: 10.1016/j.neuron.2004.05.001

Oberhauser, A., Alvarez, O., and Latorre, R. (1988). Activation by divalent cations of a Ca^{2+}-activated K^+ channel from skeletal muscle membrane. *J. Gen. Physiol.* 92, 67–86. doi: 10.1085/jgp.92.1.67

Orio, P., Rojas, P., Ferreira, G., and Latorre, R. (2002). New disguises for an old channel: MaxiK channel beta-subunits. *News Physiol. Sci.* 17, 156–161. doi: 10.1152/nips.01387.2002

Pallotta, B. S., Magleby, K. L., and Barrett, J. N. (1981). Single channel recordings of Ca^{2+}-activated K^+ currents in rat muscle cell culture. *Nature* 293, 471–474. doi: 10.1038/293471a0

Pantazis, A., Gudzenko, V., Savalli, N., Sigg, D., and Olcese, R. (2010a). Operation of the voltage sensor of a human voltage- and Ca2+-activated K+ channel. *Proc. Natl. Acad. Sci. U.S.A.* 107, 4459–4464. doi: 10.1073/pnas.0911959107

Pantazis, A., Kohanteb, A. P., and Olcese, R. (2010b). Relative motion of transmembrane segments S0 and S4 during voltage sensor activation in the human BKCa channel. *J. Gen. Physiol.* 136, 645–657. doi: 10.1085/jgp.201010503

Pantazis, A., and Olcese, R. (2012). Relative transmembrane segment rearrangements during BK channel activation resolved by structurally assigned fluorophore-quencher pairing. *J. Gen. Physiol.* 140, 207–218. doi: 10.1085/jgp.201210807

Payandeh, J., Scheuer, T., Zheng, N., and Catterall, W. A. (2011). The crystal structure of a voltage-gated sodium channel. *Nature* 475, 353–358. doi: 10.1038/nature10238

Perez, G. J., Bonev, A. D., Patlak, J. B., and Nelson, M. T. (1999). Functional coupling of ryanodine receptors to K_{Ca} channels in smooth muscle cells from rat cerebral arteries. *J. Gen. Physiol.* 113, 229–238. doi: 10.1085/jgp.113.2.229

Petersen, O. H., and Maruyama, Y. (1984). Calcium-activated potassium channels and their role in secretion. *Nature* 307, 693–696. doi: 10.1038/307693a0

Piskorowski, R. A., and Aldrich, R. W. (2006). Relationship between pore occupancy and gating in BK potassium channels. *J. Gen. Physiol.* 127, 557–576. doi: 10.1085/jgp.200509482

Pluger, S., Faulhaber, J., Furstenau, M., Lohn, M., Waldschutz, R., Gollasch, M., et al. (2000). Mice with disrupted BK channel beta1 subunit gene feature abnormal Ca^{2+} spark/STOC coupling and elevated blood pressure. *Circ. Res.* 87, E53–E60. doi: 10.1161/01.RES.87.11.e53

Qian, X., Niu, X., and Magleby, K. L. (2006). Intra- and intersubunit cooperativity in activation of BK channels by Ca2+. *J. Gen. Physiol.* 128, 389–404. doi: 10.1085/jgp.200609486

Ramsey, I. S., Moran, M. M., Chong, J. A., and Clapham, D. E. (2006). A voltage-gated proton-selective channel lacking the pore domain. *Nature* 440, 1213–1216. doi: 10.1038/nature04700

Roberts, W. M., Jacobs, R. A., and Hudspeth, A. J. (1990). Colocalization of ion channels involved in frequency selectivity and synaptic transmission at presynaptic active zones of hair cells. *J. Neurosci.* 10, 3664–3684.

Robitaille, R., and Charlton, M. P. (1992). Presynaptic calcium signals and transmitter release are modulated by calcium-activated potassium channels. *J. Neurosci.* 12, 297–305.

Robitaille, R., Garcia, M. L., Kaczorowski, G. J., and Charlton, M. P. (1993). Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron* 11, 645–655. doi: 10.1016/0896-6273(93)90076-4

Rosenblatt, K. P., Sun, Z. P., Heller, S., and Hudspeth, A. J. (1997). Distribution of Ca^{2+}-activated K^+ channel isoforms along the tonotopic gradient of the chicken's cochlea. *Neuron* 19, 1061–1075. doi: 10.1016/S0896-6273(00)80397-9

Rothberg, B. S. (2012). The BK channel: a vital link between cellular calcium and electrical signaling. *Protein Cell.* 3, 883–892. doi: 10.1007/s13238-012-2076-8

Rothberg, B. S., and Magleby, K. L. (1999). Gating kinetics of single large-conductance Ca^{2+}-activated K^+ channels in high Ca^{2+} suggest a two-tiered allosteric gating mechanism [published erratum appears in J Gen Physiol 1999 Aug;114(2):337] [see comments]. *J. Gen. Physiol.* 114, 93–124. doi: 10.1085/jgp.114.1.93

Rothberg, B. S., and Magleby, K. L. (2000). Voltage and Ca^{2+} activation of single large-conductance Ca^{2+}- activated K^+ channels described by a two-tiered allosteric gating mechanism [In Process Citation]. *J. Gen. Physiol.* 116, 75–100. doi: 10.1085/jgp.116.1.75

Salkoff, L., Butler, A., Ferreira, G., Santi, C., and Wei, A. (2006). High-conductance potassium channels of the SLO family. *Nat. Rev. Neurosci.* 7, 921–931. doi: 10.1038/nrn1992

Sasaki, M., Takagi, M., and Okamura, Y. (2006). A voltage sensor-domain protein is a voltage-gated proton channel. *Science* 312, 589–592. doi: 10.1126/science.1122352

Sausbier, M., Hu, H., Arntz, C., Feil, S., Kamm, S., Adelsberger, H., et al. (2004). Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+-activated K+ channel deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9474–9478. doi: 10.1073/pnas.0401702101

Schreiber, M., and Salkoff, L. (1997). A novel calcium-sensing domain in the BK channel. *Biophys. J.* 73, 1355–1363. doi: 10.1016/S0006-3495(97)78168-2

Schreiber, M., Wei, A., Yuan, A., Gaut, J., Saito, M., and Salkoff, L. (1998). Slo3, a novel pH-sensitive K^+ channel from mammalian spermatocytes. *J. Biol. Chem.* 273, 3509–3516. doi: 10.1074/jbc.273.6.3509

Schubert, R., Krien, U., and Gagov, H. (2001). Protons inhibit the BK(Ca) channel of rat small artery smooth muscle cells. *J. Vasc. Res.* 38, 30–38. doi: 10.1159/000051027

Schubert, R., and Nelson, M. T. (2001). Protein kinases: tuners of the BKCa channel in smooth muscle. *Trends Pharmacol. Sci.* 22, 505–512. doi: 10.1016/S0165-6147(00)01775-2

Seibold, M. A., Wang, B., Eng, C., Kumar, G., Beckman, K. B., Sen, S., et al. (2008). An african-specific functional polymorphism in KCNMB1 shows sex-specific association with asthma severity. *Hum. Mol. Genet.* 17, 2681–2690. doi: 10.1093/hmg/ddn168

Seoh, S. A., Sigg, D., Papazian, D. M., and Bezanilla, F. (1996). Voltage-sensing residues in the S2 and S4 segments of the Shaker K^+ channel. *Neuron* 16, 1159–1167. doi: 10.1016/S0896-6273(00)80142-7

Shelley, C., Niu, X., Geng, Y., and Magleby, K. L. (2010). Coupling and cooperativity in voltage activation of a limited-state BK channel gating in saturating Ca2+. *J. Gen. Physiol.* 135, 461–480. doi: 10.1085/jgp.200910331

Shi, J., and Cui, J. (2001). Intracellular Mg^{2+} enhances the function of BK-type Ca^{2+}-activated K^+ channels. *J. Gen. Physiol.* 118, 589–606. doi: 10.1085/jgp.118.5.589

Shi, J., Krishnamoorthy, G., Yang, Y., Hu, L., Chaturvedi, N., Harilal, D., et al. (2002). Mechanism of magnesium activation of calcium-activated potassium channels. *Nature* 418, 876–880. doi: 10.1038/nature00941

Silverman, W. R., Roux, B., and Papazian, D. M. (2003). Structural basis of two-stage voltage-dependent activation in K^+ channels. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2935–2940. doi: 10.1073/pnas.0636603100

Singh, A. K., McMillan, J., Bukiya, A. N., Burton, B., Parrill, A. L., and Dopico, A. M. (2012a). Multiple cholesterol recognition/interaction amino acid consensus (CRAC) motifs in cytosolic C tail of Slo1 subunit determine cholesterol sensitivity of Ca2+- and voltage-gated K+ (BK) channels. *J. Biol. Chem.* 287, 20509–20521. doi: 10.1074/jbc.M112.356261

Singh, H., Stefani, E., and Toro, L. (2012b). Intracellular BK(Ca) (iBK(Ca)) channels. *J. Physiol.* 590, 5937–5947. doi: 10.1113/jphysiol.2011.215533

Sontheimer, H. (2008). An unexpected role for ion channels in brain tumor metastasis. *Exp. Biol. Med. (Maywood).* 233, 779–791. doi: 10.3181/0711-MR-308

Squire, L. G., and Petersen, O. H. (1987). Modulation of Ca^{2+}- and voltage-activated K^+ channels by internal Mg^{2+} in salivary acinar cells. *Biochim. Biophys. Acta* 899, 171–175. doi: 10.1016/0005-2736(87)90397-X

Stefani, E., Ottolia, M., Noceti, F., Olcese, R., Wallner, M., Latorre, R., et al. (1997). Voltage-controlled gating in a large conductance Ca^{2+}-sensitive K^+ channel (hslo). *Proc. Natl. Acad. Sci. U.S.A.* 94, 5427–5431. doi: 10.1073/pnas.94.10.5427

Storm, J. F. (1987). Action potential repolarization and a fast after-hyperpolarization in rat hippocampal pyramidal cells. *J. Physiol. (Lond.)* 385, 733–759. doi: 10.1113/jphysiol.1987.sp016517

Sweet, T.-B., and Cox, D. H. (2008). Measurements of the BK_{Ca} channel's high-affinity Ca^{2+} binding constants: effects of membrane voltage. *J. Gen. Physiol.* 132, 491–505. doi: 10.1085/jgp.200810094

Takeshita, K., Sakata, S., Yamashita, E., Fujiwara, Y., Kawanabe, A., Kurokawa, T., et al. (2014). X-ray crystal structure of voltage-gated proton channel. *Nat. Struct. Mol. Biol.* 21, 352–357. doi: 10.1038/nsmb.2783

Tanaka, Y., Aida, M., Tanaka, H., Shigenobu, K., and Toro, L. (1998). Involvement of maxi-K_{Ca} channel activation in atrial natriuretic peptide-induced vasorelaxation. *Naunyn. Schmiedebergs Arch. Pharmacol.* 357, 705–708. doi: 10.1007/PL00005228

Tang, Q. Y., Zhang, Z., Meng, X. Y., Cui, M., and Logothetis, D. E. (2014). Structural determinants of phosphatidylinositol 4,5-bisphosphate (PIP2) regulation of BK channel activity through the RCK1 Ca2+ coordination site. *J. Biol. Chem.* 289, 18860–18872. doi: 10.1074/jbc.M113.538033

Tang, X. D., Xu, R., Reynolds, M. F., Garcia, M. L., Heinemann, S. H., and Hoshi, T. (2003). Haem can bind to and inhibit mammalian calcium-dependent Slo1 BK channels. *Nature* 425, 531–535. doi: 10.1038/nature02003

Tano, J. Y., and Gollasch, M. (2014). Hypoxia and ischemia-reperfusion: a BiK contribution? *Am. J. Physiol. Heart Circ. Physiol.* 307, H811–H817. doi: 10.1152/ajpheart.00319.2014

Thompson, O., Edgley, M., Strasbourger, P., Flibotte, S., Ewing, B., Adair, R., et al. (2013). The million mutation project: a new approach to genetics in Caenorhabditis elegans. *Genome Res.* 23, 1749–1762. doi: 10.1101/gr.157651.113

Tiwari-Woodruff, S. K., Schulteis, C. T., Mock, A. F., and Papazian, D. M. (1997). Electrostatic interactions between transmembrane segments mediate folding of Shaker K^+ channel subunits. *Biophys. J.* 72, 1489–1500. doi: 10.1016/S0006-3495(97)78797-6

Toro, L., Wallner, M., Meera, P., and Tanaka, Y. (1998). Maxi-K_{Ca}, a unique member of the voltage-gated K channel superfamily. *News. Physiol. Sci.* 13, 112–117.

Tseng-Crank, J., Foster, C. D., Krause, J. D., Mertz, R., Godinot, N., DiChiara, T. J., et al. (1994). Cloning, expression, and distribution of functionally distinct Ca^{2+}-activated K^+ channel isoforms from human brain. *Neuron* 13, 1315–1330. doi: 10.1016/0896-6273(94)90418-9

Tseng-Crank, J., Godinot, N., Johansen, T. E., Ahring, P. K., Strobaek, D., Mertz, R., et al. (1996). Cloning, expression, and distribution of a Ca^{2+}-activated K^+ channel beta-subunit from human brain. *Proc. Natl. Acad. Sci. U.S.A.* 93, 9200–9205. doi: 10.1073/pnas.93.17.9200

Vaithianathan, T., Bukiya, A., Liu, J., Liu, P., Asuncion-Chin, M., Fan, Z., et al. (2008). Direct regulation of BK channels by phosphatidylinositol 4,5-bisphosphate as a novel signaling pathway. *J. Gen. Physiol.* 132, 13–28. doi: 10.1085/jgp.200709913

Wachter, C., and Turnheim, K. (1996). Inhibition of high-conductance, calcium-activated potassium channels of rabbit colon epithelium by magnesium. *J. Membr. Biol.* 150, 275–282. doi: 10.1007/s002329900050

Wallner, M., Meera, P., and Toro, L. (1996). Determinant for beta-subunit regulation in high-conductance voltage-activated and Ca^{2+}-sensitive K^+ channels: an additional transmembrane region at the N terminus. *Proc. Natl. Acad. Sci. U.S.A.* 93, 14922–14927. doi: 10.1073/pnas.93.25.14922

Wang, L., and Sigworth, F. J. (2009). Structure of the BK potassium channel in a lipid membrane from electron cryomicroscopy. *Nature* 461, 292–295. doi: 10.1038/nature08291

Wang, X., Inukai, T., Greer, M. A., and Greer, S. E. (1994). Evidence that Ca(2+)-activated K+ channels participate in the regulation of pituitary prolactin secretion. *Brain Res.* 662, 83–87. doi: 10.1016/0006-8993(94)90798-6

Weaver, A. K., Liu, X., and Sontheimer, H. (2004). Role for calcium-activated potassium channels (BK) in growth control of human malignant glioma cells. *J. Neurosci. Res.* 78, 224–234. doi: 10.1002/jnr.20240

Webster, S. M., Del Camino, D., Dekker, J. P., and Yellen, G. (2004). Intracellular gate opening in Shaker K^+ channels defined by high-affinity metal bridges. *Nature* 428, 864–868. doi: 10.1038/nature02468

Wellman, G. C., and Nelson, M. T. (2003). Signaling between SR and plasmalemma in smooth muscle: sparks and the activation of Ca^{2+}-sensitive ion channels. *Cell. Calcium.* 34, 211–229. doi: 10.1016/S0143-4160(03)00124-6

Wilkens, C. M., and Aldrich, R. W. (2006). State-independent block of BK channels by an intracellular quaternary ammonium. *J. Gen. Physiol.* 128, 347–364. doi: 10.1085/jgp.200609579

Williams, S. E., Brazier, S. P., Baban, N., Telezhkin, V., Muller, C. T., Riccardi, D., et al. (2008). A structural motif in the C-terminal tail of slo1 confers carbon monoxide sensitivity to human BK Ca channels. *Pflugers. Arch.* 456, 561–572. doi: 10.1007/s00424-007-0439-4

Williams, S. E., Wootton, P., Mason, H. S., Bould, J., Iles, D. E., Riccardi, D., et al. (2004). Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. *Science* 306, 2093–2097. doi: 10.1126/science.1105010

Wu, Y. C., Art, J. J., Goodman, M. B., and Fettiplace, R. (1995). A kinetic description of the calcium-activated potassium channel and its application to electrical tuning of hair cells. *Prog. Biophys. Mol. Biol.* 63, 131–158. doi: 10.1016/0079-6107(95)00002-5

Wu, Y., Yang, Y., Ye, S., and Jiang, Y. (2010). Structure of the gating ring from the human large-conductance Ca^{2+}-gated K^+ channel. *Nature* 466, 393–397. doi: 10.1038/nature09252

Xia, X. M., Zeng, X., and Lingle, C. J. (2002). Multiple regulatory sites in large-conductance calcium-activated potassium channels. *Nature* 418, 880–884. doi: 10.1038/nature00956

Yan, J., and Aldrich, R. W. (2010). LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. *Nature* 466, 513–516. doi: 10.1038/nature09162

Yan, J., and Aldrich, R. W. (2012). BK potassium channel modulation by leucine-rich repeat-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7917–7922. doi: 10.1073/pnas.1205435109

Yang, H., and Cui, J. (2015). "BK channels," in *Handbook of Ion Channels*, eds J. Zheng and C. Matthew (Boca Raton, FL: CRC). 227–240.

Yang, H., Hu, L., Shi, J., and Cui, J. (2006). Tuning magnesium sensitivity of BK channels by mutations. *Biophys. J.* 91, 2892–2900. doi: 10.1529/biophysj.106.090159

Yang, H., Hu, L., Shi, J., Delaloye, K., Horrigan, F. T., and Cui, J. (2007). Mg^{2+} mediates interaction between the voltage sensor and cytosolic domain to activate BK channels. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18270–18275. doi: 10.1073/pnas.0705873104

Yang, H., Shi, J., Zhang, G., Yang, J., Delaloye, K., and Cui, J. (2008). Activation of Slo1 BK channels by Mg2+ coordinated between the voltage sensor and RCK1 domains. *Nat. Struct. Mol. Biol.* 15, 1152–1159. doi: 10.1038/nsmb.1507

Yang, J., Krishnamoorthy, G., Saxena, A., Zhang, G., Shi, J., Yang, H., et al. (2010). An epilepsy/dyskinesia-associated mutation enhances BK channel activation by potentiating Ca2+ sensing. *Neuron* 66, 871–883. doi: 10.1016/j.neuron.2010.05.009

Yang, J., Yang, H., Sun, X., Delaloye, K., Yang, X., Moller, A., et al. (2013). Interaction between residues in the Mg2+-binding site regulates BK channel activation. *J. Gen. Physiol.* 141, 217–228. doi: 10.1085/jgp.201210794

Yuan, A., Santi, C. M., Wei, A., Wang, Z. W., Pollak, K., Nonet, M., et al. (2003). The sodium-activated potassium channel is encoded by a member of the Slo gene family. *Neuron* 37, 765–773. doi: 10.1016/S0896-6273(03)00096-5

Yuan, C., Chen, M., Covey, D. F., Johnston, L. J., and Treistman, S. N. (2011). Cholesterol tuning of BK ethanol response is enantioselective, and is a function of accompanying lipids. *PLoS ONE.* 6:e27572. doi: 10.1371/journal.pone.0027572

Yuan, P., Leonetti, M. D., Hsiung, Y., and MacKinnon, R. (2012). Open structure of the Ca2+ gating ring in the high-conductance Ca2+-activated K+ channel. *Nature* 481, 94–97. doi: 10.1038/nature10670

Yuan, P., Leonetti, M. D., Pico, A. R., Hsiung, Y., and MacKinnon, R. (2010). Structure of the human BK channel Ca^{2+}-activation apparatus at 3.0 A resolution. *Science* 329, 182–186. doi: 10.1126/science.1190414

Zagotta, W. N., Hoshi, T., Dittman, J., and Aldrich, R. W. (1994). Shaker potassium channel gating. II: transitions in the activation pathway. *J. Gen. Physiol.* 103, 279–319. doi: 10.1085/jgp.103.2.279

Zamoyski, V. L., Serebryakov, V. N., and Schubert, R. (1989). Activation and blocking effects of divalent cations on the calcium- dependent potassium channel of high conductance. *Biomed. Biochim. Acta* 48, S388–S392.

Zeng, X. H., Xia, X. M., and Lingle, C. J. (2005). Divalent cation sensitivity of BK channel activation supports the existence of three distinct binding sites. *J. Gen. Physiol.* 125, 273–286. doi: 10.1085/jgp.200409239

Zhang, G., Huang, S. Y., Yang, J., Shi, J., Yang, X., Moller, A., et al. (2010b). Ion sensing in the RCK1 domain of BK channels. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18700–18705. doi: 10.1073/pnas.1010124107

Zhang, G., Huang, S.-Y., Yang, J., Shi, J., Yang, X., Moller, A., et al. (2010a). Ion sensing in the RCK1 domain of BK channels. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18700–18705. doi: 10.1073/pnas.1010124107

Zhang, G., Yang, H., Liang, H., Yang, J., Shi, J., McFarland, K., et al. (2014). A charged residue in S4 regulates coupling among the activation gate, voltage, and Ca2+ sensors in BK channels. *J. Neurosci.* 34, 12280–12288. doi: 10.1523/JNEUROSCI.1174-14.2014

Zhang, X., Puil, E., and Mathers, D. A. (1995). Effects of intracellular Mg^{2+} on the properties of large-conductance, Ca^{2+}-dependent K^{+} channels in rat cerebrovascular smooth muscle cells. *J. Cereb. Blood Flow Metab.* 15, 1066–1074. doi: 10.1038/jcbfm.1995.133

Zhang, X., Solaro, C. R., and Lingle, C. J. (2001). Allosteric regulation of BK channel gating by Ca^{2+} and Mg^{2+} through a nonselective, low affinity divalent cation site. *J. Gen. Physiol.* 118, 607–636. doi: 10.1085/jgp.118.5.607

Zhou, Y., Xia, X. M., and Lingle, C. J. (2011). Cysteine scanning and modification reveal major differences between BK channels and Kv channels in the inner pore region. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12161–12166. doi: 10.1073/pnas.1104150108

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 January 2015; paper pending published: 16 January 2015; accepted: 19 January 2015; published online: 06 February 2015.

*Citation: Yang H, Zhang G and Cui J (2015) BK channels: multiple sensors, one activation gate. Front. Physiol. **6**:29. doi: 10.3389/fphys.2015.00029*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 21 January 2015
doi: 10.3389/fphys.2014.00532

Single-channel kinetics of BK (Slo1) channels

Yanyan Geng*[1] *and* ***Karl L. Magleby*[1,2]***

[1] *Department of Physiology and Biophysics, University of Miami Miller School of Medicine, Miami, FL, USA*
[2] *Neuroscience Program, University of Miami Miller School of Medicine, Miami, FL, USA*

Edited by:
Alex M. Dopico, The University of Tennessee Health Science Center, USA

Reviewed by:
Jianmin Cui, Washington University, USA
Daniel H. Cox, Tufts Medical School, USA

****Correspondence:***
Yanyan Geng and Karl L. Magleby, Department of Physiology and Biophysics-R430, University of Miami Miller School of Medicine, 1600 NW 10th Ave., Miami, FL 33136, USA
e-mail: ygeng@med.miami.edu; kmagleby@med.miami.edu

Single-channel kinetics has proven a powerful tool to reveal information about the gating mechanisms that control the opening and closing of ion channels. This introductory review focuses on the gating of large conductance Ca^{2+}- and voltage-activated K^+ (BK or Slo1) channels at the single-channel level. It starts with single-channel current records and progresses to presentation and analysis of single-channel data and the development of gating mechanisms in terms of discrete state Markov (DSM) models. The DSM models are formulated in terms of the tetrameric modular structure of BK channels, consisting of a central transmembrane pore-gate domain (PGD) attached to four surrounding transmembrane voltage sensing domains (VSD) and a large intracellular cytosolic domain (CTD), also referred to as the gating ring. The modular structure and data analysis shows that the Ca^{2+} and voltage dependent gating considered separately can each be approximated by 10-state two-tiered models with five closed states on the upper tier and five open states on the lower tier. The modular structure and joint Ca^{2+} and voltage dependent gating are consistent with a 50 state two-tiered model with 25 closed states on the upper tier and 25 open states on the lower tier. Adding an additional tier of brief closed (flicker states) to the 10-state or 50-state models improved the description of the gating. For fixed experimental conditions a channel would gate in only a subset of the potential number of states. The detected number of states and the correlations between adjacent interval durations are consistent with the tiered models. The examined models can account for the single-channel kinetics and the bursting behavior of gating. Ca^{2+} and voltage activate BK channels by predominantly increasing the effective opening rate of the channel with a smaller decrease in the effective closing rate. Ca^{2+} and depolarization thus activate by mainly destabilizing the closed states.

Keywords: Markov models, channel gating, 50-state model, dependency

INTRODUCTION

Large conductance Ca^{2+}- and voltage activated K^+ channels (also referred to as Slo1 or maxi K^+ channels) are widely distributed. BK channels (for big conductance) have an unusually high conductance for such a K^+ selective channel of ∼300 pS in symmetrical 150 mM KCl. Their joint activation by depolarization and Ca^{2+} (Marty, 1981; Pallotta et al., 1981; Latorre et al., 1982) provides a negative feed-back system to drive the membrane potential more negative, which would then close both the open BK channels and also the voltage dependent Ca^{2+} channels that are often co-localized with BK channels (Robitaille et al., 1993; Wang et al., 2001). Through this negative feedback mechanism, BK channels are involved in many physiological processes (Vergara et al., 1998). Dysfunction of BK channels can lead to diseases such as autism and mental retardation (Laumonnier et al., 2006), epilepsy (Du et al., 2005), asthma (Seibold et al., 2008), cerebellar ataxia (Sausbier et al., 2004), and hypertension (Sausbier et al., 2005).

There have been a number of recent reviews on the gating mechanisms of BK channels (Magleby, 2003; Cox, 2005, 2007; Latorre and Brauchi, 2006; Cui et al., 2009; Latorre et al., 2010; Lee and Cui, 2010; Horrigan, 2012; Contreras et al., 2013; Hoshi et al., 2013). To supplement these reviews, we will take a different approach for our contribution to the Frontiers in Physiology Research Topic: BK channels: integrators of cellular signals in health and disease. Our review will focus specifically on what selected single-channel studies have revealed about the Ca^{2+}- and voltage-dependent gating of BK channels. The level of presentation will be introductory. Beta subunits, Mg^{2+} and other modulators, as well as channel conductance, and selectivity are not considered. Representative figures of single-channel data and analysis are included so that the review is relatively self-contained. Those who seek further information about gating mechanism after reading this introductory single-channel review can start with the extensive reference lists found in the reviews listed above.

THE QUESTION

Figure 1A presents the current through a single BK channel in a small patch of membrane, recorded with the patch clamp technique (Hamill et al., 1981). In this case, the inside of the excised patch of membrane in the tip of a glass pipette is exposed to the bath solution, allowing the solution at the inner membrane

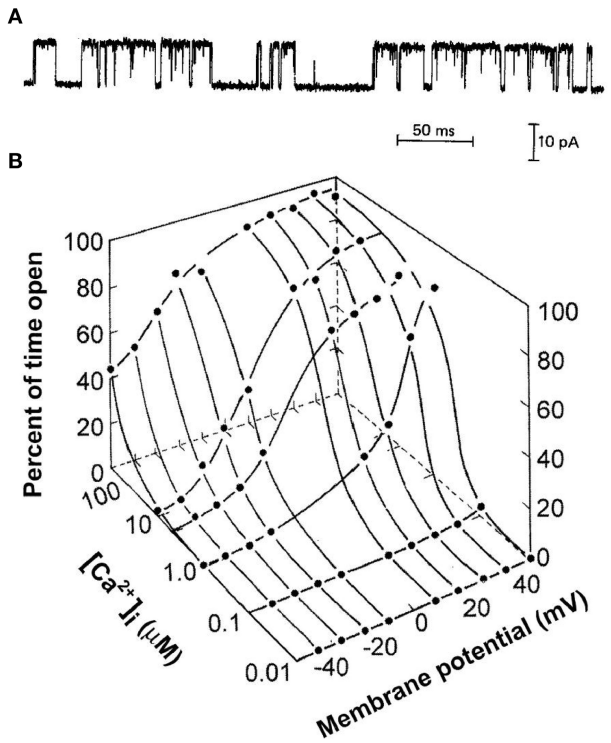


FIGURE 1 | Synergistic activation of BK channels by Ca^{2+} and depolarization. (A) A current record from a membrane patch with a single BK channel for 7.5 μM intracellular Ca^{2+} and +30 mV (from McManus and Magleby, 1988). Channel opening is indicated by upward current steps and channel closing by downward current steps. **(B)** Plot of open probability Po as a function of Ca^{2+} and voltage (from Barrett et al., 1982). All references to Ca^{2+} in this paper are for Ca^{2+} at the intracellular membrane.

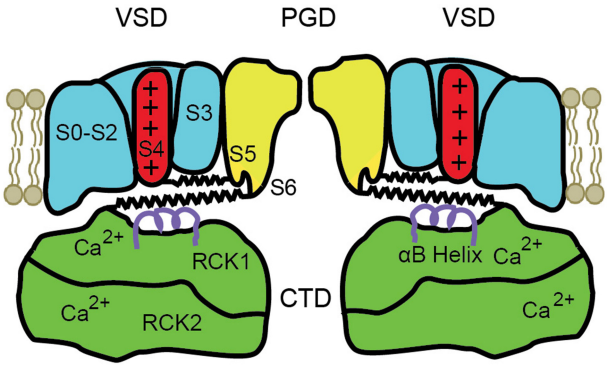


FIGURE 2 | Schematic of the modular structure of a BK channel. The central pore-gate domain (PGD) formed from S5 to S6 of each of the four subunits forms the gates and conducting pore of the channel. Two of the four voltage sensor domains (VSD) that surround the central PGD are shown. The cytosolic domain (CTD) forms a large intracellular gating ring comprised of four RCK1 domains and four RCK2 domains, of which only two of each are shown. Each RCK1 domain contains a high affinity RCK1 Ca^{2+} binding site and each RCK2 domain contains a high affinity Ca^{2+} bowl binding site. Four S4–S5 linkers connect S4 in the VSDs to the PGD (upper springs) and four RCK1-S6 linkers connect the gating ring to the PGD (lower springs), shown for two subunits. Ca^{2+} binding expands the gating ring to pull on the RCK1-S6 linkers and can also elevate the αB helices (purple helices) under the VSDs (Yuan et al., 2012). See text for references and detailed explanations.

surface to be easily changed. The upward and downward steps in the current reflect channel opening and channel closing, respectively. Measuring the current record at half open amplitude gives a record of successive open and closed interval durations that allow the calculation of open probability, Po, from the total open time divided by the sum of the total open time plus the total closed time. The successive interval durations also give information about the underlying gating mechanism of the channel. The gating is complex with intervals over wide ranges of durations. **Figure 1B** shows that BK channels are activated synergistically by both Ca^{2+} and depolarization. In resting skeletal muscle with voltage of −80 mV and intracellular Ca^{2+} < 0.1 μM, BK channels seldom open. Increasing both Ca^{2+} and depolarization are then required to activate the channels under physiological conditions. This review considerers the question of what types of kinetic gating mechanisms can account for the single-channel current records for the activation of BK channels by Ca^{2+} and voltage.

MODULAR STRUCTURE OF BK CHANNELS

A schematic diagram of the modular structure of a BK channel is shown in **Figure 2**. Only two of the four subunits are shown with front and back subunits removed. BK channels are comprised of four transmembrane voltage sensor domains (VSD) surrounding a central pore gate domain (PGD), and a large cytosolic domain (CTD), as shown schematically in **Figure 2**. The CTD is also referred to as the gating ring (Jiang et al., 2002). Each VSD is formed from S0 to S4 transmembrane segments of a single subunit. The central PGD is formed from S5 and S6 from each of the four subunits, with the pore loops between S5 and S6 forming the selectivity filter that excludes anions, Na^{2+}, Ca^{2+}, and Mg^{2+}, allowing K^+ to pass through the channel at a high rate. The gating ring is assembled from the RCK1 and RCK2 domains (regulators of the conductance of K^+) of each of the four subunits (Yuan et al., 2010), forming a ring with a central opening. The gating ring is attached to the PGD by four RCK1-S6 linkers, indicated for two subunits by the lower spring on each side of the drawing. The RCK1 domain of each subunit contains a high affinity Ca^{2+} binding site termed the RCK1 site ($K_{0.5}$ 4–17 μM), and the RCK2 domain contains a second high affinity Ca^{2+} binding site called the Ca^{2+} bowl ($K_{0.5}$ 2–4 μM) (Schreiber and Salkoff, 1997; Bao et al., 2002; Shi et al., 2002; Xia et al., 2002; Zeng et al., 2005; Zhang et al., 2010). Removing the gating ring by severing the RCK1-S6 linkers at the RCK1 end removes all Ca^{2+} and Mg^{2+} sensitivity, converting the BK channel into a purely voltage sensitive channel (Budelli et al., 2013). Ca^{2+} binding to the gating ring effectively expands the gating ring, pulling on the RCK1-S6 linkers to activate the channel (Pico, 2003; Niu et al., 2004; Yuan et al., 2010, 2012). The above information indicates that the gating ring is the module of the channel responsible for Ca^{2+} sensitivity.

Depolarization of BK channels elevates the S4 transmembrane segment in the VSD, pulling on the S4–S5 linker, which acts on the PGD to open the channel (Ma et al., 2006; Hoshi et al., 2013; Zhang et al., 2014). S4–S5 linkers for two subunits are shown as

the upper springs in **Figure 2**. Charges in S1–S3 also contribute to voltage sensitivity (Ma et al., 2006). The convergence of the action of depolarization and Ca^{2+} on the PGD through the RCK1-S6 linkers and the S4–S5 linkers provides an explanation for the synergistic activation of BK channels by both depolarization and Ca^{2+}. Mg^{2+} modulates the activity of BK channels through four low affinity sites ($K_{0.5}$ 2–4 mM), each located between the cytosolic side of a VSD and the top of an RCK1 domain in the gating ring (Hu et al., 2003; Yang et al., 2007). This review will focus on Ca^{2+} and voltage activation.

Where is the gate? The term gating is used to refer to all the processes involved in channel activity, including Ca^{2+} and voltage induced conformational changes as well as the actual opening and closing of the gate(s) in the conduction pathway. The physical location and mechanism by which the gate blocks the pore in BK channels is not clear, but the gate may be located deep in the inner vestibule of the PGD just below the selectivity filter, such that closing arises from movement (possible rotation) of the four S6 transmembrane segments to form a hydrophobic region that physically obstructs the deep pore to close or almost close the channel (Wilkens and Aldrich, 2006; Chen et al., 2014). As a possible second step, the hydrophobic region may then exclude water, leading to the formation of a water free region (a bubble) that completely blocks the movement of ions (Roth et al., 2008). Consistent with this two-step possibility, Ferguson et al. (1993) found that transitions from fully open to fully closed (and also transitions from fully closed to fully open) often proceeded through a very brief duration almost closed subconductance state with a conductance about 5% of the fully open state. The channel could also reopen from the brief duration subconductance states giving rise to very brief flicker (almost) closed states.

DISCRETE STATE MARKOV MODELS AS GATING MECHANISMS

Discrete state Markov (DSM) models, after Russian mathematician Andrey Markov, have proven highly useful to describe single-channel gating (Colquhoun and Hawkes, 1982, 1995b; Magleby and Pallotta, 1983b; McManus and Magleby, 1991; Gil et al., 2001; Lape et al., 2008). These models assume that channels gate by moving among different conformational and/or agonist bound states of the channel protein. Open and closed states of the channel represent two different conformational states. A channel with all four voltage sensors deactivated (down) is in a different conformational state than a channel with three voltage sensors deactivated and one activated (up). A channel with no bound Ca^{2+} is in a different bound state than a channel with one bound Ca^{2+}, which is in a different state than a channel with two bound Ca^{2+}.

Three examples of DSM models are shown by kinetic Schemes 1–3 in **Figure 3**, where open (O) and closed (C) states are indicated and each state is identified by a state number. Scheme 1 is the simplest DSM model that gates. Whereas most WT channels gate in large numbers of states, Cukierman et al. (1997) constructed a channel from two gramicidin A molecules covalently linked with a dioxolane ring that gates as the two-state model in Scheme 1. Schemes 2 and 3 each have two open and three closed states, but are different gating mechanisms because

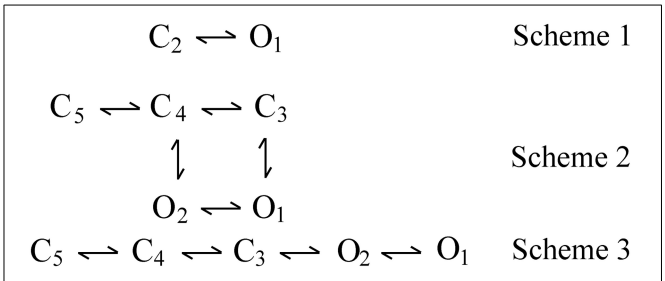


FIGURE 3 | Examples of discrete state Markov (DSM) models with limited numbers of states. C and O are closed and open states, respectively. Scheme 1 is the simplest model that gates. Scheme 2 with two gateway states (C_3 and C_4) connecting open and closed states would typically generate correlations between adjacent open and closed interval durations. Scheme 3 with one gateway state (C_3) would not generate such correlations (Fredkin et al., 1985; Magleby and Song, 1992; Colquhoun and Hawkes, 1995b).

the connections (transition pathways) between the states are different.

The assumptions for DSM models as applied to gating of BK channels are: (1) the time required for the transition between two connected states is insignificant compared to the time spent in either state, (2) the rate constants for the transitions between states remain constant (in time) for constant experimental conditions, and (3) voltage and Ca^{2+} gate the channel by changing the effective rates for the transitions between states. It follows from (2) that the rate constants for leaving a state are independent of the previous sequence of events before entering the state, and are also independent of the duration of time already spent in the state. For this reason Markov models are often referred to as memory less processes. However, it is not the model but the rate constants that are memory less. Markov models can have memories defined by the kinetic schemes and rate constants, leading to correlations in the data, as will be explained later.

DWELL-TIMES IN STATES ARE EXPONENTIALLY DISTRIBUTED IN DSM MODELS

For DSM models (and also for the decay of radioisotopes), the dwell-times (lifetimes) in a state are a random (stochastic) variable, described by an exponential distribution, where the probability (density), $f(t)$, of observing a dwell-time with duration t decays exponentially with t, such that

$$f(t) = (1/\tau)\exp(-t/\tau) \quad (1)$$

where t is the duration of a dwell-time in the state, τ is the time constant of the distribution, given by the time for the distribution to decline to 1/e (0.368) of its initial magnitude, and $1/\tau$ is the magnitude of the distribution at time 0. τ is also given by the mean of all the intervals in the exponential distribution. The product of τ times the magnitude of the exponential at time 0 gives the area of the exponential. The probability (density) function given by Equation (1) is normalized to an area of 1.0 by setting the magnitude to $1/\tau$. Increasing τ 10-fold then reduces the magnitude 10-fold to retain an area of 1.0.

Why are dwell-times in a Markov state exponentially distributed? Converting a rate constant to a probability indicates a small probability of leaving a state in each small increment of time. For example, a rate constant of 1/s away from a state converts to a probability for leaving the state of 10^{-3}/ms, 10^{-6}/μs, or 10^{-9}/ns. A small but constant probability of leaving a state per unit time leads to an exponential distribution of the times spent in the state (Colquhoun and Hawkes, 1995b). As a thought experiment, imagine that the channel possesses a true die with 10^9 sides which it throws once every nanosecond. If side 1 out of the 10^9 sides comes up, then the channel makes a conformational change. If any of the other 999,999,999 sides come up, then the channel throws the die again without memory of previous throws, and continues to do so until side 1 comes up, at which point it makes a conformational change. Mechanistically, during each brief moment of time (10^{-9} s), the thermal fluctuations in the channel have only a low probability of providing sufficient energy to move the present conformation over a thermally fluctuating energy barrier into the next state, or alternatively, during each brief moment of time the channel very rapidly samples many different conformations due to thermal fluctuations, with only a very low probability of reaching a different stable conformation state. See Frauenfelder (2014) for discussion of protein motions.

The engineered two-state channel of Cukierman et al. (1997) discussed in the previous section was identified as gating in two states from the single exponential distributions for open and closed intervals. The distributions of open times for sodium channels are described by a single exponential, suggesting a single open state (Horn and Vandenberg, 1984). For channels with multiple open and closed states, dwell-times in any given state are still exponentially distributed for DSM models, but the separate dwell-time distributions of all open and all closed interval durations are now comprised of the sums of exponentials, as will be described in later sections.

CALCULATION OF STATE LIFETIMES AND PROBABILITIES FOR DIFFERENT TRANSITIONS

Rate constants for DSM models are identified by state numbers, such that k_{i-j} is the rate constant for the transition from state i to state j. The mean lifetime (dwell-time) in any state i can be calculated from the rate constants (Colquhoun and Hawkes, 1995b) using

$$\text{Mean lifetime of state } i = 1/\left(\sum \text{ rate constants away from state } i\right) \quad (2)$$

where (Σ rate constants away from state i) gives the effective rate constant for leaving state i. For example, the mean lifetime of C_2 in Scheme 1 is given by $1/k_{2-1}$, the mean lifetime of C_5 in Scheme 2 is given by $1/k_{5-4}$, and the mean lifetime of C_4 in Scheme 2 is given by $1/(k_{4-2} + k_{4-3} + k_{4-5})$. It follows from rearranging Equation (2) that the rate constants can be determined directly for the two-state model given by Scheme 1 for data in which all open and closed intervals are detected from the inverse of the mean lifetimes of the open and closed intervals

$$k_{1-2} = 1/O_1 \quad (3)$$

$$k_{2-1} = 1/C_2 \quad (4)$$

For Scheme 1, with only one transition pathway between the states, which state is entered next is a given. For more complex models like Schemes 2 and 3, the probability of making a transition from state i to state j, P_{i-j} is

$$P_{i-j} = k_{i-j}/\left(\sum \text{ rate constants away from state } i\right) \quad (5)$$

For Scheme 2, P_{5-4}equals k_{5-4}/k_{5-4} equals 1, and P_{4-2} equals $k_{4-2}/(k_{4-2} + k_{4-3} + k_{4-5})$. For Scheme 3 P_{2-1} equals $k_{2-1}/(k_{2-1} + k_{2-3})$.

The connected states C_5-C_4-C_3 in Schemes 2 and 3 form compound closed states, with each of the states in the compound state having the same closed current level, so that the state transitions within the compound closed states are hidden from direct experimental observations. Examples of hidden transitions within the compound closed state in Scheme 2 would be: O_1-C_3-C_4-O_2 and O_2-C_4-C_3-C_4-C_5-C_4-O_2. The same would apply to transitions among the compound open states, O_1-O_2, which in BK channels would have the same open current level. Consequently, compound openings such as C_4-O_2-O_1-O_2-C_4 for Scheme 2 and C_3-O_2-O_1-O_2-C_3 for Scheme 3, would all have the same open current levels with the open–open transitions hidden. In spite of the hidden transitions, information about the numbers of closed and open states, the transition pathways among the states, the probabilities of the various transitions pathways, and the mean lifetimes of the states are reflected in the successive open and closed interval durations, which reveal the closed and open dwell-time distributions and also the 2D dwell-time distributions and the correlations between open and closed interval durations (see graphical demonstrations in (Magleby and Song, 1992; Colquhoun and Hawkes, 1995b; Rothberg et al., 1997; Rothberg and Magleby, 1998a). Because state transitions are hidden within compound states, DSM models are also referred to as hidden Markov models.

DESCRIBING DWELL-TIME DISTRIBUTIONS BY THE SUMS OF EXPONENTIALS

Even though transitions among states in a compound state are hidden, each of these states will lead to the generation of an exponential component in the distribution of interval durations (Colquhoun and Hawkes, 1982, 1995b). For n_c closed and m_o open states in a DSM model, the dwell-time distribution of all closed intervals would then consist of the sum of n_c exponential components, and the dwell-time distribution of all open intervals would consist of the sum of m_o open exponentials components, although all exponentials may not be detected because of closely spaced or identical time constants, or areas too small to detect. Distributions described by sums of exponentials are often referred to as being described by mixtures of exponentials because it is not possible to assign any single interval in the summed distribution to any given exponential component, except in terms of probabilities. A first step often used in the analysis of single-channel data is to fit open and closed dwell-time distributions

with sums of exponentials to determine the numbers of significant exponential components, which then provides an estimate of the minimum number of open and closed states required in a DSM, such that,

$$f(t) = \sum_{k=1}^{N} (a_k/\tau_k)\exp(-t/\tau_k) \quad (6)$$

where $f(t)$ is the dwell-time distribution arising from the sums of exponential components, N is the number of summed exponentials, a_k is the area (the fraction of the total area of the dwell-time distribution) of exponential k, τ_k is the time constant of exponential k, and the quotient a_k/τ_k gives the magnitude of exponential k at 0 time (Landowne et al., 2013).

A common misconception in the interpretation of dwell-time distributions is to assume a one-for-one relationship between exponential components and states, such that, the dwell-times in a specified state give rise to the dwell-times in a single exponential component. Such a relationship is seldom the case when compound states are involved, as each state in a compound state can make contributions to all of the exponential components arising from the compound state. The relationship between components and states is fascinating, being both paradoxical or predictable depending on the ratios of the various rate constants connecting the states (Shelley and Magleby, 2008). Fortunately, the methods used to determine rate constants and rank models circumvent the need to know the complexities of these relationships. Nevertheless, to discuss exponential components in terms of states, which is often done, requires understanding the relationship between components and compound states.

ESTIMATING PARAMETERS AND RANKING KINETIC GATING MECHANISMS

This section illustrates by example how kinetic parameters can be estimated for a two-state model and then describes the process for more complex models. **Figure 4A** presents a segment of a simulated single-channel record for Scheme 1 with rate constants for opening, k_{C2-O1}, and for closing, k_{O1-C2}, of 1000/s. The variation in successive dwell-times and the apparent drift in activity over time reflects stochastic variation in dwell-times. Measuring the durations of 10^6 simulated closed intervals and plotting them as a frequency histogram gives the linear plot of the dwell-time distributions in part **C**, which is well-described by the exponential function (continuous line)

$$f(t) = 1000\exp(-t/\tau) \quad (7)$$

where $f(t)$ is the number of intervals per μs of bin width, t is interval duration, and τ is 1 ms, given by the time required for the exponential to fall to 1/e of its initial magnitude. **Figure 4B** is a Sigworth and Sine (1987) plot of the same data in **Figure 4C**. In this transform, the distribution peaks at the time constant of the exponential, providing a quick visual indication of the mean interval duration in the distribution. Log binning is used in both parts **B** and **C**, with the duration of the bins increasing logarithmically with dwell-time. With log binning the bin width remains constant on a logarithmic abscissa. The Sigworth and Sine transform plots the square root of the number of intervals per bin vs. the log of the mean duration of the intervals in each bin, and the linear plot presents the number of intervals per μs of bin width (to correct for the log binning) vs. the mean duration of intervals per bin. Log binning allows binning and plotting of

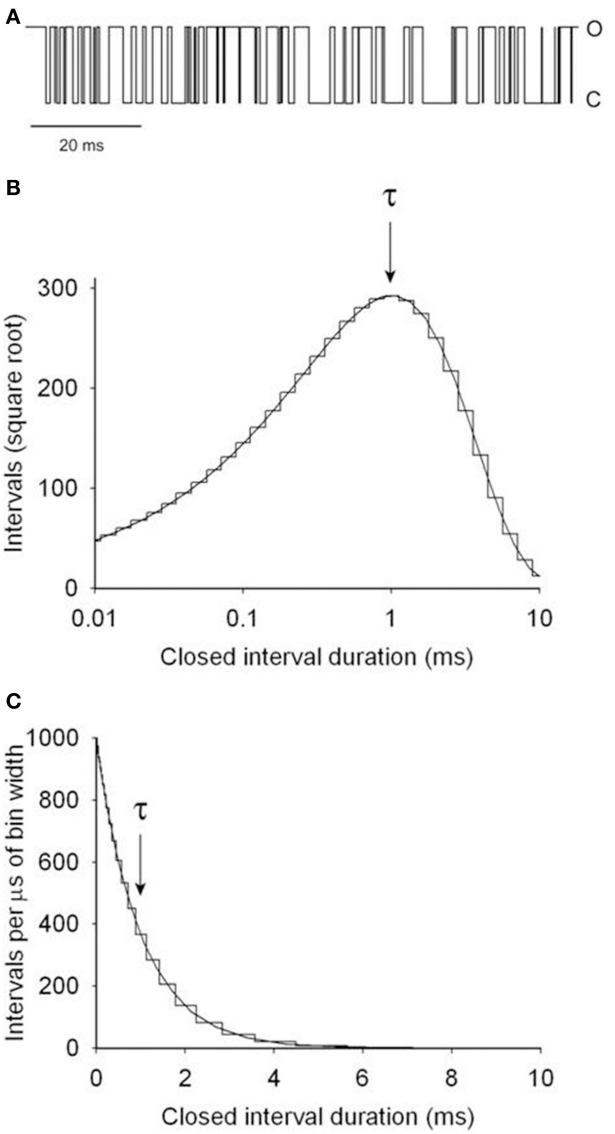


FIGURE 4 | Simulated single channel kinetics for the two-state Scheme 1. (A) A simulated single-channel current record for Scheme 1 with opening and closing rate constants of 1000/s. **(B)** Dwell-time distribution of the closed interval durations plotted with the Sigworth and Sine transformation (Sigworth and Sine, 1987). The abscissa has log binning with 10 bins per log unit. The distribution peaks at the mean interval duration of 1 ms, indicated by τ. The rate constant for opening is given by the inverse of τ. **(C)** Dwell-time distribution of the same data in part **B** on a linear plot. The intervals were corrected for the increasing bin width that occurs with log binning. The mean interval duration τ is given by the time to decay to 1/e (1/2.718 = 0.368) of the initial amplitude. The same distributions would be obtained for the open intervals. 10^6 intervals were simulated and analyzed (from Shelley et al., 2010).

dwell-times with durations ranging from microseconds to hours using less than 100 bins with constant time resolution (McManus et al., 1987). Sigworth and Sine plots, because of the uncorrected increasing bin width with log binning, can give the mistaken impression that the frequency of intervals in a dwell-time distribution first increase and then decrease, but this is not the case, as can be seen by comparison to the liner plot in **Figure 4C** which has been corrected for the log binning.

The 1 ms time constant of decay in **Figures 4B,C** indicates a rate constant for channel opening, k_{2-1}, of 1000/s (Equation 4). The analysis would be the same for the open intervals, giving a rate constant for channel closing, k_{1-2}, of 1000/s. Hence, for a two state model and ideal data with unlimited time resolution, the rate constants can be determined directly from the inverse of either the time constants or the mean interval durations.

As a general experimental approach, the rate constants for Scheme 1 and for more complicated schemes would be obtained by maximum likelihood fitting of the single-channel data using Q-matrix or other methods (Colquhoun and Hawkes, 1982, 1995a,b; McManus and Magleby, 1991; Rothberg and Magleby, 1998b; Colquhoun et al., 2003; Qin, 2014). In one approach, single-channel data, in the form of 2D dwell-time distributions obtained over a range of Ca^{2+} and voltage, but with fixed conditions for each distribution, are simultaneously fitted (global fitting) with the gating mechanisms of interest (Rothberg and Magleby, 1998a). Such fitting determines the most likely rate constants and their voltage and Ca^{2+} dependence for each tested gating mechanism. Ranking the examined gating mechanisms in terms of the likelihood that the observed single-channel data were generated by the gating mechanisms, then identifies the most likely model. When ranking models, a penalty is applied for increases in the number of free parameters in the models (Rothberg and Magleby, 1999). Such global fitting of data over a range of Ca^{2+} and voltage as well as taking into account the correlation information (see later section) is essential to define models and rate constants. Without global fitting the rate constants and ranking of models can be poorly defined. The highest ranking model is not the "correct" model, which is not known, but is the most likely of all the examined models.

It is possible to determine if there is a model with the same number of states that ranks higher than the highest ranking model. The data are fitted with a generic model (Kienker, 1989) that includes all possible models for the specified number of states. Global fitting is not possible with the generic model, so each experimental condition is fitted separately with the generic model and then the log likelihoods for each experimental condition are added. If the likelihood of the highest ranked model is equal to the likelihood of the generic model for the same data, then no other model with the same number of states will be found that has likelihood higher than the highest ranked model (Rothberg and Magleby, 1998b, 1999). A comparison of the log likelihoods of the generic models to the examined models also quantifies how well the examined models account for the data (Rothberg and Magleby, 1999).

For experimental single-channel records, very brief intervals can be sufficiently attenuated by the low pass filtering used to reduce high frequency noise in the current record that the brief intervals are not detected. These missed intervals distort the single-channel data. For example, if a very brief closed interval between two longer open intervals is missed, then the missed closed interval plus the two adjacent open intervals would be detected as a single longer open interval. Alternatively, if a very brief open interval is missed, then the missed open interval plus the two adjacent closed intervals would appear as a single longer closed interval in the experimental record. Mathematical corrections for these missed events are applied when fitting experimental data to correct for the effects of the limited time resolution (Blatz and Magleby, 1986; Crouzy and Sigworth, 1990; Hawkes et al., 1992; Qin et al., 1996).

The DSM models developed in the previous sections would indicate the numbers of states, the transition pathways among the states, the rate constants for the transitions, and the voltage and Ca^{2+} dependence of the rate constants. Given such models, it is possible to calculate the expected dwell-time distributions and the macro currents over wide ranges of voltage and Ca^{2+} using Q-matrix methods (Colquhoun and Hawkes, 1982, 1995a). Macro currents are currents recorded from hundreds to thousands of channels in a macro patch of membrane or from the membrane of an intact cell. Single-channel current records can also be calculated from the DSM models through simulation using the rate constants and a random number generator to make the stochastic decisions as to specific dwell-times and state transitions. Whereas each simulated run would give a unique example of single-channel gating, combining many such runs would give a second method to calculate average single-channel and macro current kinetics and steady-state responses.

DSM models are simplifications of actual protein function. Each state in a DSM model represents large numbers of conformational substates in very rapid equilibrium due to thermally induced protein motions (McManus and Magleby, 1989; Frauenfelder, 2014). In the DSM models used here the transitions between any two connected states and the binding and unbinding of agonist (Ca^{2+}) are represented with single effective rate constants, rather than with the short lifetime multi-state processes that underlie these transitions. These multistep processes are too rapid to be separated in the single-channel data. Whereas the DSM models used for gating among states do not include the multi-step processes of state transitions directly, they do give information about the dwell-times before the transitions and the different probabilities for exiting via the different transitions pathways. This information defines the DSM models and also delimits the information that will have to be accounted for by transition state theory.

In spite of the simplifications mentioned above, critical tests of Markov gating for BK and NMDA receptor channels have found that the single-channel kinetics are consistent with DSM models. The time constants of the exponentials describing the open and closed dwell-time distributions were independent of adjacent interval durations for fixed experimental conditions, as required for DSM models (McManus and Magleby, 1989; Gibb and Colquhoun, 1992). These observations of time constants independent of adjacent interval durations also indicate that the time constants of the exponentials can be accurately determined from the experimental data. Further support for Markov gating are the observations that dwell-time distributions are well-described by

either single exponentials or sums of exponentials, as expected for DSM models. Fractal models can be ruled out for four examined channels, BK channels, fast Cl-channels, nicotinic AChR receptor channels, and $GABA_A$ channels, as DSM models ranked significantly higher than fractal models for these four different ion channels (McManus et al., 1988).

DSM models will be referred to as gating mechanisms or schemes or simply models in the remainder of this review. Kinetic is often added to emphasize that these models can describe the dynamics of the gating over time because they contain rate constants for both forward and backward transitions. This is in contrast to equilibrium models that can only describe the steady state or equilibrium Po, and thus give no information about the time course of the gating.

GATING MECHANISMS CONSISTENT WITH THE MODULAR STRUCTURE OF BK CHANNELS

Gating mechanisms are best formulated in terms of what is known about the structure of an ion channel. The modular structure in **Figure 2** indicates that BK channels have: (1) four VSD modules, each with an S4 transmembrane segment that is assumed to be either deactivated (down) or activated (up); (2) a gating ring comprised of four pairs of intertwined RCK domains, each with two high affinity Ca^{2+} binding sites; and (3) a PGD that is gated by the four VSDs through the S4–S5 linkers and also by the four RCK1-S6 linkers from the gating ring. For simplicity, as is typically done for BK channels, the two high affinity Ca^{2+} binding sites per pair of intertwined RCK domains will be treated as a single site, giving four effective high affinity Ca^{2+} binding sites. This simplification is possible (but not necessarily justified) for BK channels because the Hill coefficients for plots of Po vs. Ca^{2+} typically approach, but seldom, exceed 4, as will be shown later.

The (mainly) independent voltage and Ca^{2+} activation systems of BK channels can be incorporated into a kinetic model by starting with separate models for each. Scheme 4 in **Figure 5** presents a gating mechanism for the voltage-dependent gating of BK channels in the absence of Ca^{2+} (Cox et al., 1997; Cui et al., 1997; Horrigan and Aldrich, 1999; Horrigan et al., 1999),

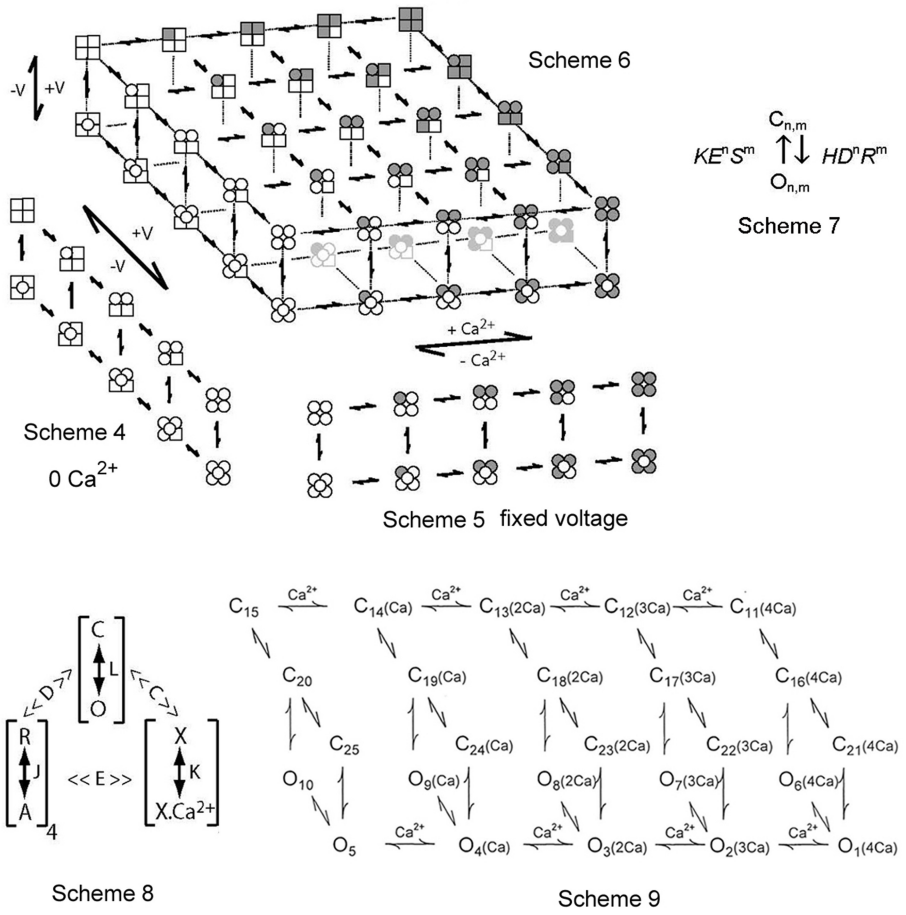


FIGURE 5 | Two-tiered gating mechanisms for BK channels. Scheme 4 is for voltage only gating, Scheme 5 is for Ca^{2+} only gating, and Scheme 6 combines the two for joint gating by both voltage and Ca^{2+}. The upper tiers are closed states and the lower tiers open states. The four subunits for each state are depicted. A filled subunit has a bound Ca^{2+} and a round subunit has an activated voltage sensor (from Rothberg and Magleby, 1999, 2000). Scheme 7 presents a constrained approach of representing the allosteric action of voltage and Ca^{2+} on the opening and closing transitions between the closed and open tiers in Schemes 4–6. Scheme 8 is a shorthand method devised by Horrigan and Aldrich (2002) to describe Scheme 6 in terms of effective equilibrium constants. Scheme 9 from Rothberg and Magleby (2000) is a subset of Scheme 6. See text for detailed descriptions of all these schemes.

and is similar to models that have been considered for other voltage gated channels (Marks and Jones, 1992; Rios et al., 1993; McCormack et al., 1994). In this 10-state model, each state is comprised of four subunits, as dictated by tetrameric BK channels (Shen et al., 1994). The five states in the upper tier are closed and the five states in the lower tier are open, with an open state indicated by an open circle in the middle of the four subunits on the lower tier. A square subunit indicates that the voltage sensor in that subunit is relaxed (deactivated), whereas a circular subunit indicates that the voltage sensor is activated. The same 10-state Scheme 4 would also apply for voltage dependent gating at 95 μM Ca^{2+} by shading all the squares and circles to indicate that all subunits have bound Ca^{2+} (Shelley et al., 2010). Notice in Scheme 4 that the voltage sensors can activate and deactivate for both open and closed channels, and that there is not an obligatory coupling between voltage sensor movement and channel opening and closing, as channels can open and close with 0, 1, 2, 3, and 4 activated voltage sensors, with Po increasing with an increase in the number of activated voltage sensors (Horrigan and Aldrich, 1999; Horrigan et al., 1999; Rothberg and Magleby, 1999, 2000; Shelley et al., 2010). Further support for Scheme 4 comes from the single-channel observations of Talukder and Aldrich (2000) of multiple components in both open and closed dwell-time distributions at high voltages (+180 to +260 mV) with zero Ca^{2+}. They suggest that the multiple components indicate states or combinations of states with different numbers of activated voltage sensors.

Scheme 5 presents a gating mechanism for the Ca^{2+}-dependent gating of BK channels (McManus and Magleby, 1991). This scheme parallels the voltage activation shown in Scheme 4 except that activation is by binding Ca^{2+}, indicated by a shaded (gray) subunit. Scheme 5 as depicted is for all four voltage sensors activated. The same Scheme 5 with no voltage sensors activated would be indicated by replacing all the round subunits in Scheme 5 with square subunits. Scheme 5 for Ca^{2+}-dependent gating has features in common with the Monod, Wyman and Changeux (MWC) model (Changeux and Edelstein, 1998) widely considered for agonist activation of receptor channels. Scheme 5 for Ca^{2+} activation has many of the same general features as Scheme 4 for voltage activation. Both are two-tiered, with five closed states on the upper tier and five open states on the lower tier. There is not an obligatory coupling between Ca^{2+} binding and channel opening and closing, as channels can open and close with 0, 1, 2, 3, and 4 bound Ca^{2+}, with Po increasing with the number of bound Ca^{2+} (Magleby and Pallotta, 1983a,b; McManus and Magleby, 1991; Cox et al., 1997; Cui et al., 1997; Rothberg and Magleby, 1998b, 1999, 2000; Horrigan and Aldrich, 2002).

Scheme 6 combines the voltage and Ca^{2+}-activation into one model (Rothberg and Magleby, 1999, 2000; Cui and Aldrich, 2000). Scheme 6 includes five repeats of Scheme 4, with 0, 1, 2, 3, and 4 bound Ca^{2+} per channel (left to right). Alternatively, Scheme 6 can be viewed as five repeats of Scheme 5, with 0, 1, 2, 3, and 4 activated voltage sensors per channel (top to bottom). The upper left most tier of one closed and one open state has 0 Ca^{2+} bound and 0 voltage sensors activated. The lower right most tier of one closed and one open state has four bound Ca^{2+} and four voltage sensors activated. Scheme 6 shows that with one Ca^{2+} sensor and one voltage sensor per subunit, the channel could potentially enter 25 closed states on the upper tier and 25 open states on the lower tier during gating, each defined by different combinations of activated voltage sensors and bound Ca^{2+} sites. If the relative positions (adjacent or diagonal) of Ca^{2+} binding and voltage sensor activation on the various subunits in a channel alter the gating, then there would be additional states (Cox et al., 1997; Rothberg and Magleby, 1999; Qian et al., 2006), which will not be considered here. Gating in all 50 states would require changing the Ca^{2+} and voltage over wide ranges. Voltage and Ca^{2+} jointly activate the channel, by shifting the gating from states in the upper left region (low Ca^{2+} and hyperpolarized voltage) of Scheme 6 to states in the lower right region (high Ca^{2+} and large depolarization). For a fixed Ca^{2+} and a fixed voltage, the channel would gate in only a subset of the states in the 50-state scheme.

One highly constrained conceptual method of representing the allosteric action of voltage and Ca^{2+} sensors on the opening and closing transitions between the upper tiers and the lower tiers in Schemes 4–6 can be represented as Scheme 7 in **Figure 5**, where n is the number of voltage sensors activated, m is the number of bound Ca^{2+}, H and K are the opening and closing rate constants when n and $m = 0$ (no activated voltage or Ca^{2+} sensors), D and E are the allosteric accelerators for voltage on the opening and closing rate constants, respectively, and R and S are the allosteric accelerators for bound Ca^{2+} on the opening and closing rates, respectively, (Chen et al., 2012). Scheme 6 with rate constants and expanded to include the allosteric action of both voltage and Ca^{2+} on the opening and closing rates using (Scheme 7) is an extension of earlier models with equilibrium constants (Marks and Jones, 1992; Rios et al., 1993; McCormack et al., 1994; Horrigan and Aldrich, 2002). For Scheme 7, a channel with two voltage sensors activated and three Ca^{2+} bound would have an allosteric acceleration in opening of D^2R^3 and the opening rate would be HD^2R^3.

Scheme 8 represents a clever notation developed by Horrigan and Aldrich (2002) to describe the steady-state Po (equilibrium gating) predicted by the 50-state model in Scheme 6. In Scheme 8, L, J, and K represent the equilibrium constants for the transitions between the closed and open states (C–O), the resting and activated voltage sensors (R–A), and the unbound and bound Ca^{2+} sensors ($X–X_{Ca2+}$), respectively. The four's reflect four voltage and four Ca^{2+} sensors, D and C are allosteric equilibrium factors for the actions of the voltage sensors and Ca^{2+} sensors on the opening-closing equilibrium, and E is a coupling factor for interactions between voltage and Ca^{2+} sensors. Note that the parameters in Scheme 8 have different meanings than those in Scheme 7 and further schemes to be presented in this review. Scheme 8 as shown has equilibrium constants, and consequently gives no information about the time course (kinetics) of gating or single-channel kinetics. Nevertheless, Scheme 8 provides a powerful tool to study gating mechanism of BK channels by indicating the fundamental principles of the gating (Horrigan and Aldrich, 2002), and Scheme 8 can be expanded to use rate constants rather than equilibrium constants, becoming Scheme 6.

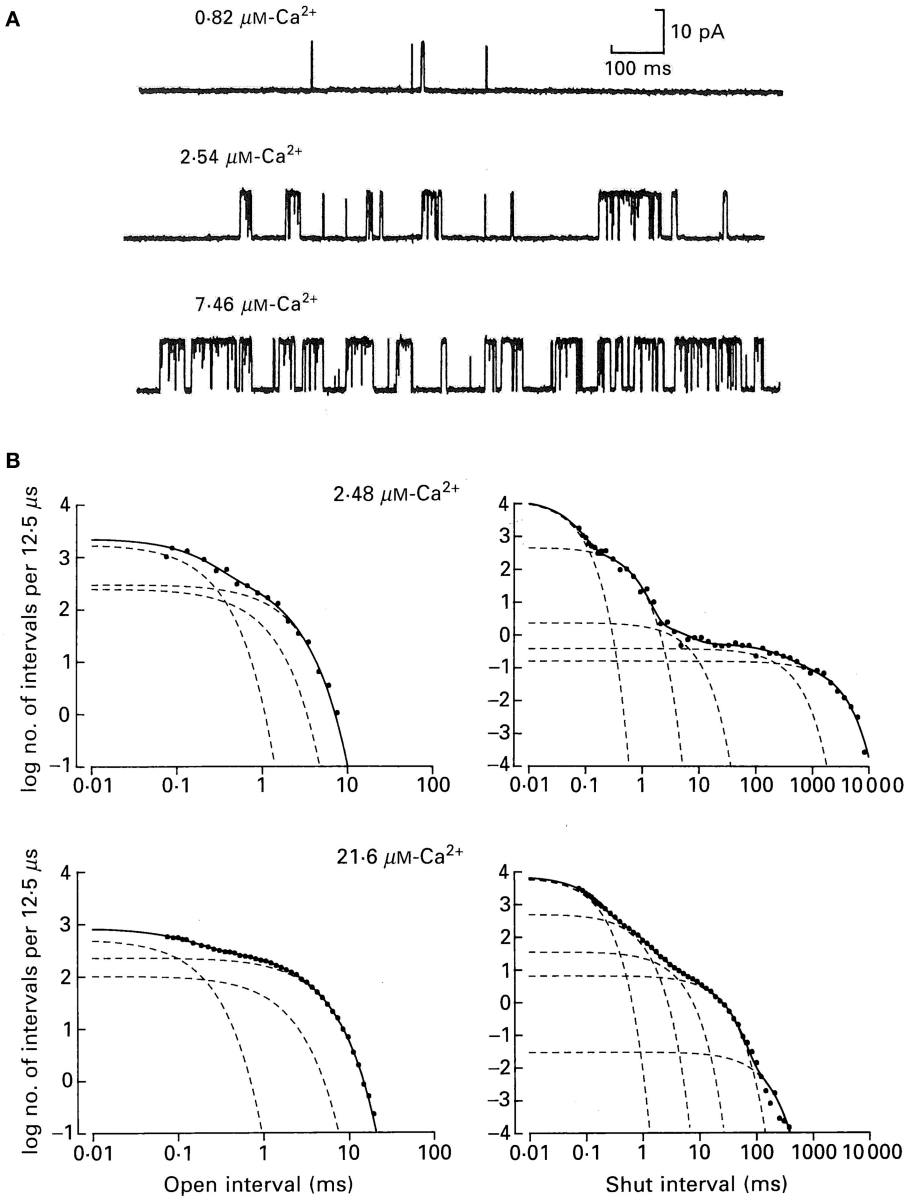


FIGURE 6 | A BK channel gating in a minimum of three open and five closed states. (A) Single channel records at the indicated Ca^{2+}. Opening is upward. **(B)** Open and closed dwell-time distributions at the indicated Ca^{2+} plotted on log–log coordinates for a different channel than those in part **A**. The open distributions were described by the sums of three significant open exponentials (dashed lines summing to make the continuous lines through the data), and the closed distributions were described by the sums of five significant closed exponentials from McManus and Magleby (1991).

BK CHANNELS TYPICALLY GATE IN A MINIMUM OF 3–4 OPEN STATES AND 5–6 CLOSED STATES FOR FIXED EXPERIMENTAL CONDITIONS

Figure 6 shows how the minimum number of kinetic states entered during gating can be estimated from the number of significant exponentials required to describe the dwell-time distributions. **Figure 6A** presents single-channel recordings obtained from a single BK channel at three different Ca^{2+}. The Ca-induced increase in channel activity is readily apparent. Measuring open and closed interval durations from long records of stable data (McManus and Magleby, 1988) and plotting them as frequency histograms on log–log coordinates gave the open (left) and closed (right) dwell-time distributions for the two indicated Ca^{2+} (**Figure 6B**). These are yet a third type of plot for dwell-time distributions, in addition to those in **Figure 4**. The ordinate is expressed as log of the number of intervals per 12.5 μs of bin width to correct for the effect of log binning the interval durations, and the abscissa is expressed as the log of the mean duration of the intervals in each log bin. In the log–log plots in **Figure 6** each additional exponential is indicated by an inflection in the dwell-time distribution.

From **Figure 6B** it can be seen that the observed durations of intervals can extend over five orders of magnitude and that the frequencies of intervals can extend over seven orders of magnitude. Such extended information is seldom seen in biology, but is routine for single-channel data because of the huge kinetic space of gating and the fact that frequency (counting) and duration (time) are two physical parameters readily measured by computer with high accuracy.

The open distributions were well-described (continuous line) by the sum of three significant exponential components (dashed lines) and the closed distributions by the sum of five significant exponential components. For experiments of this type for data from single BK channels over a range of Ca^{2+} and voltage (−100 to +100 mV), but with fixed Ca^{2+} and voltage for each determinations, the open distributions were typically described by 3–4 significant open exponential components and the closed distributions by 4–6 significant closed exponential components, suggesting gating in a minimum of 3–4 open states and 4–6 closed states (McManus and Magleby, 1988, 1989, 1991; Rothberg and Magleby, 1999). Detecting 3–4 open states and 4–6 closed states appear more consistent with the 10-state Schemes 4 and 5 than the 50-state Scheme 6. If the 50-state Scheme 6 represents the proposed gating mechanism, then why aren't 25 significant closed and 25 significant open exponentials detected, one for each of the 50 states? One reason presented earlier is that the 50 states are potential states based on the modular structure of BK channels. For any given Ca^{2+} and voltage, the gating would be effectively limited to only a small subset of the 50 states, so only that subset would generate exponentials with sufficiently large areas to detect. Furthermore, calculations on large multistate models show that many of the expected time constants for 50-state models can be nearly identical or too close to detect. It will be shown in sections below, that Ca^{2+} dependent gating at fixed voltage, and voltage dependent gating at fixed Ca^{2+}, can each be approximated by the 10-state Schemes 5 and 4, respectively, consistent with the numbers of significant detected exponential components.

The observation of typically 5–10 significant exponentials in the dwell-time distributions of BK channels (Magleby and Pallotta, 1983b; McManus and Magleby, 1988) providing experimental evidence for gating in a minimum of 5–10 states, was met with skepticism by many back at the time, even though it had been previously proposed for decades that agonist activated channels and also voltage activated channels would be expected to gate in 5–10 or more states (Hodgkin and Huxley, 1952; Monod et al., 1965; Nonner, 1980). To avoid the need for large numbers of states, even though expected, the much simpler fractal model gained interest until it was shown to be inconsistent with the experimental data for four different ion channels (McManus et al., 1988).

INVERSE RELATIONSHIP BETWEEN THE DURATIONS OF ADJACENT OPEN AND CLOSED INTERVALS

General information about gating mechanisms can be obtained from the relationships between adjacent open and closed interval durations (McManus et al., 1985; Colquhoun and Hawkes, 1987; Magleby and Song, 1992; Rothberg and Magleby, 1998b). To generate correlations there needs to be more than one gateway state in the kinetic scheme, where the number of gateway states in a kinetic scheme is given by the minimal number of states that have to be removed to sever all connections between open and closed states (Colquhoun and Hawkes, 1987). For Schemes 1 and 3 there would be no correlation between the durations of adjacent open and closed intervals because there is only one gateway state connecting open and closed states. In Schemes 2, 4, 5, and 6, there are 2, 5, 5, and 25 gateways between open and closed states so that these schemes could show correlations between adjacent open and closed interval durations. To test for correlations, McManus et al. (1985) plotted the mean durations of open intervals adjacent to specified ranges of closed intervals (**Figure 7A**). There was an inverse relationship between the durations of adjacent open and closed intervals. This inverse relationship can be observed directly in the single-channel current records in **Figure 6A**, where brief open intervals are typically adjacent to longer closed intervals and longer open intervals are typically adjacent to briefer closed intervals.

A more rigorous demonstration of this inverse relationship for essentially all possible pairs of adjacent open and closed interval durations, including showing the significance of the inverse relationship, is presented in **Figures 7B–D** (Rothberg and Magleby, 1998b). Part **B** shows 2D dwell-time distributions of all theoretically possible pairs of adjacent interval durations, plotted in the Sigworth and Sine transform. Part **C** shows a dependency plot of the data in part **B** which indicates which pairs of adjacent open and closed intervals are in excess and which are in deficit over that expected by chance pairing alone. Part **D** presents a dependency significance plot of the data in part C indicating the significance of the excesses and deficits of intervals. (These plots are described in-depth in the legend of **Figure 7**). Plots **C** and **D** would be a flat plane at 0.0 if adjacent open and closed intervals paired by chance alone, as would be the case if there were only one gateway state. The significant correlations rule out gating mechanisms like Scheme 3, with one gateway state between open and closed intervals. The observed inverse correlations between adjacent open and closed interval durations suggest gating mechanisms like Schemes 4–6 in which the lifetimes of the closed states on the upper tiers progressively decrease and the lifetimes of the open states on the lower tiers progressively increase as more voltage sensors are activated and/or more Ca^{2+} is bound. For a graphical demonstration of this concept, see Magleby and Song (1992).

GATING OF BK CHANNELS IS CONSISTENT WITH MICROSCOPIC REVERSIBILITY

To develop a gating mechanism for an ion channel it is necessary to know whether there is an external energy source driving the gating. In an excised patch of membrane held at a moderate voltage such as +30 mV, BK channels can gate with stable kinetics for many hundreds of thousands of open and closed transitions over tens of minutes. Where is the energy source for the gating? In order to record single channel currents there has to be an electrochemical (voltage and/or concentration) gradient to drive K^+ through the channel. If this energy source provided energy for gating, then the single-channel kinetics for the same current record analyzed in the forwards and backwards directions could

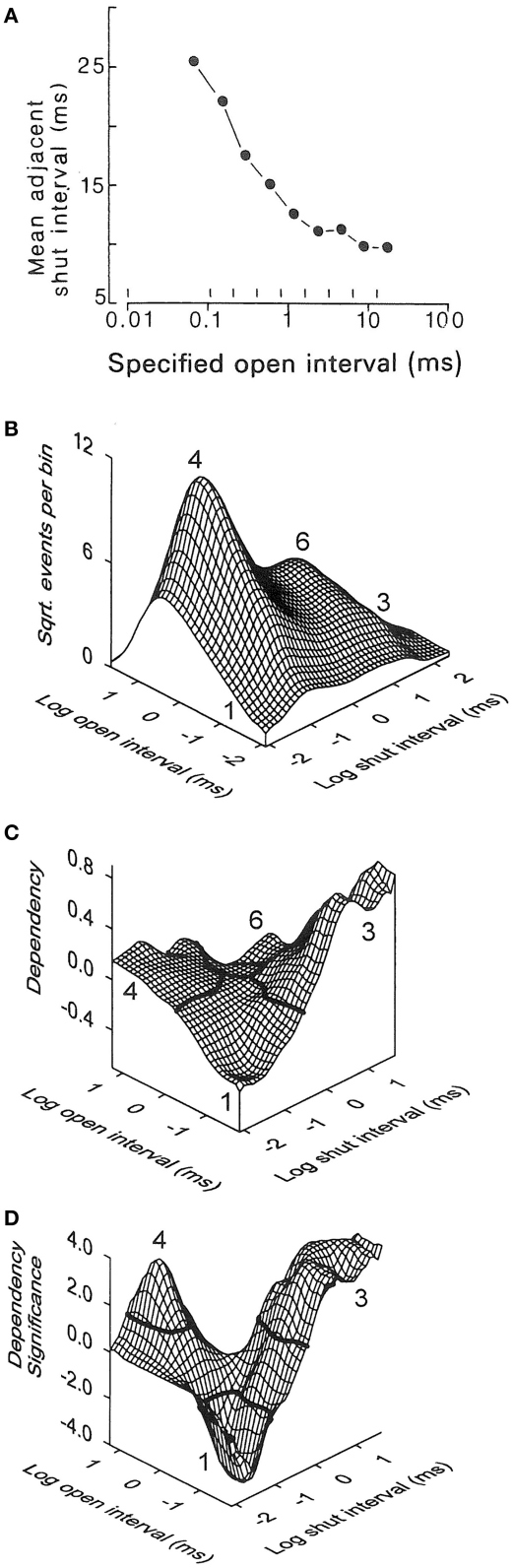


FIGURE 7 | The inverse relationship between the durations of adjacent open and closed intervals suggests two-tiered gating with the lifetimes of the open states on the lower tier inversely related to the ***(Continued)***

FIGURE 7 | Continued
lifetimes of the closed states above them on the upper tier. (A) Inverse correlation between the mean durations of closed intervals adjacent to open intervals of specified durations delineated by the vertical lines near the abscissa. 0.7 μM Ca^{2+}, +30 mV, 64,000 open and closed intervals were analyzed (from McManus et al., 1985). **(B-D)** Kinetic structure of a BK channel details the inverse relationship between durations of adjacent open and closed intervals. **(B)** 2D dwell-time distribution of adjacent open and closed interval pairs presented in a Sigworth and Sine transformation. The *x* and *y* axes indicate the logs of the means of the adjacent open and closed interval durations in each 2D bin. The ordinate gives the square root of the number of adjacent interval pairs in each 2D bin defined by the *x* and *y* axis. The greatest frequency is at number 4 for longer openings adjacent to briefer closings (12.3 μM Ca^{2+}, +30 mV). **(C)** Dependency plot of the excess frequency of interval pairs over that expected if open and closed intervals pair at random. There were 45% more longer closed intervals adjacent to briefer open intervals (number 3), 15% more longer open intervals adjacent to briefer closed intervals (number 4), and 40% fewer briefer open intervals adjacent to briefer closed intervals (number 1), than would be expected by chance pairing. Dependency for any given bin is defined as the difference between the observed number of intervals in that bin minus the number of intervals expected for independent pairing, divided by the number expected for independent pairing. **(D)** The excesses of paired intervals in part **C** are significant ($P < 0.05$) for interval pairs above the solid black lines at numbers 3 and 4, and the deficits are significant for interval pairs below the solid black line at number 1. A significant deficit of longer open intervals adjacent to longer closed intervals (at number 6) cannot be seen on the back side of the plot. 28,560 intervals were analyzed (from Rothberg and Magleby, 1998b). The observed inverse relationships between the durations of adjacent open and closed interval pairs would be consistent with Schemes 4–6 if the closed state lifetimes in these schemes progressively decreased from left to right, and the open state lifetimes progressively increased from left to right.

differ (Steinberg, 1987; Song and Magleby, 1994; Colquhoun and Hawkes, 1995b). No differences were found in single-channel records analyzed in the forwards and backwards directions for BK channels (Song and Magleby, 1994), indicating that the gating for BK channels was consistent with microscopic reversibility. Consequently when fitting data for BK channels, microscopic reversibility is maintained by keeping the product of the rate constants in one direction around each loop of states equal to the product of the rate constants in the opposite direction around the same loop of states for every loop in the model (Colquhoun and Hawkes, 1995b). For Scheme 2 the product of $k_{3-1} \times k_{1-2} \times k_{2-4} \times k_{4-3}$ would need to equal the product of $k_{1-3} \times k_{3-4} \times k_{4-2} \times k_{2-1}$. Hence one of the rate constants in each loop when fitting is not free but is determined by the other seven.

ACTIVATION OF BK CHANNELS BY Ca^{2+}

Activation of a BK channel by Ca^{2+} at the single-channel level was shown in **Figure 6A**. Data obtained from the analysis of four different patches, each containing a single BK channel, are shown in **Figure 8A** (Rothberg and Magleby, 1999). (All data in **Figure 8** were collected at +30 mV.) There was a steep dependence of Po on Ca_i^{2+}, with a maximal Po of 0.95, a half activation, $K_{0.5}$, at 11.1 μM Ca_i^{2+}, and a Hill coefficient of 3.5, (thick line from fitting the Hill equation), suggesting four or more bound Ca^{2+} are required for maximal activation of the channel. Note that four bound Ca^{2+} would be consistent with Schemes 5 and 6. The Hill coefficient of 3.5 indicates a cooperative action of Ca^{2+}

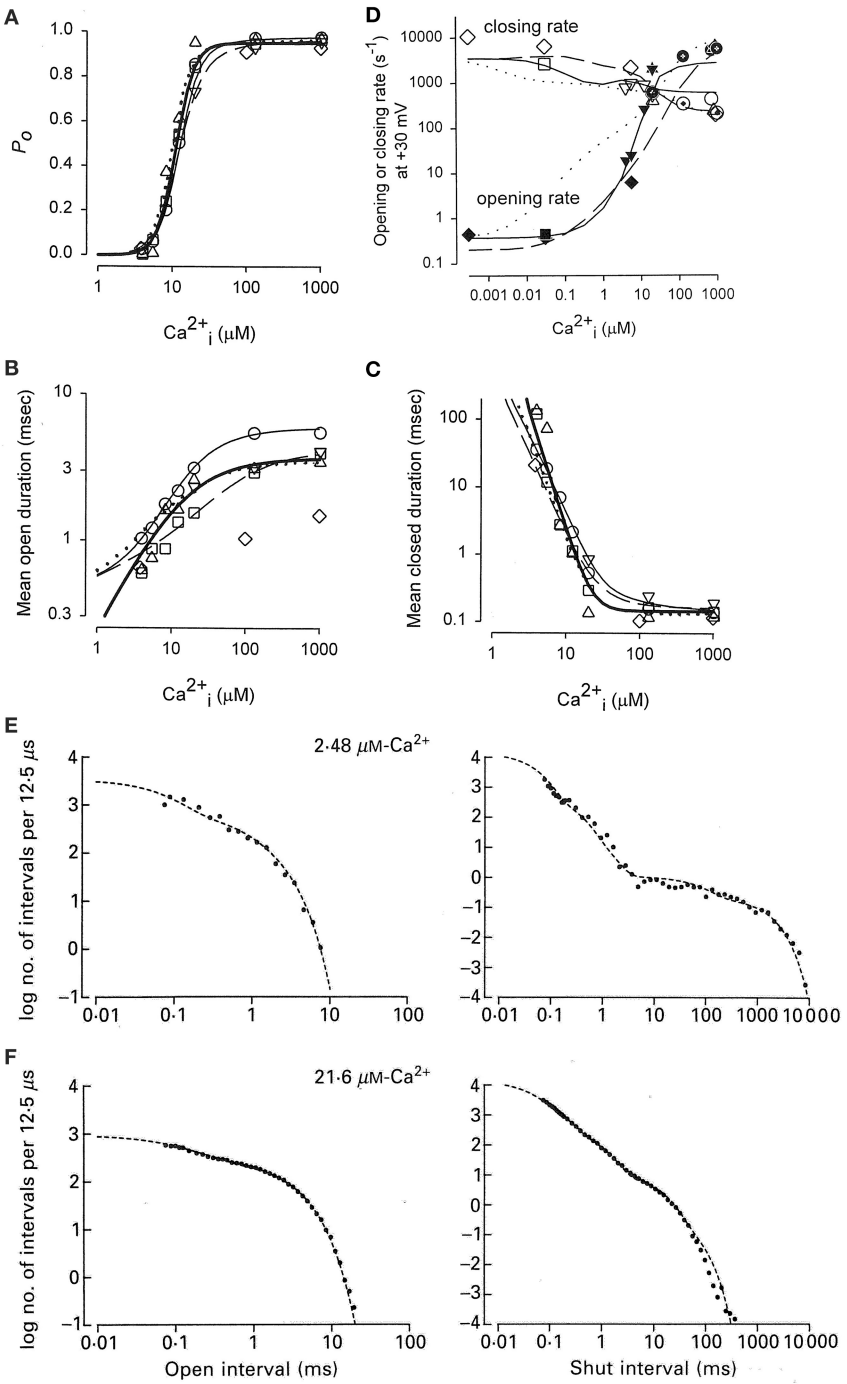


FIGURE 8 | Accounting for the Ca^{2+} dependent gating of BK channels with Scheme 5. (A) Ca^{2+} activates BK channels with a Hill coefficient of 3.5 (heavy black line), indicating cooperative action of Ca^{2+} in opening the channel. **(B)** Ca^{2+} increases mean open interval duration with a Hill coefficient of 1.0 (thick black lines) and **(C)** decreases mean closed interval duration with a Hill coefficient of −3.5. **(D)** Effective mean opening and closing rates as a function of Ca^{2+}. The major action of Ca^{2+} binding is to cooperatively increase the opening rate. The thin lines are the description of the data for models based on subsets of the states in Scheme 6 (Rothberg and Magleby, 1999, 2000). **(E,F)** Predicted dwell-time distributions for Scheme 5 with the two lower left-most open states not included, as they were entered too infrequently to alter the likelihood of the model. Parts **A–C** are from Rothberg and Magleby (1999). Part **D** is from Rothberg and Magleby (2000). Parts **E** and **F** are from McManus and Magleby (1991). All data at +30 mV.

in activating the channel, with at least four sites bound for full activation. Niu and Magleby (2002) found that decreasing the number of Ca^{2+} bowls (by mutation) decreased the Hill coefficient, with Hill coefficients of 4.1, 3.5, 2.6, 1.8, and 1.4 observed for 4, 3, 2, 1, and 0 functional Ca^{2+} bowls. Their observations reveal directly that BK channels can gate with 0–4 high affinity allosteric Ca^{2+} bowl activators. This would be consistent with the two-tiered Schemes 5 and 6 where the channel can open with 0–4 Ca^{2+} sensors bound. Niu and Magleby (2002) also found that a model with 0–4 Ca^{2+} bowl sensors together with four RCK1 Ca^{2+} sensors could describe the Po vs. Ca^{2+} and Hill slope data.

Increasing Ca^{2+} increased observed mean open interval duration 10-fold with a Hill coefficient of 1.02 (**Figure 8B**) and decreased observed mean closed interval duration about 1000-fold with a Hill coefficient of −3.48 (thick lines), for changes in Ca^{2+} from 3 to 1000 μM (**Figure 8C**). Determining the effective mean opening and closing rate constants from the inverse of the mean closed and open dwell-times (Equations 3 and 4) over a wider range of Ca^{2+} gave the plots in **Figure 8D**, where increasing Ca^{2+} from <0.1 to 1000 μM increased the effective opening rate constant 10,000-fold and decreased the effective closing rate constant about 30-fold. Hence, the major action of Ca^{2+} is a highly cooperative increase in the effective rate constant for opening (leaving the closed states), indicating destabilization of the closed states, with a much smaller increase of slowing the effective rate constant for leaving the open states (closing), which gives a much smaller stabilization of the open states.

To explore what types of gating mechanisms could describe the Ca^{2+} activation of BK channels, McManus and Magleby (1991) tested 11 models that were subsets of Scheme 5 and also an extension of Scheme 3 with five closed and four open states with one gateway state. They collected single channel data at 3–4 different Ca^{2+} for each of five patches, each containing a single BK channel. Typically >100,000 intervals were recorded from each channel. All data were collected at +30 mV. Simultaneous maximum likelihood fitting of the data for each channel then gave the most likely estimates for the rate constants and likelihood values. Ranking of the models then indicated that Scheme 5 gave excellent descriptions of the single-channel kinetics and Ca^{2+} activation of the channels. For this channel the two left most open states on the lower tier did not improve the likelihood so they were omitted, consistent with the detection of only three significant open states for this channel. This eight-state Scheme 5 could describe the Ca^{2+} dependence of the single channel kinetics (**Figures 8E,F**). The Po vs. Ca^{2+} curve was also well-described and the correlations between open and closed interval durations was approximated (not shown). The principle activation–deactivation pathway was C_{0Ca}-C_{1Ca}-C_{2Ca}-C_{3Ca}-O_{3Ca}-O_{4Ca}. The closed states C_{0Ca}-C_{1Ca}-C_{2Ca}-C_{3Ca} greatly decreased their lifetimes with each successive Ca^{2+} binding, and the open states O_{2Ca}-O_{3Ca}-O_{4Ca} moderately increased their lifetimes.

Adding an intermediate tier of brief closed states (flickers) significantly improved the description of the Ca-dependent gating (Rothberg and Magleby, 1999). More recently, such intermediate flicker states, termed flip states (Lape et al., 2008) or intermediate states (Mukhtasimova et al., 2009) have been described in the gating of nicotinic receptor channels. Thus, Scheme 5 gave reasonable descriptions of the Ca^{2+}-dependence of the single-channel kinetics for a fixed voltage, with the addition of a tier of flicker closed states improving the description of the data.

ACTIVATION OF BK CHANNELS BY VOLTAGE

Shelley et al. (2010) studied the activation of BK channels by voltage at the single-channel level. The activation of a single BK channel by voltage is shown in **Figure 9A** for voltages ranging from −70 to +100 mV with Ca^{2+} fixed at 95 μM Ca^{2+}. Po ranged from 2×10^{-4} at −70 mV to 0.94 at +100 mV. The fixed 95 μM Ca^{2+} was used to left shift the activation of the channel to voltage ranges where the channel could be fully activated (0.95) without the need to apply such high voltages that the membrane would be destroyed for the longer duration recordings required for single-channel analysis. The RCK1 Ca^{2+} site and the Mg^{2+} site were removed by the mutations D362A/D367A and E399A, respectively (Shi et al., 2002; Xia et al., 2002; Zeng et al., 2005), leaving the Ca-bowl, which would be fully saturated with the 95 μM Ca^{2+}, more than 20 times the apparent K_D (Bao et al., 2002; Xia et al., 2002). The saturating Ca^{2+} would shift the gating to the 10-state model described by the right most 10-states in Scheme 6. This Ca^{2+} saturated 10-state scheme would be indicated by filling in all of the subunits in Scheme 4 to indicate that each subunit has a bound Ca^{2+}.

Data from four different patches, each containing a single channel is shown in **Figures 9B–D**. Fitting the Po vs. voltage data with a Boltzman equation in **Figure 9B** indicated an 11.4 mV depolarization for an e-fold (2.7 times) increase in Po with an effective partial charge movement across the electric field of the membrane of 2.3 units of elementary charge (e_o) for activation (Shelley et al., 2010). This can be compared to Shaker K_V channels, which are more voltage sensitive, with a 2.4 mV depolarization for an e-fold change in Po and an effective partial charge movement of 12.3 e_o for activation (Schoppa et al., 1992). The decreased voltage sensitivity of BK channels arises because only the lowest of the four arginines in S4 of each voltage sensor contributes to the voltage dependence of BK (Ma et al., 2006). Changing the voltage from −100 to +100 mV decreased the observed mean closed time 4500-fold (**Figure 9D**), while increasing the observed mean open time 12-fold (**Figure 9C**). Hence, the predominant action of depolarization is to destabilize the closed states, with a much smaller action on stabilizing the open states.

To examine whether Scheme 4 could account for the voltage activation at the single-channel level, specific Schemes 10, 11, and 12 (**Figure 10A**) were examined by Shelley et al. (2010). Rate constants *A* and *B* for voltage sensor activation and deactivation, respectively, and rate constants *H* and *K* for channel opening and closing, respectively, were exponentially dependent on voltage such that

$$Rate(V) = Rate(V_o)\exp(qV/eo25.5\,mV) \quad (8)$$

where voltage (V) is in mV, V_o is 0 mV, and q is partial charge displacement in units of e_o for the conformational change described by the rate constant, and 25.5 mV (at 23°C) indicates that the rate constant will change e-fold for each elementary unit of charge moved through 25.5 mV of membrane potential (Hille, 2001).

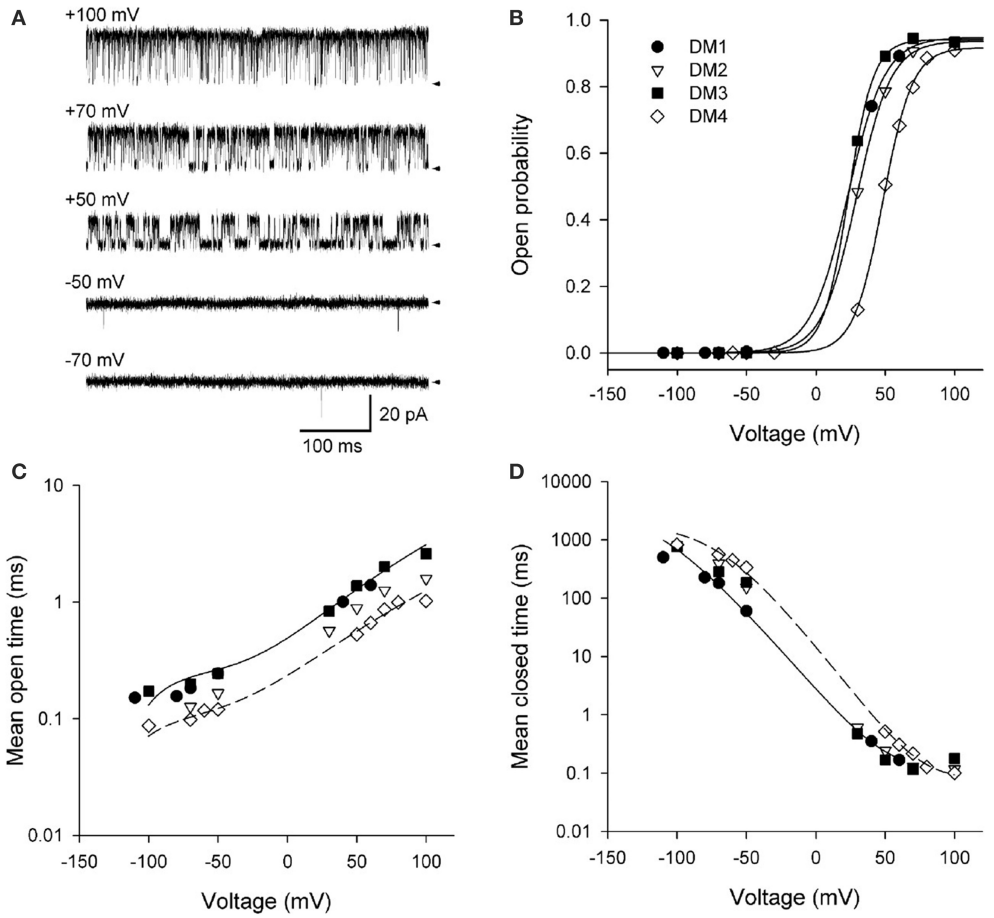


FIGURE 9 | Depolarization activates BK channels. (A) Single-channel records at the indicated voltages. Depolarization activates BK channels, as indicated by the increased time spent open with increasing depolarization. The arrows indicate the closed current level. The channel has the high affinity RCK1 Ca^{2+} site (D362A/D367A) and the low affinity Mg^{2+} site (E399A) on each subunit removed by mutation (Xia et al., 2002). Ninety five micromolar Ca^{2+} was used to saturate the Ca^{2+} bowls. **(B)** Po vs. voltage plots for four different single BK channels. The lines are Boltzman fits indicating an e-fold (2.72) increase in Po for each additional 11.4 mV of depolarization. **(C,D)** Depolarization from −100 to +100 mV increased mean open time 12-fold and decreased mean closed time 4500-fold. The thin lines are the predicted response for DM1 (continuous lines) and DM4 (dashed lines) with Scheme 12. Figures are from Shelley et al. (2010).

For example, for q of 0.3 e_o and Rate (V_o) = 100/s, then the rates at −100 and +100 mV would be 30.8/s and 324/s. The 4, 3, 2, and implied 1 preceding each rate constant above the forward arrows indicates the number of subunits with deactivated voltage sensors, and the implied 1 and 2, 3, and 4 preceding each rate constant below the backwards arrows indicates the number of subunits with activated voltage sensors. Partial Scheme *F* would add a tier of flicker closed states beneath the other schemes with subscript *F* added to the scheme designation.

Single-channel data collected over a range of voltages were simultaneously fit to each of the kinetic Schemes in **Figure 10A**. Scheme 12_F (with the tier of flicker closed states) ranked first followed by Schemes 12, 11_F, 11, and 10. Schemes 12_F and 12 gave excellent description of the voltage dependence of the distributions (**Figures 10B,C**). As different as these Sigworth and Sine plots may look from the log–log plots for Ca^{2+} activation in **Figures 8E,F**, if the same transforms were used for the plots, those with the same Po would look relatively similar. Scheme 10 did not describe the closed dwell-time distributions or the shift in the closed dwell-time distribution with voltage and can be rejected outright. Predictions of all the schemes superimposed for the open distributions and also for Schemes 12 and 12_F for the closed distributions. Interestingly, all of the schemes including Scheme 10 gave excellent descriptions of the Po vs. V curve (equivalent to the continuous lines in **Figure 9B**), indicating that the ability of a model to describe Po vs. voltage is not a critical test of gating mechanism.

For Schemes 12_F and 12 there was cooperativity (U) in subunit activation with activation of deactivated voltage sensors about $U = 4.5$ times faster for every activated S4, with little cooperativity in subunit deactivation ($W = 1.3$). Whether there is actual cooperativity in gating or whether the cooperativity is just a product of the idealized relationships between rate constants in Scheme 12_F and 12 will require additional experiments to resolve. In these schemes, the major voltage dependence of the gating arises from two separate steps. In the first step there is a voltage

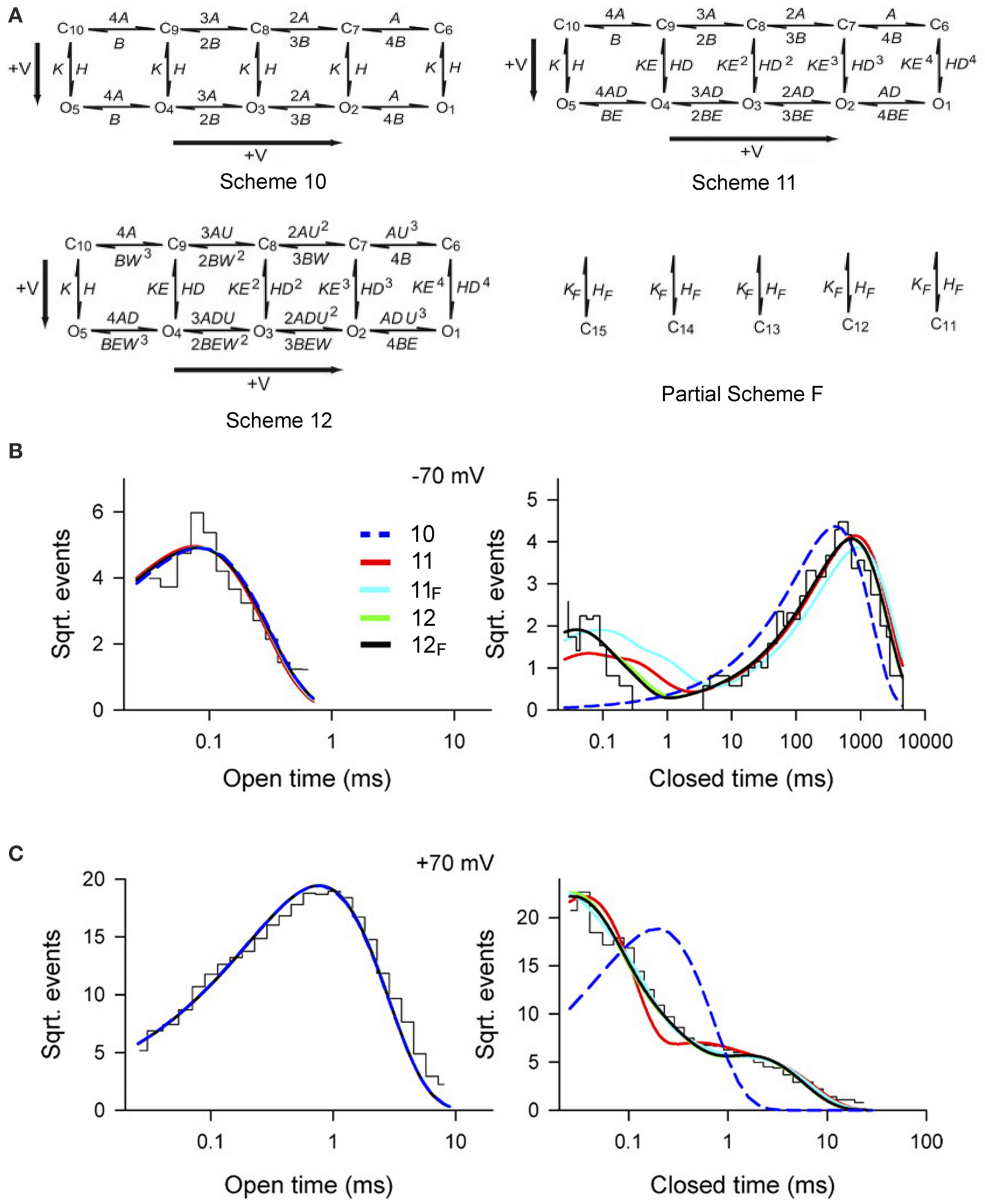


FIGURE 10 | Accounting for the voltage dependent gating of BK channels with Scheme 4, as detailed by Schemes 11 and 12. (A) Examined gating mechanisms. See text for details. **(B,C)** Open and closed dwell-time distributions at −70 and +70 mV, as indicated, for experimental conditions as specified in **Figure 9**. The various lines are the predicted descriptions for the various examined schemes, as indicated. Schemes 12_F and 12 gave excellent descriptions of the data. Schemes 11_F and 11 approximated the data. Scheme 10 could be rejected outright. Predictions of all the examined schemes essentially superimposed for the open distributions, and Schemes 12 and 12_F typically superimposed for the closed distributions. Figures from Shelley et al. (2010).

dependent increase in the rate of voltage sensor activation (mean $q_A = 0.34\ e_o$) and a voltage dependent slowing of voltage sensor deactivation (mean $q_B = -0.18\ e_o$), where q is the partial charge movement associated with these conformations. In the second step this voltage dependent change in the number of activated voltage sensors is then allosterically coupled to the opening and closing transitions in Schemes 12_F, 12, 11_F, and 11 through the coupling factors *D* and *E*. Each activated voltage sensor changes the opening rate *D*-fold and the closing rate *E*-fold. The allosteric acceleration in opening rate *D* for each activated voltage sensor was 22.9, 28.3, 17.9, 17.4 for Schemes 11, 11_F, 12, and 12_F, respectively, and the allosteric acceleration in closing rate, *E*, ranged from 1 to 1.4 for channel closing. A value of *D* of 20 would indicate a 20-, 400-, 8000-, and 160,000-fold increases in opening rate for 1–4 activated voltage sensors, respectively, compared with little effect on the closing rate. Hence, depolarization activates predominantly by accelerating the opening rate, destabilizing the closed states.

A smaller voltage dependence of increasing the opening rate constant *H* with depolarization (q_H ~0.2 e_o) and decreasing the channel closing rate constant *K* (q_K ~ -0.2 e_o) was also observed (Shelley et al., 2010). The voltage dependence of opening *H* and closing *K* rate constants is thought to reflect the further movement of the S4 voltage sensors as the channels open or close (Horrigan and Aldrich, 1999, 2002; Horrigan et al., 1999). To a first approximation, the estimates of partial charge determined from the single-channel analysis (Rothberg and Magleby, 2000; Shelley et al., 2010) are consistent with those estimated from gating current and macro current analysis following voltage steps (Horrigan and Aldrich, 1999, 2002; Horrigan et al., 1999).

The data of Shelley et al. (2010) then show that voltage activation of BK channels can be described at the single-channel level by the 10-state two tiered Scheme 4 as detailed in Scheme 12 (and approximated by Scheme 11), and that an added tier of flicker closed states (Partial Scheme F) improves the description of the data (**Figures 10B,C**). The voltage dependence arises predominantly from each activated voltage sensor greatly accelerating the opening rate.

SYNERGISTIC ACTIVATION OF BK CHANNELS BY Ca^{2+} AND VOLTAGE

The previous sections showed that Schemes 4 and 5 could account separately for the voltage and Ca^{2+} activation of BK channels at the single-channel level. Combining these two schemes gives the 50-state model in Scheme 6. This section reviews the work of Rothberg and Magleby (2000) showing that Scheme 6 can account for the synergistic activation of BK channels by Ca^{2+} and voltage. The joint activation of a BK channel by voltage and Ca^{2+} is shown in **Figure 11**. Increasing either Ca^{2+} (left to right comparison) or depolarization (upper to lower comparison) increased Po. Jointly increasing Ca^{2+} and depolarization (diagonal from upper left to lower right) led to synergistic increases in Po. Po vs. voltage plots for data from seven single-channel patches are plotted over a range of voltage and Ca^{2+} in **Figure 11B** on linear (B) and semi-logarithmic (C) coordinates. The thick solid lines are Boltzman fits, indicating an effecting partial charge movement of 2.3 e_o in determining the Po, which was little affected by Ca^{2+}, as indicated by the near parallel shifts (**Figures 11B,C**). Further support that Ca^{2+} has little effect on the charge movement is the observation by Shelley et al. (2010) that with 95 μM Ca^{2+} their estimate of the partial charge movement was the same as in **Figures 11B,C**. Increasing Ca^{2+} gave approximately parallel left shifts in the Po vs. voltage curves, suggesting independent activation by Ca^{2+} and voltage (Horrigan and Aldrich, 2002), consistent with the modular model of separate voltage and Ca^{2+} activation systems. For essentially zero Ca^{2+} (0.001 μM), a voltage of +150 mV was required to half activate the channel. With 20.3 μM Ca^{2+} the voltage required to half activate the channel was +15 mV, for a left shift of −135 mV by 20.3 μM Ca^{2+}. For BK channels, facilitation or inhibition of activation by various agents or mutations is often quantified in terms of the equivalent left or right shifts in the voltage, respectively, required to restore half activation.

As was shown in the previous sections for Ca^{2+} activation (**Figures 8B–D**) and voltage activation (**Figures 9C,D**) acting separately, activation over a range of Ca^{2+} and voltage also occurs predominately by decreasing closed interval duration with a smaller increase in open interval duration (**Figures 11D,E**). With increased Ca^{2+} and depolarization, the mean closed interval durations decreased to an apparent steady-state value of about 0.18 ms, reflecting mainly the briefest closed intervals (the flickers within bursts) that dominate at high Po. The reciprocals of the thick lines in **Figures 11D,E** indicate a partial charge movement for channel closing of −0.5 e_o and for channel opening of 1.8 e_o. Summing these and changing the sign for channel closing to correcting for the fact that slowing channel closing increases Po, gives a net partial charge for channel activation of 2.3 e_o, as determined from the Po vs. voltage plots in **Figures 11B,C**. These observations indicate that the major effect of depolarization on Po is through an increase in the channel opening rate.

To examine whether the 50-state Scheme 6 that combines Ca^{2+} and voltage activation could account for the joint activation of BK channels by Ca^{2+} and voltage, Rothberg and Magleby (2000) determined whether Scheme 9, a subset of the 50-state scheme, could account for the joint activation. The rational for the reduced state scheme was two-fold: (1) if the reduced state scheme could account for the single-channel kinetics, then the full 50-state scheme would also be able to account for the kinetics because Scheme 9 is contained within the 50-state scheme; and (2) the reduced state scheme decreased the numbers of rate constants sufficiently that they could be determined through simultaneous fitting of data obtained over a range of Ca^{2+} and voltage. **Figure 12** presents experimental open and closed dwell-time distributions for six different combinations of Ca^{2+} and voltage with Po ranging from 0.0061 to 0.82. The reduced state Scheme 9 gave good to excellent descriptions of the data (thick lines). Hence, the more complex 50-state scheme would also be able to account for the single-channel kinetics equivalently or better. Chen et al. (2012) made a direct test of the 50-state Scheme 6 using Scheme 7 to constrain rate constants to greatly reduce the number of free parameters, and found that the highly constrained model could approximate the description of the data, and that the addition of a tier of flicker closed states improved the likelihood of the description. Hence, the 50 state Scheme 6 is consistent with the voltage and Ca^{2+} dependent activation of the BK channel.

ACCOUNTING FOR THE BURSTING BEHAVIOR OF BK CHANNELS

Inspection of the single-channel records in **Figures 1**, **6**, **9**, **11** show that gating of BK channels (as with most other channels) occurs in bursts of openings, with each burst comprised of typically longer openings separated by briefer closings (flickers). The bursts are then separated from each other by longer duration closings (Magleby and Pallotta, 1983a; Nimigean and Magleby, 1999, 2000). To examine whether Scheme 9 captures the single-channel bursting behavior, single-channel records predicted by Scheme 9 were simulated with filtering and noise and plotted in **Figure 13** (From Figure S1 in Rothberg and Magleby, 2000). Comparison of the experimental single-channel current records (**Figure 11A**) with the predicted single-channel records (**Figure 13**) shows that Scheme 9, and consequently Scheme 6, can generate single-channel current records with bursting behavior that mimics the experimental data.

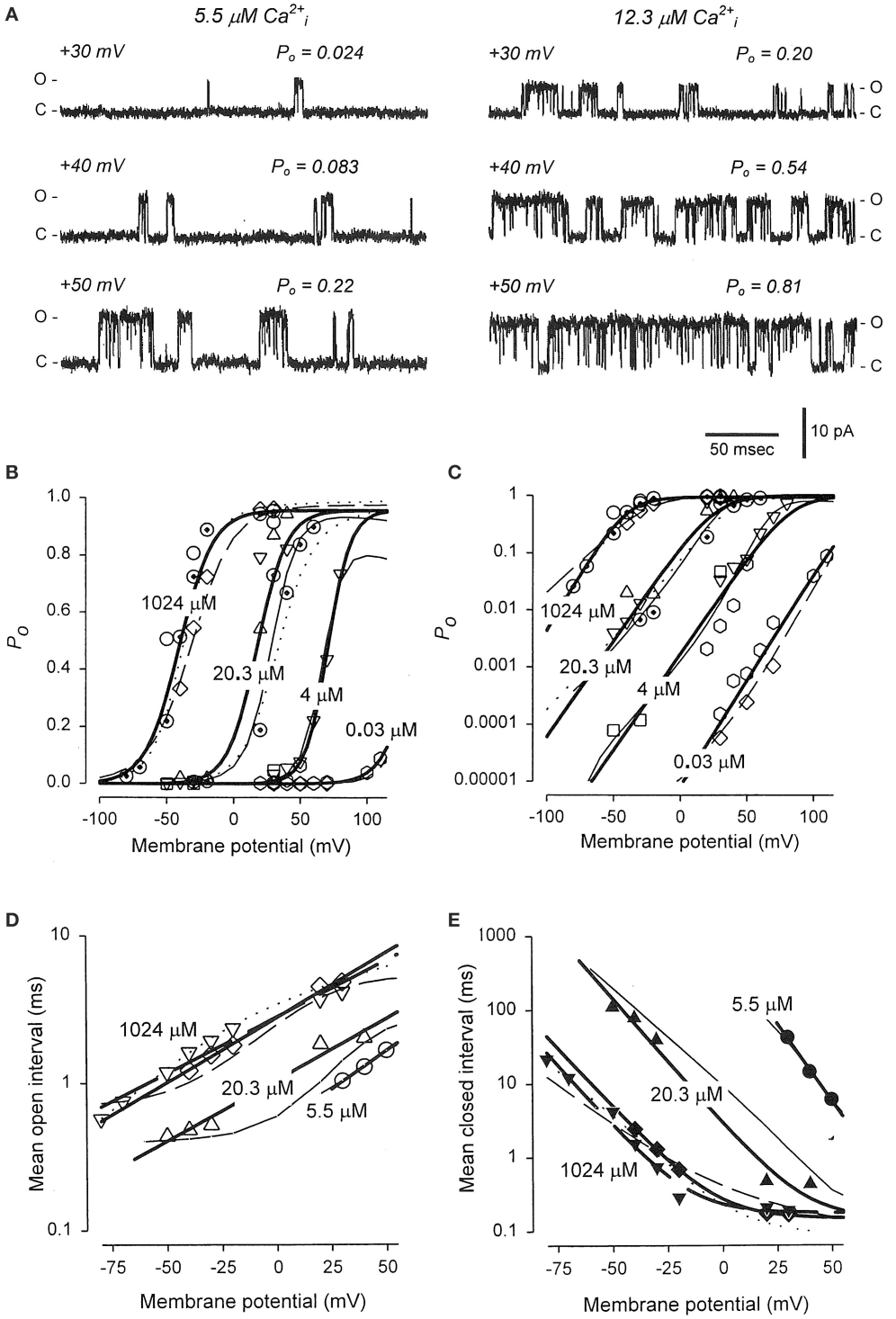


FIGURE 11 | Joint activation of BK channels by Ca^{2+} and depolarization. **(A)** Single-channel records showing that increasing Ca^{2+} or depolarization increased Po, and increasing both together gave a synergistic increase in Po. Currents for six different combinations of Ca^{2+} and voltage are shown. Open (o) and closed (c) current levels are indicated. **(B,C)** Linear and semi-logarithmic plots of Po vs. membrane potential for seven different single-channel patches at four different Ca^{2+}. Thick solid lines are the mean response. Increasing Ca^{2+} left shifts the plots to more negative potentials, indicating that less depolarization is required to activate the channel as Ca^{2+} is increased. **(D,E)** The predominant action of Ca^{2+} and voltage is to decrease mean closed interval duration (part **E**), with a much smaller effect on increasing mean open interval duration (part **D**), and this is the case over a range of Ca^{2+} and voltage. Thick solid lines are the mean response. The thin continuous, dotted, and dashed lines are the predicted responses with Scheme 9, a subset of the 50 state Scheme 6. Figures from Rothberg and Magleby (2000).

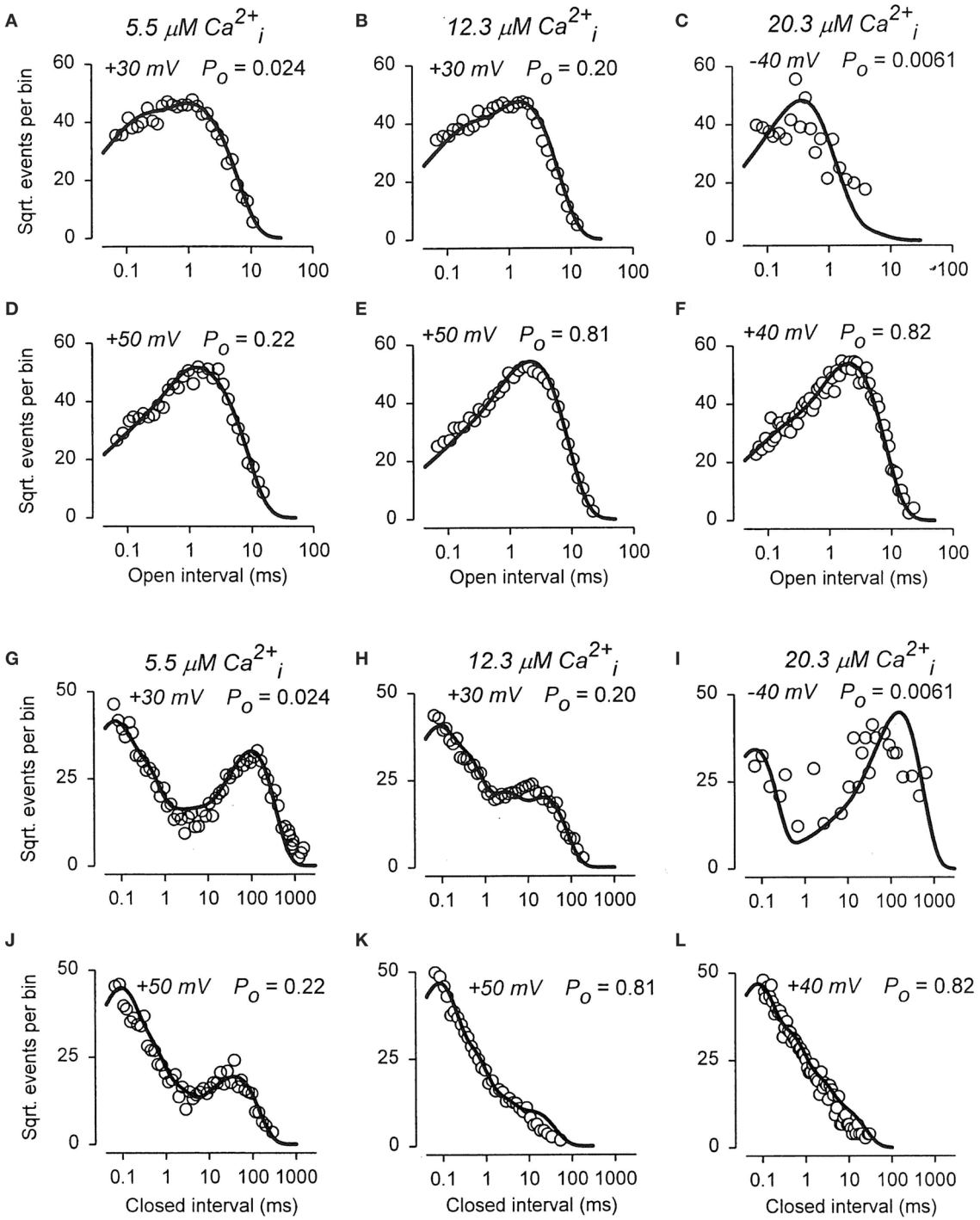


FIGURE 12 | The joint Ca^{2+} and voltage dependent gating of BK channels is consistent with a 50 state two-tiered gating mechanism. **(A–L)** Open and closed dwell-time distributions presented in the Sigworth and Sine transform are plotted for six different combinations of Ca^{2+} and voltage. The heavy lines are the predicted distributions with Scheme 9 with a single set of gating parameters. Because Scheme 9 is a subset of the 50 state Scheme 6, then Scheme 6 should describe the data equivalent or better than Scheme 9. Figure from Rothberg and Magleby (2000).

FUNCTIONS OF THE GATING RING (CTD)

As indicated previously in the Modular Structure Section, extensive mutational studies have suggested two high affinity Ca^{2+} binding sites on each subunit of BK channels, the Ca-bowl and the RCK1 site, located in the RCK2 and RCK1 domains, respectively, of each of the four subunits that form the gating ring. Extensive mutational studies also indicated that a low affinity Mg^{2+} site is sandwiched between the top of the gating ring and the cytosolic

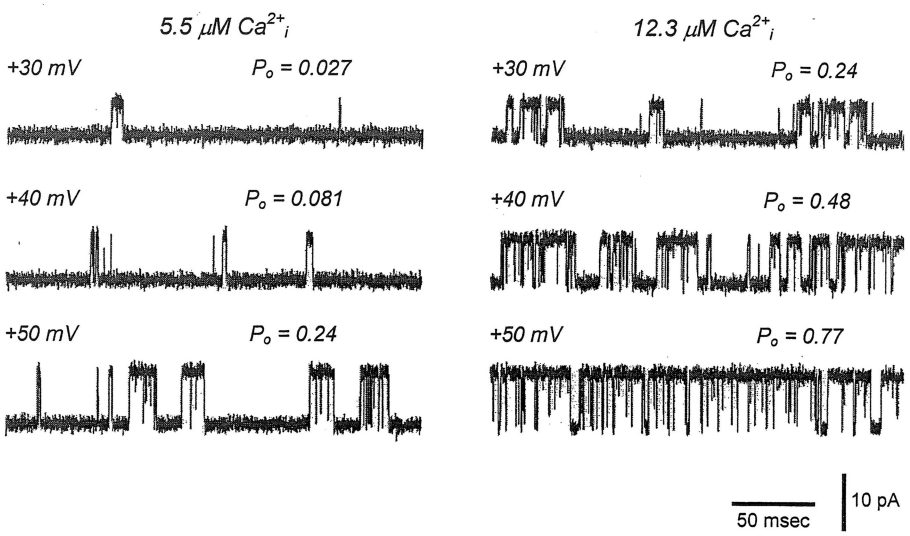


FIGURE 13 | Accounting for the bursting behavior of BK channels with Schemes 9 and 6. Simulated single-channel currents from Figure S1 of Rothberg and Magleby (2000) for comparison to the experimental data in **Figure 11A**. The currents were simulated with Scheme 9 with filtering and noise equivalent to that of the experimental data. The values of Po were obtained from 10,000 simulated intervals at each of the indicated Ca^{2+} and voltage. Opening is upward. Scheme 9 predicts single-channel Po and bursting behavior records that are remarkably similar to the experimental data. Because Scheme 9 is a subset of Scheme 6, then Scheme 6 would also be able to predict the single-channel data equivalently or better.

side of each of the voltage sensors (see **Figure 2** and associated references). On this basis, removing the gating ring should remove all of the Ca^{2+} and Mg^{2+} sensitivity. Budelli et al. (2013) found that removing the 827 amino acid gating ring and replacing it with a short 11 amino acid tail removed all of the Ca^{2+} and Mg^{2+} sensitivity from the BK channel. Hence, as indicated in the modular diagram in **Figure 2**, the Ca^{2+} sensors are located in the gating ring and the Mg^{2+} sensors require the gating ring.

To explore if the gating ring acts by pulling on S6 in the pore gate domain through the RCK1-S6 linkers, Niu et al. (2004) shortened and lengthened the linkers. In the absence of Ca^{2+}, shortening the linkers by -1 or -3 amino acids increased channel activity (**Figures 14A–C**), shifting the Po vs. voltage curves to the left (**Figure 14D**), and lengthening the linkers by +3, +6, or +12 amino acids decreased channel activity (**Figures 14A–C**), shifting the Po vs. voltage curves to the right (**Figure 14D**). A possible explanation for these observations is that lengthening the linkers decreased Po by decreasing the pull of the linkers on the PGD. The Po could then be restored by increasing the depolarization which increased the pull the voltage sensors applied to the PGD through the S4–S5 linkers (**Figure 2**). Conversely, shortening the RCK1-S6 linkers would increase the pull on the PGD, which could then be compensated for by applying less depolarization.

In the absence of Ca^{2+}, Niu et al. (2004) observed a near linear relationship between $V_{0.5}$ and changes in linker length (**Figure 14E**). This relationship could be modeled by assuming that the linker-gating ring complex acts as a passive spring in the absence of Ca^{2+}, passively pulling on the PGD to bias the channel toward opening in the absence of Ca^{2+}. If a passive opening force by the gating ring were not the case then lengthening the linkers should have had little effect on Po, in contrast to the marked decrease in activity that was observed. Further support that the linker-gating ring complex applies a passive opening force to the channel comes from the observations of Budelli et al. (2013) that BK channels without a gating ring have a right shifted Po vs. voltage curve, indicating that more voltage is required to open the channel in the absence of the gating ring, presumably to replace the passive opening force that was applied by the gating ring before it was removed. Budelli et al. also found that removing the gating ring in the absence of Ca^{2+} decreased mean open interval duration, consistent with the proposal that the gating ring applies a passive opening force to the gates in the absence of Ca^{2+}. A major structural contributor to the passive spring may be the AC region of the RCK1 domain (Krishnamoorthy et al., 2005).

In the presence of Ca^{2+}, changing the RCK1-S6 linker length had a rather different effect than in the absence of Ca^{2+}. Niu et al. (2004) observed that lengthening the linkers in the presence of Ca^{2+} decreased the Ca^{2+} dependent increase in Po over a range of Ca^{2+} (**Figure 14F**). These observations suggest that the linkers are involved in transmitting the action of Ca^{2+} to the PGD. Their further observation that in higher Ca^{2+} that lengthening the linkers gave the least decrease in Po (**Figure 14F**) and the greatest decrease at low Ca^{2+} suggests that high Ca^{2+} may provide a greater range of movement in the gating ring which could compensate to partially overcome the extra length of the RCK1-S6 linkers. From macro-current analysis Pico (2003) has made some similar observations for the effects of changes in linker length on Ca^{2+} activation.

The observations in the previous sections are consistent with the RCK1-S6 linkers transmitting force from the gating ring to the PGD to activate the channel. In the absence of Ca^{2+} the gating ring applies a passive opening force to the PGD, and in the

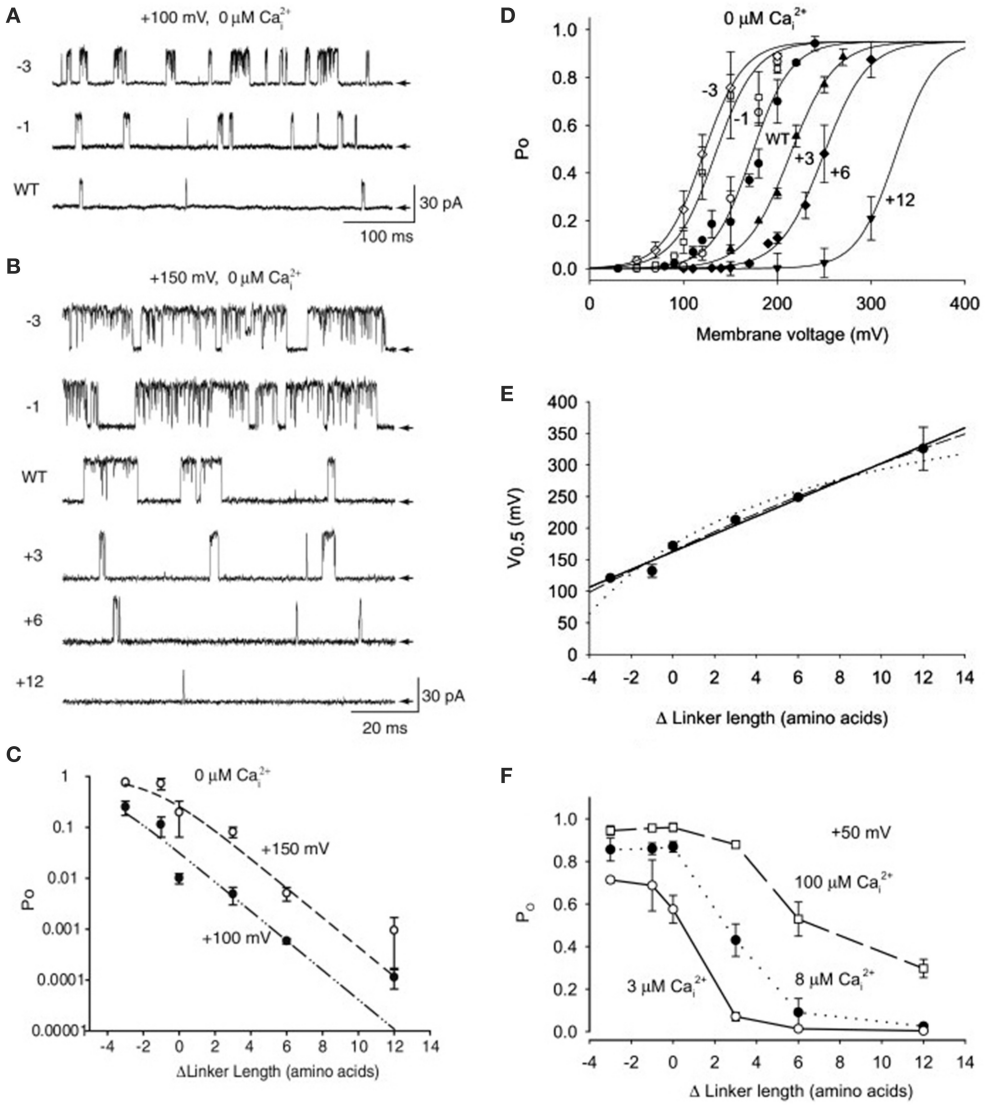


FIGURE 14 | The linker-gating ring complex applies passive spring force to open the channel in 0 Ca^{2+} and active machine force to open the channel in the presence of Ca^{2+}. (A,B) In the absence of Ca^{2+} shortening the RCK1-S6 linkers connecting the gating ring to the PGD (diagrammed in **Figure 2**) by 1 or 3 amino acids (−1, −3) increased channel activity. Two examples are presented, at +100 and +150 mV. Lengthening the linkers by +3, +6, or +12 amino acids progressively decreased channel activity. The arrows indicate the closed level of the single channel currents. **(C)** Plot of Po vs. change in linker length at two different potentials for data like that in parts **A** and **B. (D)** Shortening the RCK1-S6 linkers shifted the Po vs. voltage plots to the left and lengthening the linkers shifted the plots to the right. The shift in the voltage for half activation gives a quantitative measure of the change in force associated with the change in linker length. **(E)** The plot of the voltage for half activation vs. linker length at 0 Ca^{2+} is approximated by a straight line, indicating that the linker gating ring complex acts as a passive spring. **(F)** Increasing linker length decreased the action of Ca^{2+}, with the greatest decrease at low Ca^{2+}, suggesting that the linker transmits opening force from the gating ring to the PGD, and that increasing Ca^{2+} increased the force and/or distance of the Ca^{2+}-induced movement of the RCK1-S6 linker. Data from Niu et al. (2004).

presence of Ca^{2+} the gating ring applies active opening force. Yuan et al. (2012) have constructed an interpolative movie using a BK gating ring with MthK and K_V transmembrane domains to show how Ca^{2+}-induced movement in the gating ring may be transmitted to the PGD through the RCK1-S6 linkers.

SUMMARY OF GATING MECHANISM

This brief review of single-channel gating kinetics has presented a few selected single-channel experiments as an introduction to gating mechanism for BK channels. The Ca^{2+}-dependent gating and the voltage dependent gating can each be approximated by two-tiered 10-state models with five closed states on the upper tier and five open states on the lower tier (Schemes 4 and 5). The joint activation by Ca^{2+} and voltage is obtained with five repeats of the 10-state voltage activation mechanism, where each repeat has 0, 1, 2, 3, or 4 bound Ca^{2+}. Alternatively, the joint activation can be obtained with five repeats of the 10-state Ca^{2+} activation mechanism, where each repeat has 0, 1, 2, 3, or 4 activated voltage

sensors. Both of these approaches give the same 50-state two-tiered model comprised of 25 closed states on an upper tier and 25 open states on a lower tier. Adding a tier of flicker closed states to extend the 10-state models to three tiers and 15 states and the 50-state model to three tiers and 75 states improves the description of all the models. Ca^{2+} and depolarization activate BK channels by predominantly increasing the rate of channel opening, with a much smaller decrease in the rate of channel closing. Hence, Ca^{2+} and depolarization activate BK channels by mainly destabilizing the closed states.

To a first approximation, voltage and Ca^{2+} can act relatively independently to activate the channel, consistent with separate modules for voltage activation (VSDs) and Ca^{2+} activation (gating ring), with both allosterically coupling at the PGD (**Figure 2**). Nevertheless, detailed analysis suggests that there may be interactions within and among the various sensors (Horrigan and Aldrich, 2002; Qian et al., 2006; Shelley et al., 2010). Such interaction might be anticipated because the voltage sensors would be coupled to each other through their attachment to the PGD, so that movement in one VSD might influence movement in the other VSDs. Similarly the Ca^{2+} sensor domains, RCK1 and RCK2, in the gating ring could interact through the gating ring itself and also through their attachment to the PGD. Because VSDs and the Ca^{2+} sensors are both attached to the PGD, then they could interact allosterically through the PGD. Such interaction would be expected for allosteric models of activation and has been observed (Horrigan and Aldrich, 2002).

This review has presented only a brief introduction to gating of BK channels with observations and references restricted in most part to selected single-channel recording and analysis papers. For those who have made it this far and would like to delve deeper into the details of gating mechanism, some of the following papers could be consulted (Cox et al., 1997; Cui et al., 1997; Rothberg et al., 1997; Rothberg and Magleby, 1998a,b, 1999, 2000; Horrigan and Aldrich, 1999, 2002; Horrigan et al., 1999; Cui and Aldrich, 2000; Shi and Cui, 2001; Zhang et al., 2001; Magleby, 2003; Chen et al., 2012), as well as the reviews listed at the beginning of this review.

NEEDED STUDIES

A full 50-state model (75 states with the flicker closed states), rather than the subset of states from the 50-state model (Scheme 9), will be needed with rate constants to fully describe the kinetics underlying gating for the joint activation of BK channels by voltage and Ca^{2+}. A preliminary study has shown that a highly constrained 50-state model can approximate the single-channel data (Chen et al., 2012). A viable model would describe both the single-channel kinetics and the kinetics of the macro current data, as the average of the single-channel responses gives the macro currents.

Future studies need to address to what extent the gating parameters determined by single-channel analysis can account for the gating currents and the pseudo mono-exponential relaxations of macro currents associated with activation and deactivation following voltage steps (Cox et al., 1997; Horrigan and Aldrich, 1999; Horrigan et al., 1999). To account for mono-exponential relaxations, it was proposed that Ca^{2+} binding and voltage sensing must be fast compared to channel opening and closing (Horrigan and Aldrich, 1999; Horrigan et al., 1999). A test of this proposal and also of the ability of single-channel analysis to estimate parameters typically obtained from voltage jumps could be obtained by using gating parameters estimated from single-channel analysis to predict macro currents for comparison to the observed macro current data.

BK models need to be expanded to take into account the two high affinity Ca^{2+} binding sites on each subunit rather than the usual assumption of one high affinity site. This extension seems essential toward understanding the gating mechanism, as the RCK1 site and Ca^{2+} bowl site may well-act through different mechanisms. This would increase the number of states to 250 and to 375 with a tier of flicker states, but experimental approaches and analysis techniques are available to study these more complete models. Some view such large state models with incredulity, but gating in structurally realistic models with the expected number of states is the same process as gating in simplified models with a limited number of states. No matter the number of states, the channel occupies only one state at any time, and then enters the next state based on the probabilities of the various transition pathways away from the occupied state. If BK channels threw up their voltage sensors and gave up on gating because of disbelief in the large number of potential states available to enter, then our health would surely suffer. With powerful experimental and computational techniques, and fast desktop computers, certainly we can stretch our sights out at least as far as our BK channels do. A 250-state model is just three 10-state models working simultaneously, each of which can be studied separately to obtain the parameters and then combined. Differences between the experimentally observed gating and the gating predicted by a 250-state model would then indicate potential interactions within and among the various domains of the channel, providing further insight into gating mechanism.

AUTHOR CONTRIBUTIONS

Both authors contributed to writing, revising, and approval of the manuscript.

ACKNOWLEDGMENTS

The authors are indebted to the previous members of the laboratory who carried out much of the single-channel data collection and presented in this review. This work was supported in part by the National Institutes of Health Grant AR032805 to Karl L. Magleby.

REFERENCES

Bao, L., Rapin, A. M., Holmstrand, E. C., and Cox, D. H. (2002). Elimination of the BK(Ca) channel's high-affinity Ca^{2+} sensitivity. *J. Gen. Physiol.* 120, 173–189. doi: 10.1085/jgp.20028627

Barrett, J. N., Magleby, K. L., and Pallotta, B. S. (1982). Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol.* 331, 211–230. doi: 10.1113/jphysiol.1982.sp014370

Blatz, A. L., and Magleby, K. L. (1986). Correcting single channel data for missed events. *Biophys. J.* 49, 967–980. doi: 10.1016/S0006-3495(86)83725-0

Budelli, G., Geng, Y., Butler, A., Magleby, K. L., and Salkoff, L. (2013). Properties of Slo1 K^+ channels with and without the gating ring. *Proc. Natl. Acad. Sci. U.S.A.* 110, 16657–16662. doi: 10.1073/pnas.1313433110

Changeux, J. P., and Edelstein, S. J. (1998). Allosteric receptors after 30 years. *Neuron* 21, 959–980. doi: 10.1016/S0896-6273(00)80616-9

Chen, R.-S., Geng, Y., and Magleby, K. L. (2012). Voltage- and Ca^{2+} activation of BK channels studied with highly constrained allosteric gating mechanisms. *Biophys. J.* 102(Suppl. 1), p684a. doi: 10.1016/j.bpj.2011.11.3720

Chen, X., Yan, J., and Aldrich, R. W. (2014). BK channel opening involves side-chain reorientation of multiple deep-pore residues. *Proc. Natl. Acad. Sci. U.S.A.* 111, E79–E88. doi: 10.1073/pnas.1321697111

Colquhoun, D., Hatton, C. J., and Hawkes, A. G. (2003). The quality of maximum likelihood estimates of ion channel rate constants. *J. Physiol.* 547, 699–728. doi: 10.1113/jphysiol.2002.034165

Colquhoun, D., and Hawkes, A. G. (1982). On the stochastic properties of bursts of single ion channel openings and of clusters of bursts. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 300, 1–59. doi: 10.1098/rstb.1982.0156

Colquhoun, D., and Hawkes, A. G. (1987). A note on correlations in single ion channel records. *Proc. R. Soc. Lond. B Biol. Sci.* 230, 15–52. doi: 10.1098/rspb.1987.0008

Colquhoun, D., and Hawkes, A. G. (1995a). "A Q-matrix cookbook," in *Single-Channel Recording, 2nd Edn.*, eds B. Sakmann and E. Neher (New York, NY: Plenum Press), 589–633. doi: 10.1007/978-1-4419-1229-9_20

Colquhoun, D., and Hawkes, A. G. (1995b). "The principles of the stochastic interpretation of ion-channel mechanisms," in *Single-Channel Recording, 2nd Edn.*, eds B. Sakmann and E. Neher (New York, NY: Plenum Press), 397–482.

Contreras, G. F., Castillo, K., Enrique, N., Carrasquel-Ursulaez, W., Castillo, J. P., Milesi, V., et al. (2013). A BK (Slo1) channel journey from molecule to physiology. *Channels (Austin)* 7, 442–458. doi: 10.4161/chan.26242

Cox, D. H. (2005). The BK_{Ca} channel's Ca^{2+}-binding sites, multiple sites, multiple ions. *J. Gen. Physiol.* 125, 253–255. doi: 10.1085/jgp.200509270

Cox, D. H. (2007). "BK_{Ca}-channel structure and function," in *Biological Membrane Ion Channels*, eds S.-H. Chung, O. Anderson, and V. Krishnamvrthy (New York, NY: Springer), 171–218. doi: 10.1007/0-387-68919-2_5

Cox, D. H., Cui, J., and Aldrich, R. W. (1997). Allosteric gating of a large conductance Ca-activated K^+ channel. *J. Gen. Physiol.* 110, 257–281. doi: 10.1085/jgp.110.3.257

Crouzy, S. C., and Sigworth, F. J. (1990). Yet another approach to the dwell-time omission problem of single-channel analysis. *Biophys. J.* 58, 731–743. doi: 10.1016/S0006-3495(90)82416-4

Cui, J., and Aldrich, R. W. (2000). Allosteric linkage between voltage and Ca^{2+}-dependent activation of BK-type mslo1 K^+ channels. *Biochemistry* 39, 15612–15619. doi: 10.1021/bi001509+

Cui, J., Cox, D. H., and Aldrich, R. W. (1997). Intrinsic voltage dependence and Ca^{2+} regulation of mslo large conductance Ca-activated K^+ channels. *J. Gen. Physiol.* 109, 647–673. doi: 10.1085/jgp.109.5.647

Cui, J., Yang, H., and Lee, U. S. (2009). Molecular mechanisms of BK channel activation. *Cell. Mol. Life Sci.* 66, 852–875. doi: 10.1007/s00018-008-8609-x

Cukierman, S., Quigley, E. P., and Crumrine, D. S. (1997). Proton conduction in gramicidin A and in its dioxolane-linker dimer in different lipid bilayers. *Biophys. J.* 73, 2489–2502. doi:10.1016/S0006-3495(97)78277-8

Du, W., Bautista, J. F., Yang, H., Diez-Sampedro, A., You, S. A., Wang, L., et al. (2005). Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat. Genet.* 37, 733–738. doi: 10.1038/ng1585

Ferguson, W. B., McManus, O. B., and Magleby, K. L. (1993). Opening and closing transitions for BK channels often occur in two steps via sojourns through a brief lifetime subconductance state. *Biophys. J.* 65, 702–714. doi: 10.1016/S0006-3495(93)81097-X

Frauenfelder, H. (2014). Ask not what physics can do for biology-ask what biology can do for physics. *Phys. Biol.* 11, 053004. doi: 10.1088/1478-3975/11/5/053004

Fredkin, D. R., Montal, M., and Rice, J. A. (1985). "Identification of aggregated Markovian models: application to the nicotinic acetylcholine receptor," in *Proceedings of the Berkeley Conference in Honor of Jerzy Neyman and Jack Kiefer*, eds L. M. LeCam and R. A. Olshen (Belmont: Wadsworth Press), 269–289.

Gibb, A. J., and Colquhoun, D. (1992). Activation of N-methyl-D-aspartate receptors by L-glutamate in cells dissociated from adult rat hippocampus. *J. Physiol.* 456, 143–179. doi: 10.1113/jphysiol.1992.sp019331

Gil, Z., Magleby, K. L., and Silberberg, S. D. (2001). Two-dimensional kinetic analysis suggests nonsequential gating of mechanosensitive channels in Xenopus oocytes. *Biophys. J.* 81, 2082–2099. doi: 10.1016/S0006-3495(01)75857-2

Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391, 85–100. doi: 10.1007/BF00656997

Hawkes, A. G., Jalali, A., and Colquhoun, D. (1992). Asymptotic distributions of apparent open times and shut times in a single channel record allowing for the omission of brief events. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 337, 383–404. doi: 10.1098/rstb.1992.0116

Hille, B. (2001). *Ion Channels of Excitable Membranes, 3rd Edn.* Sunderland: Sinauer.

Hodgkin, A. L., and Huxley, A. F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* 117, 500–544. doi: 10.1113/jphysiol.1952.sp004764

Horn, R., and Vandenberg, C. A. (1984). Statistical properties of single sodium channels. *J. Gen. Physiol.* 84, 505–534. doi: 10.1085/jgp.84.4.505

Horrigan, F. T. (2012). Conformational coupling in BK potassium channels. *J. Gen. Physiol.* 140, 625–634. doi: 10.1085/jgp.201210849

Horrigan, F. T., and Aldrich, R. W. (1999). Allosteric voltage gating of potassium channels II. Mslo channel gating charge movement in the absence of Ca^{2+}. *J. Gen. Physiol.* 114, 305–336. doi: 10.1085/jgp.114.2.305

Horrigan, F. T., and Aldrich, R. W. (2002). Coupling between voltage sensor activation, Ca^{2+} binding and channel opening in large conductance (BK) potassium channels. *J. Gen. Physiol.* 120, 267–305. doi: 10.1085/jgp.20028605

Horrigan, F. T., Cui, J., and Aldrich, R. W. (1999). Allosteric voltage gating of potassium channels I. Mslo ionic currents in the absence of Ca^{2+}. *J. Gen. Physiol.* 114, 277–304. doi: 10.1085/jgp.114.2.277

Hoshi, T., Pantazis, A., and Olcese, R. (2013). Transduction of voltage and Ca^{2+} signals by Slo1 BK channels. *Physiology (Bethesda)* 28, 172–189. doi: 10.1152/physiol.00055.2012

Hu, L., Shi, J., Ma, Z., Krishnamoorthy, G., Sieling, F., Zhang, G., et al. (2003). Participation of the S4 voltage sensor in the Mg^{2+}-dependent activation of large conductance (BK) K^+ channels. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10488–10493. doi: 10.1073/pnas.1834300100

Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T., and MacKinnon, R. (2002). Crystal structure and mechanism of a calcium-gated potassium channel. *Nature* 417, 515–522. doi: 10.1038/417515a

Kienker, P. (1989). Equivalence of aggregated Markov models of ion-channel gating. *Proc. R. Soc. Lond. B Biol. Sci.* 236, 269–309. doi: 10.1098/rspb.1989.0024

Krishnamoorthy, G., Shi, J., Sept, D., and Cui, J. (2005). The NH2 terminus of RCK1 domain regulates Ca^{2+}-dependent BK(Ca) channel gating. *J. Gen. Physiol.* 126, 227–241. doi: 10.1085/jgp.200509321

Landowne, D., Yuan, B., and Magleby, K. L. (2013). Exponential sum-fitting of dwell-time distributions without specifying starting parameters. *Biophys. J.* 104, 2383–2391. doi: 10.1016/j.bpj.2013.04.030

Lape, R., Colquhoun, D., and Sivilotti, L. G. (2008). On the nature of partial agonism in the nicotinic receptor superfamily. *Nature* 454, 722–727. doi: 10.1038/nature07139

Latorre, R., and Brauchi, S. (2006). Large conductance Ca^{2+}-activated K^+ (BK) channel: activation by Ca^{2+} and voltage. *Biol. Res.* 39, 385–401. doi: 10.4067/S0716-97602006000300003

Latorre, R., Morera, F. J., and Zaelzer, C. (2010). Allosteric interactions and the modular nature of the voltage- and Ca^{2+}-activated (BK) channel. *J. Physiol.* 588, 3141–3148. doi: 10.1113/jphysiol.2010.191999

Latorre, R., Vergara, C., and Hidalgo, C. (1982). Reconstitution in planar lipid bilayers of a Ca^{2+}-dependent K^+ channel from transverse tubule membranes isolated from rabbit skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.* 79, 805–809. doi: 10.1073/pnas.79.3.805

Laumonnier, F., Roger, S., Guerin, P., Molinari, F., M'rad, R., Cahard, D., et al. (2006). Association of a functional deficit of the BK_{Ca} channel, a synaptic regulator of neuronal excitability, with autism and mental retardation. *Am. J. Psychiatry* 163, 1622–1629. doi: 10.1176/appi.ajp.163.9.1622

Lee, U. S., and Cui, J. (2010). BK channel activation: structural and functional insights. *Trends Neurosci.* 33, 415–423. doi: 10.1016/j.tins.2010.06.004

Ma, Z., Lou, X. J., and Horrigan, F. T. (2006). Role of charged residues in the S1-S4 voltage sensor of BK channels. *J. Gen. Physiol.* 127, 309–328. doi: 10.1085/jgp.200509421

Magleby, K. L. (2003). Gating mechanism of BK (Slo1) channels: so near, yet so far. *J. Gen. Physiol.* 121, 81–96. doi: 10.1085/jgp.20028721

Magleby, K. L., and Pallotta, B. S. (1983a). Burst kinetics of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol.* 344, 605–623. doi: 10.1113/jphysiol.1983.sp014958

Magleby, K. L., and Pallotta, B. S. (1983b). Calcium dependence of open and shut interval distributions from calcium- activated potassium channels in cultured rat muscle. *J. Physiol.* 344, 585–604. doi: 10.1113/jphysiol.1983.sp014957

Magleby, K. L., and Song, L. (1992). Dependency plots suggest the kinetic structure of ion channels. *Proc. R. Soc. Lond. B Biol. Sci.* 249, 133–142. doi: 10.1098/rspb.1992.0095

Marks, T. N., and Jones, S. W. (1992). Calcium currents in the A7r5 smooth muscle-derived cell line. An allosteric model for calcium channel activation and dihydropyridine agonist action. *J. Gen. Physiol.* 99, 367–390. doi: 10.1085/jgp.99.3.367

Marty, A. (1981). Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature* 291, 497–500. doi: 10.1038/291497a0

McCormack, K., Joiner, W. J., and Heinemann, S. H. (1994). A characterization of the activating structural rearrangements in voltage-dependent Shaker K^+ channels. *Neuron* 12, 301–315. doi: 10.1016/0896-6273(94)90273-9

McManus, O. B., Blatz, A. L., and Magleby, K. L. (1985). Inverse relationship of the durations of adjacent open and shut intervals for C1 and K channels. *Nature* 317, 625–627. doi: 10.1038/317625a0

McManus, O. B., Blatz, A. L., and Magleby, K. L. (1987). Sampling, log binning, fitting, and plotting durations of open and shut intervals from single channels and the effects of noise. *Pflugers Arch.* 410, 530–553. doi: 10.1007/BF00586537

McManus, O. B., and Magleby, K. L. (1988). Kinetic states and modes of single large-conductance calcium-activated potassium channels in cultured rat skeletal muscle. *J. Physiol.* 402, 79–120. doi: 10.1113/jphysiol.1988.sp017195

McManus, O. B., and Magleby, K. L. (1989). Kinetic time constants independent of previous single-channel activity suggest Markov gating for a large conductance Ca-activated K channel. *J. Gen. Physiol.* 94, 1037–1070. doi: 10.1085/jgp.94.6.1037

McManus, O. B., and Magleby, K. L. (1991). Accounting for the Ca^{2+}-dependent kinetics of single large- conductance Ca^{2+}-activated K^+ channels in rat skeletal muscle. *J. Physiol.* 443, 739–777. doi: 10.1113/jphysiol.1991.sp018861

McManus, O. B., Weiss, D. S., Spivak, C. E., Blatz, A. L., and Magleby, K. L. (1988). Fractal models are inadequate for the kinetics of four different ion channels. *Biophys. J.* 54, 859–870. doi: 10.1016/S0006-3495(88)83022-4

Monod, J., Wyman, J., and Changeux, J. P. (1965). On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* 12, 88–118. doi: 10.1016/S0022-2836(65)80285-6

Mukhtasimova, N., Lee, W. Y., Wang, H. L., and Sine, S. M. (2009). Detection and trapping of intermediate states priming nicotinic receptor channel opening. *Nature* 459, 451–454. doi: 10.1038/nature07923

Nimigean, C. M., and Magleby, K. L. (1999). The beta subunit increases the Ca^{2+} sensitivity of large conductance Ca^{2+}-activated potassium channels by retaining the gating in the bursting states. *J. Gen. Physiol.* 113, 425–440. doi: 10.1085/jgp.113.3.425

Nimigean, C. M., and Magleby, K. L. (2000). Functional coupling of the beta(1) subunit to the large conductance Ca^{2+}-activated K^+ channel in the absence of Ca^{2+}. Increased Ca^{2+} sensitivity from a Ca^{2+}-independent mechanism. *J. Gen. Physiol.* 115, 719–736. doi: 10.1085/jgp.115.6.719

Niu, X., and Magleby, K. L. (2002). Stepwise contribution of each subunit to the cooperative activation of BK channels by Ca^{2+}. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11441–11446. doi: 10.1073/pnas.172254699

Niu, X., Qian, X., and Magleby, K. L. (2004). Linker-gating ring complex as passive spring and Ca^{2+}-dependent machine for a voltage and Ca^{2+}-activated potassium channel. *Neuron* 42, 745–756 doi: 10.1016/j.neuron.2004.05.001

Nonner, W. (1980). Relations between the inactivation of sodium channels and the immobilization of gating charge in frog myelinated nerve. *J. Physiol.* 299, 573–603. doi: 10.1113/jphysiol.1980.sp013143

Pallotta, B. S., Magleby, K. L., and Barrett, J. N. (1981). Single channel recordings of Ca^{2+}-activated K^+ currents in rat muscle cell culture. *Nature* 293, 471–474. doi: 10.1038/293471a0

Pico, A. R. (2003). *RCK Domain Model of Calcium Activation in BK Channels*. New York, NY: Rockefeller University.

Qian, X., Niu, X., and Magleby, K. L. (2006). Intra- and intersubunit cooperativity in activation of BK channels by Ca^{2+}. *J. Gen. Physiol.* 128, 389–404. doi: 10.1085/jgp.200609486

Qin, F. (2014). Principles of single-channel kinetic analysis. *Methods Mol. Biol.* 1183, 371–399. doi: 10.1007/978-1-4939-1096-0_23

Qin, F., Auerbach, A., and Sachs, F. (1996). Estimating single-channel kinetic parameters from idealized patch-clamp data containing missed events. *Biophys. J.* 70, 264–280. doi: 10.1016/S0006-3495(96)79568-1

Rios, E., Karhanek, M., Ma, J., and Gonzalez, A. (1993). An allosteric model of the molecular interactions of excitation-contraction coupling in skeletal muscle. *J. Gen. Physiol.* 102, 449–481. doi: 10.1085/jgp.102.3.449

Robitaille, R., Garcia, M. L., Kaczorowski, G. J., and Charlton, M. P. (1993). Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron* 11, 645–655. doi: 10.1016/0896-6273(93)90076-4

Roth, R., Gillespie, D., Nonner, W., and Eisenberg, R. E. (2008). Bubbles, gating, and anesthetics in ion channels. *Biophys. J.* 94, 4282–4298. doi: 10.1529/biophysj.107.120493

Rothberg, B. S., Bello, R. A., and Magleby, K. L. (1997). Two-dimensional components and hidden dependencies provide insight into ion channel gating mechanisms. *Biophys. J.* 72, 2524–2544. doi: 10.1016/S0006-3495(97)78897-0

Rothberg, B. S., and Magleby, K. L. (1998a). Investigating single-channel gating mechanisms through analysis of two-dimensional dwell-time distributions. *Methods Enzymol.* 293, 437–456. doi: 10.1016/S0076-6879(98)93028-2

Rothberg, B. S., and Magleby, K. L. (1998b). Kinetic structure of large-conductance Ca^{2+}-activated K^+ channels suggests that the gating includes transitions through intermediate or secondary states. A mechanism for flickers. *J. Gen. Physiol.* 111, 751–780. doi: 10.1085/jgp.111.6.751

Rothberg, B. S., and Magleby, K. L. (1999). Gating kinetics of single large-conductance Ca^{2+}-activated K^+ channels in high Ca^{2+} suggest a two-tiered allosteric gating mechanism. *J. Gen. Physiol.* 114, 93–124. doi: 10.1085/jgp.114.1.93

Rothberg, B. S., and Magleby, K. L. (2000). Voltage and Ca^{2+} activation of single large-conductance Ca^{2+}-activated K^+ channels described by a two-tiered allosteric gating mechanism. *J. Gen. Physiol.* 116, 75–99. doi: 10.1085/jgp.116.1.75

Sausbier, M., Arntz, C., Bucurenciu, I., Zhao, H., Zhou, X. B., Sausbier, U., et al. (2005). Elevated blood pressure linked to primary hyperaldosteronism and impaired vasodilation in BK channel-deficient mice. *Circulation* 112, 60–68. doi: 10.1161/01.CIR.0000156448.74296.FE

Sausbier, M., Hu, H., Arntz, C., Feil, S., Kamm, S., Adelsberger, H., et al. (2004). Cerebellar ataxia and Purkinje cell dysfunction caused by Ca^{2+}-activated K^+ channel deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9474–9478. doi: 10.1073/pnas.0401702101

Schoppa, N. E., McCormack, K., Tanouye, M. A., and Sigworth, F. J. (1992). The size of gating charge in wild-type and mutant Shaker potassium channels. *Science* 255, 1712–1715. doi: 10.1126/science.1553560

Schreiber, M., and Salkoff, L. (1997). A novel calcium-sensing domain in the BK channel. *Biophys. J.* 73, 1355–1363. doi: 10.1016/S0006-3495(97)78168-2

Seibold, M. A., Wang, B., Eng, C., Kumar, G., Beckman, K. B., Sen, S., et al. (2008). An African-specific functional polymorphism in KCNMB1 shows sex-specific association with asthma severity. *Hum. Mol. Genet.* 17, 2681–2690. doi: 10.1093/hmg/ddn168

Shelley, C., and Magleby, K. L. (2008). Linking exponential components to kinetic states in Markov models for single-channel gating. *J. Gen. Physiol.* 132, 295–312. doi: 10.1085/jgp.200810008

Shelley, C., Niu, X., Geng, Y., and Magleby, K. L. (2010). Coupling and cooperativity in voltage activation of a limited-state BK channel gating in saturating Ca^{2+}. *J. Gen. Physiol.* 135, 461–480. doi: 10.1085/jgp.200910331

Shen, K. Z., Lagrutta, A., Davies, N. W., Standen, N. B., Adelman, J. P., and North, R. A. (1994). Tetraethylammonium block of Slowpoke calcium-activated potassium channels expressed in Xenopus oocytes: evidence for tetrameric channel formation. *Pflugers Arch.* 426, 440–445. doi: 10.1007/BF00388308

Shi, J., and Cui, J. (2001). Intracellular Mg^{2+} enhances the function of BK-type Ca^{2+}-activated K^+ channels. *J. Gen. Physiol.* 118, 589–606. doi: 10.1085/jgp.118.5.589

Shi, J., Krishnamoorthy, G., Yang, Y., Hu, L., Chaturvedi, N., Harilal, D., et al. (2002). Mechanism of magnesium activation of calcium-activated potassium channels. *Nature* 418, 876–880. doi: 10.1038/nature00941

Sigworth, F. J., and Sine, S. M. (1987). Data transformations for improved display and fitting of single-channel dwell time histograms. *Biophys. J.* 52, 1047–1054. doi: 10.1016/S0006-3495(87)83298-8

Song, L., and Magleby, K. L. (1994). Testing for microscopic reversibility in the gating of maxi K^+ channels using two-dimensional dwell-time distributions. *Biophys. J.* 67, 91–104. doi: 10.1016/S0006-3495(94)80458-8

Steinberg, I. Z. (1987). Relationship between statistical properties of single ionic channel recordings and the thermodynamic state of the channels. *J. Theor. Biol.* 124, 71–87. doi: 10.1016/S0022-5193(87)80253-9

Talukder, G., and Aldrich, R. W. (2000). Complex voltage-dependent behavior of single unliganded calcium- sensitive potassium channels. *Biophys. J.* 78, 761–772. doi: 10.1016/S0006-3495(00)76634-3

Vergara, C., Latorre, R., Marrion, N. V., and Adelman, J. P. (1998). Calcium-activated potassium channels. *Curr. Opin. Neurobiol.* 8, 321–329. doi: 10.1016/S0959-4388(98)80056-1

Wang, Z. W., Saifee, O., Nonet, M. L., and Salkoff, L. (2001). SLO-1 potassium channels control quantal content of neurotransmitter release at the *C. elegans* neuromuscular junction. *Neuron* 32, 867–881. doi: 10.1016/S0896-6273(01)00522-0

Wilkens, C. M., and Aldrich, R. W. (2006). State-independent block of BK channels by an intracellular quaternary ammonium. *J. Gen. Physiol.* 128, 347–364. doi: 10.1085/jgp.200609579

Xia, X. M., Zeng, X., and Lingle, C. J. (2002). Multiple regulatory sites in large-conductance calcium-activated potassium channels. *Nature* 418, 880–884. doi: 10.1038/nature00956

Yang, H., Hu, L., Shi, J., Delaloye, K., Horrigan, F. T., and Cui, J. (2007). Mg^{2+} mediates interaction between the voltage sensor and cytosolic domain to activate BK channels. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18270–18275. doi: 10.1073/pnas.0705873104

Yuan, P., Leonetti, M. D., Hsiung, Y. C., and MacKinnon, R. (2012). Open structure of the Ca^{2+} gating ring in the high-conductance Ca^{2+}-activated K^+ channel. *Nature* 481, U94–U105. doi: 10.1038/nature10670

Yuan, P., Leonetti, M. D., Pico, A. R., Hsiung, Y., and MacKinnon, R. (2010). Structure of the human BK channel Ca^{2+}-activation apparatus at 3.0 A resolution. *Science* 329, 182–186. doi: 10.1126/science.1190414

Zeng, X. H., Xia, X. M., and Lingle, C. J. (2005). Divalent cation sensitivity of BK channel activation supports the existence of three distinct binding sites. *J. Gen. Physiol.* 125, 273–286. doi: 10.1085/jgp.200409239

Zhang, G., Huang, S. Y., Yang, J., Shi, J., Yang, X., Moller, A., et al. (2010). Ion sensing in the RCK1 domain of BK channels. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18700–18705. doi: 10.1073/pnas.1010124107

Zhang, G., Yang, H., Liang, H., Yang, J., Shi, J., McFarland, K., et al. (2014). A charged residue in S4 regulates coupling among the activation gate, voltage, and Ca^{2+} sensors in BK channels. *J. Neurosci.* 34, 12280–12288. doi: 10.1523/JNEUROSCI.1174-14.2014

Zhang, X., Solaro, C. R., and Lingle, C. J. (2001). Allosteric regulation of BK channel gating by Ca^{2+} and Mg^{2+} through a nonselective, low affinity divalent cation site. *J. Gen. Physiol.* 118, 607–636. doi: 10.1085/jgp.118.5.607

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 December 2014; paper pending published: 15 December 2014; accepted: 31 December 2014; published online: 21 January 2015.

Citation: Geng Y and Magleby KL (2015) Single-channel kinetics of BK (Slo1) channels. Front. Physiol. ***5****:532. doi: 10.3389/fphys.2014.00532*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 22 August 2014
doi: 10.3389/fphys.2014.00316

The regulation of BK channel activity by pre- and post-translational modifications

Barry D. Kyle and Andrew P. Braun*

Department of Physiology and Pharmacology, Cumming School of Medicine, Libin Cardiovascular Research Institute, University of Calgary, Calgary, AB, Canada

Edited by:
Alex M. Dopico, The University of Tennessee Health Science Center, USA

Reviewed by:
Carmen Valenzuela, Instituto de Investigaciones Biomédicas CSIC-UAM, Spain
Luis MS Loura, University of Coimbra, Portugal

***Correspondence:**
Andrew P. Braun, Department of Physiology and Pharmacology, Cumming School of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, AB T2N 4N1, Canada
e-mail: abraun@ucalgary.ca

Large conductance, Ca^{2+}-activated K^+ (BK) channels represent an important pathway for the outward flux of K^+ ions from the intracellular compartment in response to membrane depolarization, and/or an elevation in cytosolic free [Ca^{2+}]. They are functionally expressed in a range of mammalian tissues (e.g., nerve and smooth muscles), where they can either enhance or dampen membrane excitability. The diversity of BK channel activity results from the considerable alternative mRNA splicing and post-translational modification (e.g., phosphorylation) of key domains within the pore-forming α subunit of the channel complex. Most of these modifications are regulated by distinct upstream cell signaling pathways that influence the structure and/or gating properties of the holo-channel and ultimately, cellular function. The channel complex may also contain auxiliary subunits that further affect channel gating and behavior, often in a tissue-specific manner. Recent studies in human and animal models have provided strong evidence that abnormal BK channel expression/function contributes to a range of pathologies in nerve and smooth muscle. By targeting the upstream regulatory events modulating BK channel behavior, it may be possible to therapeutically intervene and alter BK channel expression/function in a beneficial manner.

Keywords: calcium-activated K^+ channel, β subunit, phosphorylation, modulation, smooth muscle, neuron, contractility

INTRODUCTION: BK CHANNEL DISTRIBUTION AND ARCHITECTURE

BK channels, also called MaxiK/Slo1/K_{Ca}1.1 channels, are a class of K^+ ion channels that undergo extensive pre- and post-translational modification. BK channel α subunits are encoded by the *KCNMA1* gene, also known as *SLO,* and are ubiquitously expressed throughout mammalian tissues (e.g., neurons, smooth and skeletal muscles, exocrine cells). BK channels are assembled and strategically positioned on membrane surfaces, including the plasma membrane (Latorre et al., 1989), mitochondria and nucleus (Singh et al., 2012). Functional BK channels are multimeric structures composed of four similar pore-forming α subunits (Shen et al., 1994) and up to four regulatory β subunits can co-assemble with the tetrameric α subunit complex. The synergistic activation of BK channels by Ca^{2+} ions and depolarization causes a substantial K^+ current that exhibits a large or "big" single channel conductance (i.e., up to 250 pS under symmetric K^+ conditions). Activation of this formidable ionic current serves to drive membrane potential in the negative direction.

The transmembrane portion of the BK channel α subunit structure is thought to largely resemble that of voltage-gated K^+ (K_v) channel subunits in terms of voltage-sensing and pore-forming domains. Notably, BKα subunits contain an additional transmembrane segment, termed S0, resulting in an extracellular N-terminus. Specialized charged residues are present within the transmembrane segments S2–S4 of the BKα subunit that contribute to its voltage-sensing properties. While topologically similar to their K_v channel counterparts, BK channels display weaker or less sensitive voltage-dependent activation (i.e., the ionic conductance-voltage relation is less steep), due to an altered distribution of voltage-sensing residues within the S2–S4 segments (Ma et al., 2006). Mechanistically, membrane depolarization drives conformational re-arrangements in the voltage sensor domains, resulting in an upward twisting of the S4 segment relative to the pore domain; these conformational movements are reversed upon repolarization (Hoshi et al., 2013).

The C-terminal domain of the BKα subunit contains a considerable range of specialized structures that regulate channel function. These include several binding sites for divalent cations (i.e., Ca^{2+} and Mg^{2+}) and regions that undergo dynamic post-translational modification such as phosphorylation. Each mammalian BKα subunit contains two "regulators of K^+ conductance" (RCK) domains, arranged in tandem along the C-terminus; in the tetrameric channel complex, these RCK domains co-assemble to form an octomeric gating ring structure in the cytosol (Yuan et al., 2010). The RCK domains also have Ca^{2+}-binding regions and are crucial in conferring the channel's Ca^{2+} ion sensing properties (Cui et al., 2009). Ca^{2+} ions bind to these specialized regions within the BKα C-terminus, leading to a structural expansion of the intracellular region of the ion conduction pathway that facilitates gating and K^+ efflux (Yuan et al., 2012; Hoshi et al., 2013).

GENETIC DIVERSITY AND SPLICE VARIANTS

Unlike the K_v channel superfamily, which uses different genes to increase its genetic diversity, BK channels derive functional

diversity through the alternative post-transcriptional splicing of mRNA derived from the single *KCNMA1* gene encoding the BKα subunit (Shipston, 2001). Up to ten distinct splice sites have been described in *KCNMA1* (Poulsen et al., 2009), leading to the generation of BKα subunits with different phenotypes and various functional roles, including altered sensitivity to Ca^{2+} and/or voltage (Shipston, 2001; Johnson et al., 2011), responses to phosphorylation (Tian et al., 2001), signaling cascades (Schubert and Nelson, 2001; Tian et al., 2001, 2004), membrane expression regulation (Alioua et al., 2008; Ahrendt et al., 2014), trafficking and lipidation (Toro et al., 2006; Zarei et al., 2007; Shipston, 2014). The impressive range of phenotypic products that can result from differential splicing of the *KCNMA1* gene product contributes to diversity of BK channel function between tissues, cells and intracellular compartments.

BK CHANNEL AUXILIARY SUBUNITS

BK channels can co-assemble with modulatory auxiliary subunits BKβ1-4 (Knaus et al., 1994a; Tanaka et al., 1997; Brenner et al., 2000a; Uebele et al., 2000), as well as a newly defined family of leucine-rich repeat containing subunits (LRRCs), referred to as γ subunits (Yan and Aldrich, 2010, 2012). Both BKβ and γ subunits contain sizeable extracellular regions and it is thought that these regions physically interact with the membrane-spanning domains of the BKα subunit. In particular, BKβ subunits appear to interact mainly with the N-terminal S0–S2 segments of the pore-forming BKα subunit (Morrow et al., 2006; Liu et al., 2008; Morera et al., 2012), thereby regulating channel opening through allosteric effects on the intramolecular processes underlying Ca^{2+} and/or voltage-dependent activation. As these auxiliary subunits are expressed in a tissue-specific manner, they confer distinct functional consequences by impacting BK channel kinetics and gating behavior. For instance, BKβ1 subunits are typically expressed in smooth muscle, whereas BKβ4 are expressed in neural tissue. BKβ subunits 1, 2 and 4 are reported to stabilize the channel's voltage sensor domains in the active conformation (Contreras et al., 2012), thereby enhancing channel activity, In contrast, BKβ2 and β3 subunits confer BK channel inactivation via an N-terminal "inactivation ball" (Wallner et al., 1999; Brenner et al., 2000a; Uebele et al., 2000) (**Figure 1**), which will limit K^+ efflux and membrane hyperpolarization. To date, two functionally-distinct BKβ2 splice variants ($BK\beta2_{a-b}$) have been described in mammals, although $BK\beta2_b$ does not appear to inactivate the channel complex (Ohya et al., 2010). Similarly, four functionally-distinct BKβ3 splice variants ($BK\beta3_{a-d}$) are known, with splice variants A-C conferring partial inactivation of BK channel current (Uebele et al., 2000). BKβ4 subunits are the most distantly-related of the β subunits in terms of sequence similarity and produce mixed effects on BK channel gating, depending on the local Ca^{2+} concentration. At low Ca^{2+} concentrations, BKβ4 appears to decrease channel activation, but at high Ca^{2+} concentrations, activation is enhanced (Brenner et al., 2000a; Wang et al., 2006).

The molecular mechanisms by which γ-subunits interact with and influence BK channel gating and kinetics are currently an area of active investigation. All four known LRRC proteins (i.e., LRRC26, 38, 52, and 55) have been reported to enhance voltage-dependent activation of BK channels (Yan and Aldrich, 2010, 2012), with LRRC26 producing an impressive shift of up to −150 mV.

ROLE OF BK CHANNELS IN SMOOTH MUSCLE FUNCTION AND DISEASE

Phasic smooth muscles, such as those lining the urinary bladder, urethra and ureters, undergo action potential (AP) events, with rapid depolarization-repolarization fluctuations. APs cause

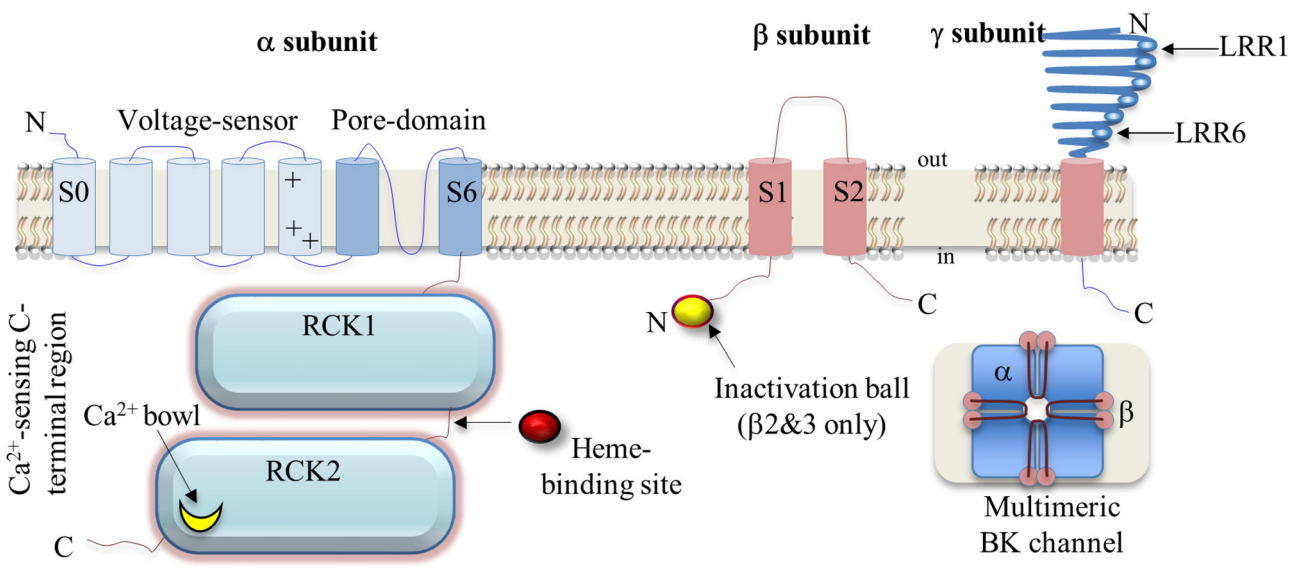


FIGURE 1 | A schematic illustration of BK channel α, β and γ subunit architecture with major structures defined. Abbreviations: N, amino-terminus; C, carboxy-terminus; LRR, leucine-rich repeat; S, transmembrane segment; RCK, regulator of K^+ conductance.

a significant global increase in intracellular [Ca^{2+}] and BK channels are largely responsible for the rapid down-stroke (repolarization) phase (Burdyga and Wray, 2005; Thorneloe and Nelson, 2005; Kyle et al., 2013b). In contrast, tonic smooth muscles, such as those found throughout vascular tissue and much of the gastrointestinal tract and airways, regulate lower magnitude changes in membrane potential by principally responding to localized elevations in intracellular [Ca^{2+}] mediated by ryanodine receptors (RyRs) (**Figure 2**). The dynamic post-translational "tuning" of BK channels permits considerable diversity in the biophysical properties of the current.

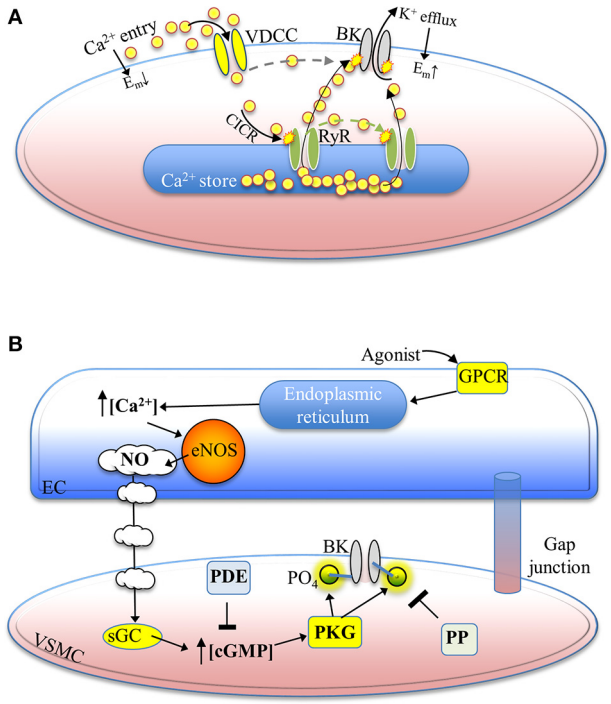


FIGURE 2 | A summary of select physiological mechanisms leading to BK channel activation and reversible phosphorylation-mediated enhancement. (A) Ca^{2+}–dependent activation of BK channels hyperpolarizes the membrane potential. Depolarization of the membrane potential activates voltage-dependent Ca^{2+} channels, leading to Ca^{2+} entry and Ca^{2+}-induced Ca^{2+} release from nearby ryanodine receptors. Released Ca^{2+} promotes BK channel activation, which drives the membrane potential in the negative (hyperpolarized) direction. Ca^{2+} influx via VDCCs may also contribute directly to BK channel activation (dotted line) as a result of the spatial proximity of these two channels within membrane nano/micro-domains. **(B)** Mechanisms underlying the generation of nitric oxide from an endothelial cell, with the NO/cGMP/PKG-mediated phosphorylation of a BK channel illustrated in an adjacent vascular smooth muscle cell. Nitric oxide release from endothelial cells binds to soluble guanylyl cyclase in smooth muscle cells, resulting in elevated intracellular cGMP concentrations. PKG is then activated and phosphorylates the BKα subunit. Phosphodiesterase activity lowers intracellular cGMP and protein phosphatase activity removes the regulatory phosphate from Ser/Thr residues of the BK channel protein. Abbreviations: VDCC, voltage-dependent Ca^{2+} channel; BK, BK channel; E_m, membrane potential; CICR, Ca^{2+}-induced Ca^{2+} release; RyR, ryanodine receptor; GPCR, GTP-binding protein-coupled receptor; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; EC, endothelial cell; sGC, soluble guanylyl cyclase; PDE, phosphodiesterase; PO_4, phosphate group; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G; PP, protein phosphatase; VSMC, vascular smooth muscle cell.

In common with many other tetrameric K^+ channels in smooth muscles, the amplitude of K^+ current carried through BK channels in smooth muscles can be dynamically regulated by post-translational modifications to the channel complex, including the reversible phosphorylation of the pore-forming BKα subunit by a number of protein kinases, as described below. Almost all phosphorylation sites are conserved in mammalian BK channel splice variants.

Many tissues have distinct macromolecular signaling complexes underlying the function of ion channels. Smooth muscles, for instance, generally have closely-associated RyRs, which periodically release Ca^{2+} and cause local elevations in $[Ca^{2+}]_i$ (i.e., 10–20 μM) (Pérez et al., 1999; ZhuGe et al., 2002) near BK channels positioned on the plasma membrane, which is sufficient to significantly raise the P_o and efflux K^+ (**Figure 2**). The RyRs themselves are often close to Ca^{2+} influx pathways, for instance voltage-gated Ca^{2+} channels, or in proximity to IP_3 receptors (Ohi et al., 2001).

The primary role of BK channels in vascular smooth muscle (VSM) is to repolarize/hyperpolarize the cell membrane potential in the face of chronic depolarizing stimuli, thereby reducing contractile activity. It is now well-recognized that enhancement of BK channel current in VSM via phosphorylation is principally-regulated by nitric oxide (NO)/cGMP/PKG signaling (Feil et al., 2003) (see Section BK Channel Modulation via Protein Phosphorylation below). NO is a gaseous second messenger synthesized mainly by the adjacent endothelial cell layer lining the lumen of all blood vessels (Fleming and Busse, 2003). Therefore, BK channel activity is considered to be closely linked with endothelial cell activity. Therapeutically, NO and synthetic NO donors are used to treat a range of vascular disorders, including angina pectoris and hypertension (Wimalawansa, 2008).

In addition to the urinary tract and VSM, BK channels are also important regulators in mediating the proper function of various other smooth muscles, including those found in the gastrointestinal tract, airway, and uterus. Their function, however, varies between cell types and layers, and generally is dependent on the associated macromolecular signaling complex. In the colon, for instance, BK channels contribute to setting the resting membrane potential in longitudinal smooth muscle, whereas in the circular layer, they limit excitatory responses (Sanders, 2008).

In VSM, a single amino acid polymorphism in the BKβ1 subunit (i.e., E65K) is reported to have a gain-of-function effect on BKs channel activation and has been associated with lower systolic and diastolic blood pressures and a decreased prevalence of diabetic hypertension in humans (Fernández-Fernández et al., 2004; Nielsen et al., 2008). In contrast, BKβ1 subunit expression is decreased in some forms of genetic hypertension (Amberg and Santana, 2003). Moreover, a point mutation (R140W) in the BKβ1 subunit that modestly impairs channel opening has been linked with asthma severity in African-American males (Seibold et al., 2008). Provocative data from Jaggar and colleagues further suggest that the majority of BKβ1 subunits reside within the cell interior and assemble with α subunits at the cell surface in a dynamic fashion (Leo et al., 2014). NO signaling appears

to promote the forward trafficking of internal BKβ1 subunits to the cell membrane, where they co-associate with BKα subunits to enhance channel activation. The authors suggest that auxiliary BKβ1 subunits undergo selective endocytosis from the plasma membrane, followed by re-insertion in response to a vasodilatory stimulus, such as NO. These data imply that native BK channels in VSM may not always contain a full complement of β1 subunits (i.e., the ratio of β1 to α subunits in a single channel complex is <1), as described in rat cremaster artery (Yang et al., 2009), and that the subunit stoichiometry of these channels is not permanent. Dynamic regulation of BK channel subunit co-assembly and interaction at the plasma membrane may thus represent a novel paradigm for the modulation of ion channel activity.

Many research groups have reported that BK channel activity is upregulated during hypertension, and its contribution is apparently enhanced compared to normotensive animals (for review, see Joseph et al., 2013). It should be noted, however, that downregulation of BK channel activity has also been reported during hypertension (Amberg et al., 2003; Amberg and Santana, 2003; Nieves-Cintrón et al., 2007; Yang et al., 2013). Investigators have speculated that this decrease may be due to reduced BKβ1 subunit expression/coupling, which would dampen the Ca^{2+} sensitivity of BK channel activation. Several research groups have reported that BK current density is positively-correlated to blood pressure in hypertensive animals (Rusch et al., 1992; England et al., 1993; Rusch and Runnells, 1994; Liu et al., 1998). Aortic smooth muscle isolated from rats with renal hypertension, spontaneously-hypertensive rats (SHR) and stroke-prone SHR (Rusch et al., 1992; England et al., 1993; Liu et al., 1998) exhibits significantly-upregulated BK channel activity, likely as a compensatory response. Collectively, these studies indicate that the expression and function of BK channels in the vasculature involves complex expression and signaling pathways, and may vary between cells, tissues, vascular beds and pathophysiological profiles.

BK channels are densely-expressed in mammalian bladder tissues (∼20 channels per square micrometer) (Ohi et al., 2001) with BKβ1 auxiliary subunits. BKα subunit knockout mice have demonstrated bladder dysfunction and exhibit a depolarized resting membrane potential in isolated bladder smooth muscle cells and intact tissues, indicating a role for BK channels in setting the membrane potential (Sprossmann et al., 2009). Inhibition of BK channel current with iberiotoxin in the bladders of healthy mice led to similar effects (Heppner et al., 1997; Hristov et al., 2011). BKβ1-knockout mice similarly display overactive bladder symptoms, and a significant decrease in BK channel activity (Petkov et al., 2001). Intriguingly, bladder smooth muscle tissue taken from patients with neurogenic bladder over-activity exhibit little to no response to BK channel inhibition by iberiotoxin, or the channel agonist NS1619, indicating severe BK channel dysfunction (Oger et al., 2010). Macroscopic current recordings from these tissues demonstrated a significantly lower BK channel current density that mirrors that reported for experimentally-induced partial urethral obstruction in rats (Aydin et al., 2012). Patients with benign prostatic hyperplasia experiencing overactive bladder symptoms also demonstrate a parallel reduction in BK channel expression (Chang et al., 2010). Overexpression of BK channel protein in rats with experimentally-induced partial urethral obstruction proved to be an effective treatment for the existing overactive bladder activity (Christ and Hodges, 2006). These data collectively indicate that BK channels are important regulators of bladder smooth muscle excitability, and a potential target for therapeutic intervention for overactive bladder conditions.

ROLE OF BK CHANNELS IN NEURONAL FUNCTION/DYSFUNCTION

BK channels are abundantly expressed in both central and peripheral neurons, with prominent expression reported in both the cell body and pre-synaptic terminals (Faber and Sah, 2003). Functionally, these channels are key regulators of neuronal excitability, as channel opening will reduce action potential (AP) amplitude and duration, increase the magnitude of the fast after-hyperpolarization (fAHP) immediately following repolarization and limit the frequency of AP burst firing (Bielefeldt and Jackson, 1993; Faber and Sah, 2003; Gu et al., 2007; Haghdoost-Yazdi et al., 2008). At the pre-synaptic nerve terminal, localized BK channel activity can modulate both the amplitude and duration of depolarization-evoked Ca^{2+} entry as a result of the rapid repolarization and deactivation of voltage-gated Cav 2.1 (i.e., P/Q-type) and 2.2 (N-type) Ca^{2+} channels (Robitaille and Charlton, 1992; Issa and Hudspeth, 1994; Marrion and Tavalin, 1998; Fakler and Adelman, 2008). Reduced Ca^{2+} influx will limit vesicle fusion at active zones, leading to decreased neurotransmitter release (Roberts et al., 1990; Hu et al., 2001; Raffaelli et al., 2004).

Dissecting the functional roles of BK channels in the nervous system has been greatly aided by the availability of highly selective toxins (i.e., iberiotoxin) (Kaczorowski and Garcia, 1999) and small molecule inhibitors (e.g., penitrem A, paxilline, lolitrem B) (Knaus et al., 1994b; Imlach et al., 2008; Nardi and Olesen, 2008), along with the generation of genetically-engineered mice lacking either BKα or β subunits (Brenner et al., 2000b, 2005; Plüger et al., 2001; Meredith et al., 2004; Sausbier et al., 2004). Such strategies have revealed that the loss of neuronal BK current, either acutely or chronically, increases membrane excitability by decreasing the magnitude of the fAHP. Reducing the fAHP facilitates more rapid membrane depolarization in response to a tonic stimulus, leading to higher frequency AP firing. Such alterations in neuronal activity are typically associated with neurological disorders in the CNS, including tremor and ataxia (Sausbier et al., 2004; Brenner et al., 2005; Imlach et al., 2008). Interestingly, a point mutation in the RCK1 domain of the BKα subunit (i.e., D434G) identified in a subset of epileptic patients has been shown to increase neuronal BK channel activity by enhancing Ca^{2+}-dependent channel gating (Du et al., 2005; Wang et al., 2009; Yang et al., 2010). Functionally, increasing BK activity and the associated fAHP may augment membrane excitability in the soma by enhancing the recovery rate of fast Na^{+} currents from voltage-dependent inactivation and reducing the absolute refractory period of neuronal firing.

In the CNS of mice and humans, genetic knockout or mutational disruption of the molecular chaperone cysteine string protein (CSPα) is linked with early onset neurodegeneration (Fernandez-Chacon et al., 2004; Donnelier and Braun, 2014),

and interestingly, these conditions are associated with a significant up-regulation of BK channel expression in mouse brain and cultured neurons (Kyle et al., 2013a; Ahrendt et al., 2014). Although the mechanistic link between increased BK expression/activity and neurodegeneration remains undefined, it is hypothesized that increased BK current density in pre-synaptic terminals and/or the soma may lead to disrupted synaptic membrane excitability and neurotransmitter release. As described below, elevated BK channel expression in the CNS is closely linked with epilepsy, strongly suggesting that increased BK current density can lead to neurological disorders and possibly synaptic dysfunction/degeneration.

POST-TRANSLATIONAL MODIFICATION

Heteromeric BK channel complexes are the subject of extensive post-translational modifications, which can significantly alter channel behavior. Some modifications are highly-complex and require prior upstream modification(s) to the channel subunits.

BK CHANNEL MODULATION VIA PROTEIN PHOSPHORYLATION

Perhaps the most studied enzymatically-driven modification of BK channels is the addition of phosphate (PO_4^{3-}) groups to functionally-important residues (Ser/Thr/Tyr) present within the channel's pore-forming α subunit. These reactions are catalyzed by select protein kinases and are reversed by the actions of protein phosphatases that dephosphorylate these sites following removal of the stimulus. Phosphorylation can be either stimulatory or inhibitory with respect to the open probability of the channel and can depend on several variables (see below).

Regulation of BK channel activity in smooth muscles by phosphorylation-dependent signaling pathways is well documented (Schubert and Nelson, 2001) and the main modifying enzymes include cAMP- and cGMP-dependent protein kinases (i.e., PKA and PKG, respectively), protein kinase C (Zhou et al., 2010) along with c-Src tyrosine kinase (Davis et al., 2001). Biochemically, PKA is comprised of 2 catalytic and 2 regulatory subunits and kinase activation occurs in response to the direct binding of the second messenger cAMP to the regulatory subunits (Taylor et al., 1990). Cyclic AMP synthesis occurs following stimulation of adenylyl cyclase by hormones (e.g., adenosine, β-adrenergic agonists, PGI_2, PGE_2, etc.) or direct activators (e.g., forskolin). In the case of PKG activation, synthesis of cGMP can occur via a soluble or a membrane-bound form of guanylyl cyclase (Münzel et al., 2003); the former is typically activated by NO and the latter by natriuretic peptides acting on the cell surface receptors NPR-A and NPR-B. Structurally, PKG exists as a homodimer in which each monomer consists of a regulatory and catalytic domain linked in a single polypeptide chain (Francis et al., 2010); holo-PKG thus closely resembles the overall structure of PKA. Generally, PKA and PKG-mediated phosphorylation leads to BK channel enhancement, whereas PKC leads to channel inhibition. It should be stressed, however, that these regulatory effects on BK channel activity depend upon contextual phosphorylation/modification at multiple sites (Zhou et al., 2010, 2012; Kyle et al., 2013c), and may be further influenced by the constitutive phosphorylation status of the channel complex (see below). Selective blockade of the phosphodiesterase enzymes responsible for cGMP metabolism by pharmacologic agents such as sildenafil will prolong cGMP effects in smooth muscle and this process has been exploited therapeutically to treat erectile dysfunction and pulmonary hypertension (Francis et al., 2010). For a comprehensive overview of early studies describing BK channel regulation by kinase-associated pathways, see Schubert and Nelson (2001).

Using a multi-faceted strategy involving protein biochemistry, site-directed mutagenesis and patch clamp recordings, our group has recently reported that NO/cGMP/PKG signaling in VSM cells leads to the modification of three distinct Ser residues in the BKα C-terminus (i.e., Ser 691, 873 and 1111–1113), which directly correlate with enhancement of channel activity (Kyle et al., 2013c). Not unexpectedly, one of these sites (i.e., Ser873) is also important for PKA-mediated enhancement of BK activity (Nara et al., 1998). The regulatory phosphorylation status of BK channels also appears to differ developmentally, as BK channels in fetal arteries display more enhanced activity compared with channels from adult VSM (Lin et al., 2005, 2006). Augmentation of BK channel activity by NO/cGMP/PKG signaling is readily reversible and this is largely due to dephosphorylation via Ser/Thr protein phosphatases. Several studies have described involvement of protein phosphatases 1 and 2A in the regulation of BK channel activity, based mainly on the selective actions of inhibitors, such as okadaic acid (Zhou et al., 1996, 2010; Sansom et al., 1997).

Activation of PKC is reported to inhibit BK channel activity in VSM via the putative phosphorylation of Ser695 and Ser1151, and these modifications also appear to interfere with the stimulatory effects mediated by PKA and PKG (Zhou et al., 2010). Interestingly, this PKC-mediated inhibition of channel activity is absent in STREX-containing BKα splice variants (Zhou et al., 2012) (see below).

Similar to VSM, neuronal BK channel activity can be enhanced in response to regulatory phosphorylation of the pore-forming BKα subunit by both PKA and PKG, which can be reversed by the actions of Ser/Thr phosphatases 1 and 2A (Reinhart et al., 1991; Reinhart and Levitan, 1995; Sansom et al., 1997; Tian et al., 1998). Interestingly, proteomic analyses of rat brain BK channels isolated under basal conditions has identified ∼30 Ser and Thr residues that appear to be constitutively phosphorylated *in vivo*, with 23 of these modified residues located within the channel's C-terminus (Yan et al., 2008). Such observations suggest that constitutive phosphorylation may help stabilize BK channel tertiary structure and/or create binding sites for interacting proteins. The various protein kinases responsible for these *in vivo* modifications are presently unknown, as is the extent to which channels from other tissues or expressed heterologously exhibit constitutive phosphorylation. Our recent data describing a role for multiple phosphorylation sites to support cGMP-dependent augmentation of BK channel activity in VSM cells (Kyle et al., 2013c) promote the idea that individual phospho-Ser/Thr residues act synergistically to enhance BK channel activity.

In neurons and neuroendocrine cells (e.g., pituitary, adrenal gland) and more recently in VSM (Nourian et al., 2014), a portion of BK channels identified by qRT-PCR contain the STREX splicing insert, a 59 amino acid insert present at splice site C2 within the C-terminus (Xie and McCobb, 1998; Shipston, 2001). In response to cAMP/PKA signaling, a Ser residue within the STREX

insert can undergo phosphorylation, which has been shown to decrease BK channel activity (Tian et al., 2001). Functionally, such a change would be expected to enhance membrane excitability in neuroendocrine cells and promote exocytosis. Interestingly, phosphorylation of the STREX domain also appears to override the positive gating effects mediated by PKA-induced phosphorylation at other C-terminal sites, leading to an overall dominant-negative effect of STREX phosphorylation on BK channel activity (i.e., a single STREX-containing α subunit within a tetrameric channel is sufficient to flip PKA-mediated phosphorylation from stimulatory to inhibitory) (Tian et al., 2004). Furthermore, this inhibitory effect of PKA on BK channel activity appears to depend upon the presence of palmitoyl fatty acid groups within the STREX insert (Shipston, 2014), as palmitoylation-incompetent BK channels do not undergo PKA-mediated phosphorylation of the STREX insert and a decrease in activity (Tian et al., 2008). Collectively, these findings suggest that presence of STREX insert will lead to association of a C-terminal domain with the plasma membrane, which appears necessary for PKA-mediated phosphorylation within the STREX insert and inhibition of channel activity. Interestingly, presence of the STREX insert also appears to prevent the inhibitory effect of protein kinase C (PKC) on BK channel opening, possibly by inducing a conformation that precludes PKC-induced phosphorylation of Ser695 within the linker joining RCK1 and RCK2 domains (Zhou et al., 2012).

In addition to Ser/Thr phosphorylation, BK channels also undergo direct Tyr phosphorylation in the presence Src family kinases (i.e., c-Src and Hck) and the Ca^{2+}-sensitive tyrosine kinase Pyk-2 (Ling et al., 2000, 2004; Alioua et al., 2002; Yang et al., 2012). Functionally, direct tyrosine phosphorylation of the BKα subunit has been reported to either increase (Ling et al., 2000, 2004; Yang et al., 2012) or decrease (Alioua et al., 2002) channel activity, although the reason(s) for this discrepancy remains unclear. Work from our group has shown that Phe substitution of Tyr766 in the C-terminus largely inhibits c-Src-induced BKα subunit phosphorylation, but does not appear to disrupt Pyk-2 mediated modification (Ling et al., 2000, 2004). Future studies examining the direct phosphorylation of native BK channels by tyrosine kinases *in situ* are needed to clarify the physiologic importance of this regulatory event.

ENDOGENOUS REGULATORY MOLECULES

Endogenous molecules (e.g., heme, carbon monoxide (CO), reactive oxygen species) have been reported to interact with the BK channel complex (for review, see Hou et al., 2009). Similarly, acidification of the cytosol (i.e., pH 6.5) is able to increase BK channel activation by left-shifting the voltage dependence by ∼45 mV, but such effects can be readily masked by physiological levels of free Mg^{2+} (i.e., 1 mM) and Ca^{2+} (i.e., 1 μM) (Avdonin et al., 2003). The importance of $[H^+]$ with regards to BK channel activity may become more apparent during pathological conditions where fluctuations in $[H^+]$ and $[Ca^{2+}]$ may occur (e.g., cerebral ischemia) (Lipton, 1999).

The linker between the RCK1 and RCK2 regions of the BKα subunit (**Figure 1**) reportedly contains a binding site for intracellular heme molecules (Hou et al., 2009). Application of heme to the cytosolic face of BK channels was found to inhibit channel opening with an IC_{50} ∼70 nM (Tang et al., 2003), likely via an allosteric process. Moreover, the direction of gating modulation by heme appears to be closely-linked to membrane potential, as BK channel P_o is enhanced at negative membrane potentials and inhibited at positive potentials. Heme regulators, transporters and degradation products (e.g., CO) are currently under investigation for their therapeutic potential in influencing BK channel activity and thus, global membrane potential (Hou et al., 2009).

Soluble guanylyl cyclase (sGC) contains an iron (heme) center that serves to bind NO, however, this site is also targeted by CO, which can activate sGC, leading to increased cytosolic [cGMP], PKG activation and enhanced BK channel activity (see **Figure 2B**). It has been further suggested that CO, along with NO, can also directly augment BK channel activity when applied at sufficiently-high concentrations (Hou et al., 2009; Leffler et al., 2011). Further examination of the physiologic contribution of such effects to BK channel regulation are warranted.

Reactive oxygen species (ROS) that are reported to influence BK channel behavior include hydrogen peroxide (H_2O_2), superoxide (O_2^-) and peroxynitrite ($ONOO^-$). Increased levels of ROS may occur under localized conditions, such as atherosclerosis (Li and Förstermann, 2009) and are particularly troublesome, as H_2O_2 and O_2^- will react with free NO to generate $ONOO^-$, thereby reducing NO bioavailability and cGMP/PKG signaling in vascular smooth muscle. For detailed discussions on impact of ROS on BK channel activity, the reader is referred to excellent review articles (Tang et al., 2004; Hou et al., 2009).

REGULATION OF BK CHANNEL EXPRESSION BY UBIQUITINATION

Protein ubiquitination has emerged as a ubiquitous quality control mechanism for the regulation of protein trafficking and turnover and has been implicated in the dynamic control of diverse cellular processes (e.g., gene transcription, synaptic development and plasticity, oncogenesis, etc.) (Hershko and Ciechanover, 1998). Protein ubiquitination functions as a tagging system to mark proteins for degradation by the 26S proteasome complex and the human genome is reported to contain >600 genes encoding E3 ubiquitin ligases (Li et al., 2008), the enzyme responsible for conjugating ubiquitin monomers to target substrates. Given this level of abundance, the ubiquitin-proteasome system (UPS) appears to enzymatically parallel protein phosphorylation, for which ∼520 putative kinase genes have been described (Manning et al., 2002), as a widespread mechanism for protein modification and the regulation of cellular function. Recent evidence indicates that BK channels also undergo ubiquitination, which appears to have important functional implications. In the CNS, interaction of BK channels with cereblon (Jo et al., 2005), a substrate receptor for the CRL4A E3 ligase, leads to ubiquitination of the BKα subunit and retention of modified channels in the endoplasmic reticulum (Liu et al., 2014). Preventing ubiquitination of BK channels by pharmacologic or genetic interference of the CRL4A enzyme complex leads to increased trafficking of BK channels to the neuronal cell membrane and a higher incidence of seizure induction and epilepsy in mice. Such data point to ubiquitination as an important quality control mechanism to limit BK channel expression in neurons, which will ultimately impact membrane excitability. Given that cereblon transcripts

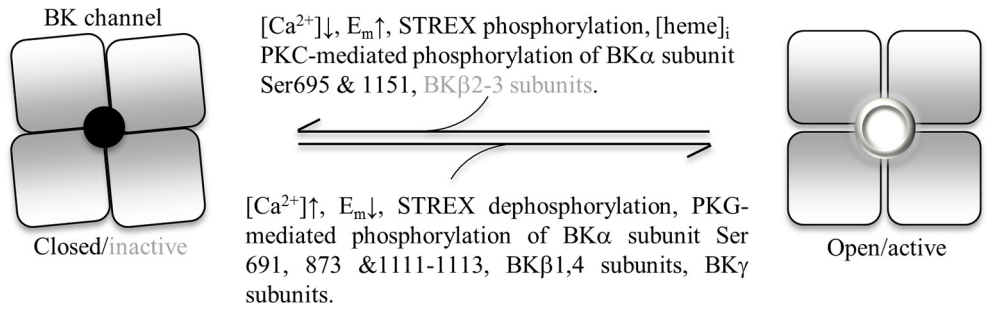


FIGURE 3 | A summary of cellular events/factors leading to BK channel activation (open pore) and deactivation/inactivation (closed pore). Abbreviations: E_m, membrane potential; STREX, stress-axis regulated exon; PKC, protein kinase C; Ser, serine.

are also widely expressed in tissues outside the CNS, this regulatory paradigm may have broader functional importance. As noted above, disruption of the neuronal chaperone CSPα in mice also elevates BK channel expression, suggesting that increased channel density be a common contributing factor to excitation-related neuropathologies.

In VSM, BKβ1 subunits are reported to undergo ubiquitination in cultured myocytes exposed to high glucose and in arteries obtained from mice made diabetic by injection of streptozotocin, a pancreatic β-cell poison. Diabetes-like conditions elevate the expression of a muscle-specific RING finger E3 ubiquitin ligase via enhanced NF-κB transcriptional activity, leading to increased BKβ1 subunit ubiquitination and proteolysis (Yi et al., 2014). As previously described, loss of the BKβ1 subunit would be expected to decrease Ca^{2+}- and voltage-dependent activation of VSM BK channels (Brenner et al., 2000b), leading to exaggerated membrane depolarization and smooth muscle contraction. As BKβ1 subunits may be capable of dynamically assembling with BKα subunits at the membrane (Leo et al., 2014), ubiquitination of BKβ1 alone may not necessarily result in a decreased cellular level of BKα subunits.

CONCLUDING REMARKS

BK channel activity is regulated both directly and indirectly through a diverse range of modulatory pathways involving covalent modifications, metabolic factors, trafficking events and transcriptional processes (see **Figure 3**). Given the formidable effect that BK channels can exert on membrane excitability, as a result of their large single channel conduction and dual activation by membrane depolarization/cytosolic free Ca^{2+}, such "fine-tuning" affords cells the ability to precisely control the impact of these channels on their function and responsiveness to both acute and chronic stimuli. As reinforced by the accompanying articles in this thematic issue, BK channels represent powerful effectors in tissue health and dysfunction and that understanding their modes of regulation may lead to novel therapeutic strategies in disease treatment.

ACKNOWLEDGMENTS

This work was supported by research funding to APB from the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council.

AUTHOR NOTE

Lingle and coworkers have demonstrated that the γ1 subunit (i.e., LRRC26) mediated leftward shift in BK channel gating occurs in an all-or-none fashion, in contrast to the incremental shifts in gating produced by stoichiometric association of BKβ1 subunits (Proc. Natl. Acad. Sci. U.S.A. 111, 4873, 2014. doi: 10.1073/pnas.1322123111). Subsequently, Evanson et al. (2014) have reported that LRRC26 is endogenously expressed in rat cerebral vascular myocytes and may function as an auxiliary γ1 subunit by altering the voltage and calcium sensitivity of BK channel gating (Circ. Res. 115, 423–431. doi: 10.1161/CIRCRESAHA.115.303407).

REFERENCES

Ahrendt, E., Kyle, B. D., Braun, A. P., and Braun, J. E. (2014). Cysteine string protein limits express of the large conductance, calcium-activated K^+ (BK) channel. *PLoS ONE* 9:e86586. doi: 10.1371/journal.pone.0086586

Alioua, A., Lu, R., Kumar, Y., Eghbali, M., Kundu, P., Toro, L., et al. (2008). Slo1 caveolin-binding motif, a mechanism of caveolin-1-Slo1 interaction regulating Slo1 surface expression. *J. Biol. Chem.* 283, 4808–4817. doi: 10.1074/jbc.M709802200

Alioua, A., Mahajan, A., Nishimaru, K., Zarei, M. M., Stefani, E., and Toro, L. (2002). Coupling of c-Src to large conductance voltage- and Ca^{2+}-activated K^+ channels as a new mechanism of agonist-induced vasoconstriction. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14560–14565. doi: 10.1073/pnas.222348099

Amberg, G. C., Bonev, A. D., Rossow, C. F., Nelson, M. T., and Santana, L. F. (2003). Modulation of the molecular composition of large conductance, Ca^{2+} activated K^+ channels in vascular smooth muscle hypertension. *J. Clin. Invest.* 112, 717–724. doi: 10.1172/JCI200318684

Amberg, G. C., and Santana, L. F. (2003). Downregulation of the BK channel β1 subunit in genetic hypertension. *Circ. Res.* 93, 965–971. doi: 10.1161/01.RES.0000100068.43006.36

Avdonin, V., Tang, X. D., and Hoshi, T. (2003). Stimulatory action of internal protons on Slo1 BK channels. *Biophys. J.* 84, 2969–2980. doi: 10.1016/S0006-3495(03)70023-X

Aydin, M., Wang, H. Z., Zhang, X., Chua, R., Downing, K., Melman, A., et al. (2012). Large-conductance calcium-activated potassium channel activity, as determined by whole-cell patch clamp recording, is decreased in urinary bladder smooth muscle cells from male rats with partial urethral obstruction. *BJU Int.* 110, E402–E408. doi: 10.1111/j.1464-410X.2012.11137.x

Bielefeldt, K., and Jackson, M. B. (1993). A calcium-activated potassium channel causes frequency-dependent action potential failures in a mammalian nerve terminal. *J. Neurophysiol.* 70, 284–298.

Brenner, R., Chen, Q. H., Vilaythong, A., Toney, G. M., Noebels, J. L., and Aldrich, R. W. (2005). BK channel β4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat. Neurosci.* 8, 1752–1759. doi: 10.1038/nn1573

Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y., and Aldrich, R. W. (2000a). Cloning and functional characterization of novel large conductance calcium-activated potassium channel β subunits, hKCNMB3 and hKCNMB4. *J. Biol. Chem.* 275, 6453–6461. doi: 10.1074/jbc.275.9.6453

Brenner, R., Perez, G., Bonev, A. D., Eckman, D. M., Kosek, J. C., Wiler, S. W., et al. (2000b). Vasoregulation by the β1 subunit of the calcium-activated potassium channel. *Nature* 407, 870–876. doi: 10.1038/35038011

Burdyga, T., and Wray, S. (2005). Action potential refractory period in ureter smooth muscle is set by Ca sparks and BK channels. *Nature* 436, 559–562. doi: 10.1038/nature03834

Chang, S., Gomes, C. M., Hypolite, J. A., Marx, J., Alanzi, J., Zderic, S. A., et al. (2010). Detrusor overactivity is assoicated with downregulation of large-conductance calcium- and voltage-activated potassium channel protein. *Am. J. Physiol. Renal Physiol.* 298, F1416–F1423. doi: 10.1152/ajprenal.00595.2009

Christ, G. J., and Hodges, S. (2006). Molecular mechanisms of detrusor and corporal myocyte contraction: identifying tragets for pharmacotherapy of bladder and erectile dysfunction. *Br. J. Pharmacol.* 147, S41–S55. doi: 10.1038/sj.bjp.0706627

Contreras, G. F., Neely, A., Alvarez, O., Gonzalez, C., and Latorre, R. (2012). Modulation of BK channel voltage gating by different auxiliary β subunits. *Proc. Natl. Acad. Sci. U.S.A.* 109, 18991–18996. doi: 10.1073/pnas.1216953109

Cui, J., Yang, H., and Lee, U. S. (2009). Molecular mechanisms of BK channel activation. *Cell. Mol. Life Sci.* 66, 852–875. doi: 10.1007/s00018-008-8609-x

Davis, M. J., Wu, X., Nurkiewicz, T. R., Kawasaki, J., Gui, P., Hill, M. A., et al. (2001). Regulation of ion channels by protein tyrosine phosphorylation. *Am. J. Physiol.* 281, H1835–H1862.

Donnelier, J., and Braun, J. E. (2014). CSPα - Chaperoning presynaptic proteins. *Front. Cell. Neurosci.* 8:116. doi: 10.3389/fncel.2014.00116

Du, W., Bautista, J. F., Yang, H., Diez-Sampdero, A., You, S. A., Wang, L., et al. (2005). Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat. Genet.* 7, 733–738. doi: 10.1038/ng1585

England, S. K., Wooldridge, T. A., Stekiel, W. J., and Rusch, N. J. (1993). Enhanced single-channel K^+ current in arterial membranes from genetically hypertensive rats. *Am. J. Physiol. Heart Circ. Physiol.* 264, H1337–H1345.

Faber, E. S., and Sah, P. (2003). Calcium-activated potassium channels: multiple contributions to neuronal function. *Neuroscientist* 9, 181–194. doi: 10.1177/1073858403009003011

Fakler, B., and Adelman, J. P. (2008). Control of K_{Ca} channels by calcium nano/microdomains. *Neuron* 59, 873–881. doi: 10.1016/j.neuron.2008.09.001

Feil, R., Lohmann, S. M., De Jonge, H. R., Walter, U., and Hofmann, F. (2003). Cyclic GMP-dependent protein kinases and the cardiovascular system: insights from genetically modified mice. *Circ. Res.* 93, 907–916. doi: 10.1161/01.RES.0000100390.68771.CC

Fernandez-Chacon, R., Wolfel, M., Nishimune, H., Tabares, L., Schmitz, F., Castellano-Munoz, M., et al. (2004). The synaptic vesicle protein CSPα prevents presynaptic degeneration. *Neuron* 42, 237–251. doi: 10.1016/S0896-6273(04)00190-4

Fernández-Fernández, J. M., Tomás, M., Vázquez, E., Orlo, P., Latorre, R., Senti, M., et al. (2004). Gain-of-function mutation in the KCNMB1 potassium channel subunit is associated with low prevalence of diastolic hypertension. *J. Clin. Invest.* 113, 1032–1039. doi: 10.1172/JCI200420347

Fleming, I., and Busse, R. (2003). Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284, R1–R12. doi: 10.1152/ajpregu.00323.2002

Francis, S. H., Busch, J. L., and Corbin, J. D. (2010). cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol. Rev.* 62, 525–563. doi: 10.1124/pr.110.002907

Gu, N., Vervaeke, K., and Storm, J. F. (2007). BK potassium channels facilitate high-frequency firing and cause early spike frequency adaptation in rat CA1 hippocampal pyramidal cells. *J. Physiol.* 580, 859–882. doi: 10.1113/jphysiol.2006.126367

Haghdoost-Yazdi, H., Janahmadi, M., and Behzadi, G. (2008). Iberiotoxin-sensitive large conductance Ca^{2+}-dependent K^+ (BK) channels regulate the spike configuration in the burst firing of cerebellar Purkinje neurons. *Brain Res.* 1212, 1–8. doi: 10.1016/j.brainres.2008.03.030

Heppner, T. J., Bonev, A. D., and Nelson, M. T. (1997). Ca^{2+}-activated K^+ channels regulate action potential repolarization in urinary bladder smooth muscle. *Am. J. Physiol. Cell Physiol.* 273, C110–C117.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–479. doi: 10.1146/annurev.biochem.67.1.425

Hoshi, T., Pantazis, A., and Olcese, R. (2013). Transduction of voltage and Ca^{2+} signals by Slo1 BK channels. *Physiology* 28, 172–189. doi: 10.1152/physiol.00055.2012

Hou, S., Heinemann, S. H., and Hoshi, T. (2009). Modulation of BKCa channel gating by endogenous signaling molecules. *Physiology* 4, 26–35. doi: 10.1152/physiol.00032.2008

Hristov, K. L., Chen, M., Kellett, W. F., Rovner, E. S., and Petkov, G. V. (2011). Large-conductance voltage- and Ca^{2+}-activated K^+ channels regulate human detrusor smooth muscle function. *Am. J. Physiol. Cell Physiol.* 301, C903–C912. doi: 10.1152/ajpcell.00495.2010

Hu, H., Shao, L.-R., Chavoshy, S., Gu, N., Trieb, M., Behrens, R., et al. (2001). Presynaptic Ca^{2+}-activated K^+ channels in glutamatergic hippocampal terminals and their role in spike repolarization and regulation of transmitter release. *J. Neurosci.* 21, 9585–9597.

Imlach, W. L., Finch, S. C., Dunlop, J., Meredith, A. L., Aldrich, R. W., and Dalziel, J. E. (2008). The molecular mechanims of "ryegrass staggers," a neurological disorder of K^+ channels. *J. Pharmacol. Exp. Ther.* 327, 657–664. doi: 10.1124/jpet.108.143933

Issa, N. P., and Hudspeth, A. J. (1994). Clustering of Ca^{2+} channels and Ca^{2+}-activated K^+ channels at fluorescently labeled presynaptic active zones of hair cells. *Proc. Natl. Acad. Sci. U.S.A.* 91, 7578–7582. doi: 10.1073/pnas.91.16.7578

Jo, S., Lee, K.-H., Song, S., Jung, Y.-K., and Park, C.-S. (2005). Identification and functional characterization of cereblon as a binding protein for large-conductance calcium-activated potassium channel in rat brain. *J. Neurochem.* 94, 1212–1224. doi: 10.1111/j.1471-4159.2005.03344.x

Johnson, B. E., Glauser, D. A., Dan-Glauser, E. S., Halling, B., Aldrich, R. W., and Goodman, M. B. (2011). Alternatively spliced domains interact to regulate BK potassium channel gating. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20784–20789. doi: 10.1073/pnas.1116795108

Joseph, B. K., Thakali, K. M., Moore, C. L., and Rhee, S. W. (2013). Ion channel remodeling in vascular smooth muscle during hypertension: implications for novel therapeutic approaches. *Pharmacol. Res.* 70, 126–138. doi: 10.1016/j.phrs.2013.01.008

Kaczorowski, G. J., and Garcia, M. L. (1999). Pharmacology of voltage-gated and calcium-activated potassium channels. *Curr. Opin. Chem. Biol.* 3, 448–458. doi: 10.1016/S1367-5931(99)80066-0

Knaus, H.-G., Folander, K., Garcia-Calvo, M., Garcia, M. L., Kaczorowski, G. J., Smith, M., et al. (1994a). Primary sequence and immunological characterization of β-subunit of high conductance Ca^{2+}-activated K^+ channel from smooth muscle. *J. Biol. Chem.* 269, 17274–17278.

Knaus, H.-G., McManus, O. B., Lee, S. H., Schmalhofer, W. A., Garcia-Calvo, M., Helms, L. M., et al. (1994b). Tremorogenic indole alkaloids potentialy inhibit smooth muscle high-conductance calcium-activated potassium channels. *Biochem* 33, 5819–5828. doi: 10.1021/bi00185a021

Kyle, B. D., Ahrendt, E., Braun, A. P., and Braun, J. E. (2013a). The large conductance, calcium-activated K^+ (BK) channel is regulated by cysteine string protein. *Sci. Rep.* 3:2447. doi: 10.1038/srep02447

Kyle, B. D., Bradley, E., Large, R., Sergeant, G. P., McHale, N. G., Thornbury, K. D., et al. (2013b). Mechanisms underlying activation of transient BK current in rabbit urethral smooth muscle cells and its modulation by IP_3-generating agonists. *Am. J. Physiol. Cell Physiol.* 305, C609–C622. doi: 10.1152/ajpcell.00025.2013

Kyle, B. D., Hurst, S., Swayze, R. D., Sheng, J.-Z., and Braun, A. P. (2013c). Specific phosphorylation sites underlie the stimulation of a large conductance, Ca^{2+}-activated K^+ channel by cGMP-dependent protein kinase. *FASEB J.* 27, 2027–2038. doi: 10.1096/fj.12-223669

Latorre, R., Oberhauser, A., Labarca, P., and Alvarez, O. (1989). Varieties of calcium-activated potassium channels. *Annu. Rev. Physiol.* 51, 385–399. doi: 10.1146/annurev.ph.51.030189.002125

Leffler, C. W., Parfenova, H., and Jaggar, J. H. (2011). Carbon monoxide as an endogenous vascular modulator. *Am. J. Physiol. Heart Circ. Physiol.* 301, H1–H11. doi: 10.1152/ajpheart.00230.2011

Leo, M. D., Bannister, J. P., Narayanan, D., Nair, A., Grubbs, J. E., Gabrick, K. S., et al. (2014). Dynamic regulation of β1 subunit trafficking controls vascular contractility. *Proc. Natl. Acad. Sci. U.S.A.* 111, 2361–2366. doi: 10.1073/pnas.1317527111

Li, H., and Förstermann, U. (2009). Prevention of atherosclerosis by interference with the vascular nitric oxide system. *Curr. Pharm. Des.* 15, 3133–3145. doi: 10.2174/138161209789058002

Li, W., Bengston, M. H., Ulbrich, A., Matsuda, A., Reddy, V. A., Orth, A., et al. (2008). Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PLoS ONE* 3:e1487. doi: 10.1371/journal.pone.0001487

Lin, M. T., Hessinger, D. A., Pearce, W. J., and Longo, L. D. (2006). Modulation of BK channel calcium affinity by differential phosphorylation in developing ovine basilar artery myocytes. *Am. J. Physiol. Heart Circ. Physiol.* 291, H732–H740. doi: 10.1152/ajpheart.01357.2005

Lin, M. T., Longo, L. D., Pearce, W. J., and Hessinger, D. A. (2005). Ca^{2+}-activated K^+ channel-associated phosphatase and kinase activities during development. *Am. J. Physiol. Heart Circ. Physiol.* 289, H414–H425. doi: 10.1152/ajpheart.01079.2004

Ling, S., Sheng, J.-Z., and Braun, A. P. (2004). The calcium-dependent activity of large-conductance, calcium-activated K^+ channels is enhanced by Pyk2- and Hck-induced tyrosine phosphorylation. *Am. J. Physiol. Cell Physiol.* 287, C698–C706. doi: 10.1152/ajpcell.00030.2004

Ling, S., Woronuk, G., Sy, L., Lev, S., and Braun, A. P. (2000). Enhanced activity of a large conductance, calcium-sensitive K^+ channel in the presence of *Src* tyrosine kinase. *J. Biol. Chem.* 275, 30683–30689. doi: 10.1074/jbc.M004292200

Lipton, P. (1999). Ischemic cell death in brain neurons. *Physiol. Rev.* 79, 1431–1568.

Liu, G., Zakharov, S. I., Yang, L., Wu, R. S., Deng, S. X., Landry, D. W., et al. (2008). Locations of the β1 transmembrane helices in the BK potassium channel. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10727–10732. doi: 10.1073/pnas.0805212105

Liu, J., Ye, J., Zou, X., Xu, Z., Feng, Y., Zou, X., et al. (2014). $CRL4A^{CRBN}$ E3 ubiquitin ligase restricts BK channel activity and prevents epileptogenesis. *Nat. Commun.* 5:3924. doi: 10.1038/ncomms4924

Liu, Y., Hudetz, A. G., Knaus, H.-G., and Rusch, N. J. (1998). Increased expression of Ca^{2+}-sensitive K^+ channels in the cerebral microcirculation of genetically hypertensive rats. evidence for their protection against cerebral vasospasm. *Circ. Res.* 82, 729–737. doi: 10.1161/01.RES.82.6.729

Ma, Z., Lou, X. J., and Horrigan, F. T. (2006). Role of charged residues in the S1-S4 voltage sensor of BK channels. *J. Gen. Physiol.* 127, 309–328. doi: 10.1085/jgp.200509421

Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002). The protein kinase complement of the human genome. *Science* 298, 1912–1934. doi: 10.1126/science.1075762

Marrion, N. V., and Tavalin, S. J. (1998). Selective activation of Ca^{2+}-activated K^+ channels by co-localized Ca^{2+} channels in hippocampal neurons. *Nature* 395, 900–905. doi: 10.1038/27674

Meredith, A. L., Thorneloe, K. S., Werner, M. E., Nelson, M. T., and Aldrich, R. W. (2004). Overactive bladder and incontinence in the absence of the BK large conductance Ca^{2+}-activated K^+ channel. *J. Biol. Chem.* 279, 36746–36752. doi: 10.1074/jbc.M405621200

Morera, F., Alioua, A., Kundu, P., Salazar, M., Gonzalez, C., Martinez, A. D., et al. (2012). The first transmembrane domain (TM1) of β2-subunit binds to the transmembrane domain S1 of α-subunit in BK potassium channels. *FEBS Lett.* 586, 2287–2293. doi: 10.1016/j.febslet.2012.05.066

Morrow, J. P., Zakharov, S. I., Liu, G., Yang, L., Sok, A. J., and Marx, S. O. (2006). Defining the BK channel domains required for β1-subunit modulation. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5096–5101. doi: 10.1073/pnas.0600907103

Münzel, T., Feil, R., Mülsch, A., Lohmann, S. M., Hofmann, F., and Walter, U. (2003). Physiology and pathophysiology of vascular signaling controlled by cyclic guanosine 3′,5′-cyclic monophosphate-dependent protein kinase. *Circulation* 108, 2172–2183. doi: 10.1161/01.CIR.0000094403.78467.C3

Nara, M., Dhulipala, P. D., Wang, Y. X., and Kotlikoff, M. I. (1998). Reconstitution of β-adrenergic modulation of large conductance, calcium-activated potassium (Maxi-K) channels in *Xenopus* oocytes. *J. Biol. Chem.* 273, 14920–14924. doi: 10.1074/jbc.273.24.14920

Nardi, A., and Olesen, S. P. (2008). BK channel modulators: a comprehensive overview. *Curr Med. Chem.* 15, 1126–1146. doi: 10.2174/092986708784221412

Nielsen, T., Sølvsten Burgdorf, K., Grarup, N., Borch-Johnsen, K., Hansen, T., Jørgensen, T., et al. (2008). The KCNMB1 Glu65Lys polymorphism associates with reduced systolic and diastolic blodd pressure in the Inter99 study of 5729 Danes. *J. Hypertension* 26, 2142–2146. doi: 10.1097/HJH.0b013e32830b894a

Nieves-Cintrón, M., Amberg, G. C., Nichols, C. B., Molkentin, J. D., and Santana, L. F. (2007). Activation of NFATc3 down-regulates the β1 subunit of large conductance, calcium-actiated K^+ channels in arterial smooth muscle and contributes to hypertension. *J. Biol. Chem.* 282, 3231–3240. doi: 10.1074/jbc.M608822200

Nourian, Z., Li, M., Leo, M. D., Jaggar, J. H., Braun, A. P., and Hill, M. A. (2014). Large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) α-subunit splice variants in resistance arteries from rat cerebral and skeletal muscle vasculature. *PLoS ONE* 9:e98863. doi: 10.1371/journal.pone.0098863

Oger, S., Behr-Roussel, D., Gorny, D., Bernabé, J., Comperat, E., Chartier-Kastler, E., et al. (2010). Efects of potassium chanel modulators on myogenic spontaneous phasic contractile activity in human detrusor from neurogenic patients. *BJU Int.* 108, 604–611. doi: 10.1111/j.1464-410X.2010.09935.x

Ohi, Y., Yamamura, H., Nagano, N., Ohya, S., Muraki, K., Watanabe, M., et al. (2001). Local Ca^{2+} transients and distribution of BK channels and ryanodine receptors in smooth muscle cells of guinea-pig vas deferens and urinary bladder. *J. Physiol.* 534, 313–326. doi: 10.1111/j.1469-7793.2001.t01-3-00313.x

Ohya, S., Fujimori, T., Kimura, T., Yamamura, H., and Imaizumi, Y. (2010). Novel spliced variants of large-conductance Ca^{2+}-activated K^+-channel β2-subunit in human and rodent pancreas. *J. Pharmacol. Sci.* 114, 198–205. doi: 10.1254/jphs.10159FP

Pérez, G. J., Bonev, A. D., Patlak, J. B., and Nelson, M. T. (1999). Functional coupling of ryanodine receptors to K_{Ca} channels in smooth muscle cells from rat cerebral arteries. *J. Gen. Physiol.* 113, 229–237. doi: 10.1085/jgp.113.2.229

Petkov, G. V., Bonev, A. D., Heppner, T. J., Brenner, R., Aldrich, R. W., and Nelson, M. T. (2001). β1-Subunit of the Ca^{2+}-activated K^+ channel regulates contractile activity of mouse urinary bladder smooth muscle. *J. Physiol. (Lond.)* 537, 443–452. doi: 10.1111/j.1469-7793.2001.00443.x

Plüger, S., Faulhaber, J., Fürstenau, M., Löhn, M., Waldschutz, R., Gollasch, M., et al. (2001). Mice with disrupted BK channel β1 subunit gene feature abnormal Ca^{2+} spark/STOC coupling and elevated blood pressure. *Circ. Res.* 87, e53–e60. doi: 10.1161/01.RES.87.11.e53

Poulsen, A. N., Wulf, H., Hay-Schmidt, A., Jansen-Olesen, I., Olesen, J., and Klaerke, D. A. (2009). Differential expression of BK channel isoforms and β-subunits in rat neuro-vascular tissues. *Biochim. Biophys. Acta* 1788, 380–389. doi: 10.1016/j.bbamem.2008.10.001

Raffaelli, G., Saviane, C., Mohajerani, M. H., Pedarzani, P., and Cherubini, E. (2004). BK potassium channels control transmitter release at CA3-CA3 synapses in rat hippocampus. *J. Physiol.* 557, 147–157. doi: 10.1113/jphysiol.2004.062661

Reinhart, P. H., Chung, S., Martin, B. L., Brautigan, D. L., and Levitan, I. B. (1991). Modulation of calcium-activated potassium channels from rat brain by protein kinase A and phosphatase 2A. *J. Neurosci.* 11, 1627–1635.

Reinhart, P. H., and Levitan, I. B. (1995). Kinase and phosphatase activities intimately associated with a reconstituted calcium-dependent potassium channel. *J. Neurosci.* 15, 4572–4579.

Roberts, W. M., Jacobs, R. A., and Hudspeth, A. J. (1990). Colocalization of ion channels involved in frequency selectivity and synaptic transmission at presynaptic active zones of hair cells. *J. Neurosci.* 10, 3664–3684.

Robitaille, R., and Charlton, M. P. (1992). Presynaptic calcium signals and transmitter release are modulated by calcium-activated potassium channels. *J. Neurosci.* 12, 297–305.

Rusch, N. J., De Lucena, R. G., Wooldridge, T. A., England, S. K., and Cowley, A. W. Jr. (1992). A Ca^{2+}-dependent K^+ current is enhanced in arterial membranes of hypertensive rats. *Hypertension* 19, 301–307. doi: 10.1161/01.HYP.19.4.301

Rusch, N. J., and Runnells, A. M. (1994). Remission of high blood pressure reverses arterial potassium channel alterations. *Hypertension* 23, 941–945. doi: 10.1161/01.HYP.23.6.941

Sanders, K. M. (2008). Regulation of smooth muscle excitation and contraction. *Neurogastroenterol. Motil.* 20, 39–53. doi: 10.1111/j.1365-2982.2008.01108.x

Sansom, S. C., Stockand, J. D., Hall, D., and Williams, B. (1997). Regulation of large conductance calcium-activated potassium channels by protein phosphatase 2A. *J. Biol. Chem.* 272, 9902–9906. doi: 10.1074/jbc.272.15.9902

Sausbier, M., Hu, H., Arntz, C., Feil, S., Kamm, S., Adelsberger, H., et al. (2004). Cerebellar ataxia and Purkinje cell dysfunction caused by Ca^{2+}-activated K^+ channel deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9474–9478. doi: 10.1073/pnas.0401702101

Schubert, R., and Nelson, M. T. (2001). Protein kinases: tuners of the BK_{Ca} channel in smooth muscle. *TIPS* 22, 505–512. doi: 10.1016/S0165-6147(00)01775-2

Seibold, M. A., Wang, B., Eng, C. E., Kumar, G., Beckman, K. B., Sen, S., et al. (2008). An african-specific functional polymorphism in *KCNMB1* shows sex-specific association with asthma severity. *Hum. Mol. Genet.* 17, 2681–2690. doi: 10.1093/hmg/ddn168

Shen, K.-Z., Lagrutta, A., Davies, N. W., Standen, N. B., Adelman, J. P., and North, R. A. (1994). Tetraethylammonium block of *Slowpoke* calcium-activated

potassium channels expressed in *Xenopus* oocytes: evidence for tetrameric channel formation. *Pflügers Arch.* 426, 440–445. doi: 10.1007/BF00388308

Shipston, M. J. (2001). Alternative splicing of potassium channels: a dynamic switch of cellular excitability. *Trends Cell Biol.* 11, 353–358. doi: 10.1016/S0962-8924(01)02068-2

Shipston, M. J. (2014). Ion channel regulation by protein S-acylation. *J. Gen. Physiol.* 143, 659–678. doi: 10.1085/jgp.201411176

Singh, H., Stefani, E., and Toro, L. (2012). Intracellular BK_{Ca} (iBK_{Ca}) channels. *J. Physiol. (Lond.)* 590, 5937–5947. doi: 10.1113/jphysiol.2011.215533

Sprossmann, F., Pankert, P., Sausbier, U., Wirth, A., Zhou, X.-B., Madlung, J., et al. (2009). Inducible knockout mutagenesis reveals compensatory mechanisms elicited by constitutive BK channel deficiency in overactive murine bladder. *FEBS Lett.* 276, 1680–1697. doi: 10.1111/j.1742-4658.2009.06900.x

Tanaka, Y., Meera, P., Song, M., Knaus, H.-G., and Toro, L. (1997). Molecular constituents of maxi-K_{Ca} channels in human coronary smooth muscle: predominant $\alpha + \beta$ subunit complexes. *J. Physiol. (Lond.)* 502, 545–557. doi: 10.1111/j.1469-7793.1997.545bj.x

Tang, X. D., Santarelli, L. C., Heinemann, S. H., and Hoshi, T. (2004). Metabolic regulation of potassium channels. *Annu. Rev. Physiol.* 66, 131–159. doi: 10.1146/annurev.physiol.66.041002.142720

Tang, X. D., Xu, R., Reynolds, M. F., Garcia, M. L., Heinemann, S. H., and Hoshi, T. (2003). Haem can bind to and inhibit mammalian calcium-dependent Slo1 BK channels. *Nature* 425, 531–535. doi: 10.1038/nature02003

Taylor, S. S., Buechler, J. A., and Yonemoto, W. (1990). cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annu. Rev. Biochem.* 59, 971–1005. doi: 10.1146/annurev.bi.59.070190.004543

Thorneloe, K. S., and Nelson, M. T. (2005). Ion channels in smooth muscle: regulators of intracellular calcium and contractility. *Can. J. Physiol. Pharmacol.* 83, 215–242. doi: 10.1139/y05-016

Tian, L., Coghill, L. S., McClafferty, H., MacDonald, S. H., Antoni, F. A., Ruth, P., et al. (2004). Distinct stoichiometry of BKCa channel tetramer phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11897–11902. doi: 10.1073/pnas.0402590101

Tian, L., Duncan, R. R., Hammond, M. S., Coghill, L. S., Wen, H., Rusinova, R., et al. (2001). Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J. Biol. Chem.* 276, 7717–7720. doi: 10.1074/jbc.C000741200

Tian, L., Jeffries, O., McClafferty, H., Molyvdas, A., Rowe, I. C., Saleem, F., et al. (2008). Palmitoylation gates phosphorylation-dependent regulation of BK potassium channels. *Proc. Natl. Acad. Sci. U.S.A.* 105, 21006–21011. doi: 10.1073/pnas.0806700106

Tian, L., Knaus, H.-G., and Shipston, M. J. (1998). Glucocorticoid regulation of calcium-activated potassium channels mediated by serine/threonine protein phosphatase. *J. Biol. Chem.* 273, 13531–13536. doi: 10.1074/jbc.273.22.13531

Toro, B., Cox, N., Wilson, R. J., Garrido-Sanabria, E., Stefani, E., Toro, L., et al. (2006). KCNMB1 regulates surface expression of a voltage and Ca^{2+}-activated K^+ channel via endocytic trafficking signals. *Neuroscience* 142, 661–669. doi: 10.1016/j.neuroscience.2006.06.061

Uebele, V. N., Lagrutta, A., Wade, T., Figueroa, D. J., Liu, Y., McKenna, E., et al. (2000). Cloning and functional expression of two families of β-subunits of the large conductance calcium-activated K^+ channel. *J. Biol. Chem.* 275, 23211–23218. doi: 10.1074/jbc.M910187199

Wallner, M., Meera, P., and Toro, L. (1999). Molecular basis f fast inactivation in voltage and Ca^{2+}-activated K^+ channels: a tranmembrane β-subunit homolog. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4137–4142. doi: 10.1073/pnas.96.7.4137

Wang, B., Rothberg, B. S., and Brenner, R. (2006). Mechanism of β4 subunit modulation of BK channels. *J. Gen. Physiol.* 127, 449–465. doi: 10.1085/jgp.200509436

Wang, B., Rothberg, B. S., and Brenner, R. (2009). Mechanism of increased BK channel activation from a channel mutation that causes epilepsy. *J. Gen. Physiol.* 133, 283–294. doi: 10.1085/jgp.200810141

Wimalawansa, S. (2008). Nitric oxide: new evidence for novel therapeutic indications. *Expert Opin. Pharmacother.* 9, 1935–1954. doi: 10.1517/14656566.9.11.1935

Xie, J., and McCobb, D. P. (1998). Control of alternative splicing of potassium channels by stress hormones. *Science* 280, 443–446. doi: 10.1126/science.280.5362.443

Yan, J., and Aldrich, R. W. (2010). LRRC26 auxiliary protein allow BK channel activation at resting voltage without calcium. *Nature* 466, 513–517. doi: 10.1038/nature09162

Yan, J., and Aldrich, R. W. (2012). BK potassium channel modulation by leucine-rich repeat-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7917–7922. doi: 10.1073/pnas.1205435109

Yan, J., Olsen, J. V., Park, K. S., Li, W., Bildl, W., Schulte, U., et al. (2008). Profiling the phospho-status of the BK_{Ca} channel α subunit in rat brain reveals unexpected patterns and complexity. *Mol. Cell. Proteomics* 7, 2188–2198. doi: 10.1074/mcp.M800063-MCP200

Yang, J., Krishnamoorthy, G., Saxena, A., Zhang, G., Shi, J., Yang, H., et al. (2010). An epilepsy/dyskinesia-associated mutation enhances BK channel activation by potentiating Ca^{2+} sensing. *Neuron* 66, 871–883. doi: 10.1016/j.neuron.2010.05.009

Yang, Y., Li, P.-Y., Cheng, J., Mao, L., Wen, J., Tan, X.-Q., et al. (2013). Function of BK_{Ca} channels is reduced in human vascular smooth muscle cells from Han Chinese patients with hypertension. *Hypertension* 61, 519–525. doi: 10.1161/HYPERTENSIONAHA.111.00211

Yang, Y., Murphy, T. V., Ella, S. R., Grayson, T. H., Haddock, R., Hwang, Y. T., et al. (2009). Heterogeneity in function of small artery smooth muscle BK_{Ca}: involvement of the β1-subunit. *J. Physiol. (Lond.)* 587, 3025–3044. doi: 10.1113/jphysiol.2009.169920

Yang, Y., Wu, X., Gui, P., Wu, J., Sheng, J.-Z., Ling, S., et al. (2012). α5β1 integrin engagement increases large conductance, Ca^{2+}-activated K^+ channel current and Ca^{2+} sensitivity through cSrc-mediated channel phosphorylation. *J. Biol. Chem.* 285, 131–141. doi: 10.1074/jbc.M109.033506

Yi, F., Wang, H., Chai, Q., Wang, X., Shen, W.-K., Willis, M., et al. (2014). Regulation of large conducatnce Ca^{2+}-activated K^+ (BK) channel β1 subunit expression by muscle RING finger protein 1 in diabetic vessels. *J. Biol. Chem.* 289, 10853–10864. doi: 10.1074/jbc.M113.520940

Yuan, P., Leonetti, M. D., Hsiung, Y., and MacKinnon, R. (2012). Open structure of the Ca^{2+} gating ring in the high-conductance Ca^{2+}-activated K^+ channel. *Nature* 481, 94–98. doi: 10.1038/nature10670

Yuan, P., Leonetti, M. D., Pico, A., Hsiung, Y., and MacKinnon, R. (2010). Structure of the human BK channel Ca^{2+}-activation apparatus at 3.0 Å resolution. *Science* 329, 182–186. doi: 10.1126/science.1190414

Zarei, M. M., Song, M., Wilson, R. J., Cox, N., Colom, L. V., Knaus, H.-G., et al. (2007). Endocytic trafficking signals in KCNMB2 regulate surface expression of a large conductance voltage and Ca^{2+}-activated K^+ channel. *Neuroscience* 147, 80–89. doi: 10.1016/j.neuroscience.2007.04.019

Zhou, X., Wulfsen, I., Korth, M., McClafferty, H., Lukowski, R., Shipston, M. J., et al. (2012). Palmitoylation and membrane association of the stress axis regulated insert (STREX) controls BK channel regulation by protein kinase C. *J. Biol. Chem.* 287, 32161–32171. doi: 10.1074/jbc.M112.386359

Zhou, X.-B., Ruth, P., Schlossmann, J., Hofmann, F., and Korth, M. (1996). Protein phosphatase 2A is essential for the activation of Ca^{2+}-activated K^+ currents by cGMP-dependent protein kinase in tracheal smooth muscle and chinese hamster ovary cells. *J. Biol. Chem.* 271, 19760–19767. doi: 10.1074/jbc.271.33.19760

Zhou, X.-B., Wulfsen, I., Utku, E., Sausbier, U., Sausbier, M., Wieland, T., et al. (2010). Dual role of protein kinase C on BK channel regulation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8005–8010. doi: 10.1073/pnas.0912029107

ZhuGe, R., Fogarty, K. E., Tuft, R. A., and Walsh, J. V. Jr. (2002). Spontaneous transient outward currents arise from microdomains where BK channels are exposed to a mean Ca^{2+} concentration on the order of 10 μM during a Ca^{2+} spark. *J. Gen. Physiol.* 120, 15–27. doi: 10.1085/jgp.20028571

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 July 2014; accepted: 02 August 2014; published online: 22 August 2014.
Citation: Kyle BD and Braun AP (2014) The regulation of BK channel activity by pre- and post-translational modifications. Front. Physiol. ***5****:316. doi: 10.3389/fphys.2014.00316*
This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 09 October 2014
doi: 10.3389/fphys.2014.00382

Current understanding of iberiotoxin-resistant BK channels in the nervous system

Bin Wang[1], David B. Jaffe[2] and Robert Brenner[1]*

[1] *Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA*
[2] *Department of Biology and the UTSA Neurosciences Institute, University of Texas at San Antonio, San Antonio, TX, USA*

Edited by:
Thomas M. Weiger, University of Salzburg, Austria

Reviewed by:
Neil V. Marrion, University of Bristol, UK
Alexi Alekov, Medizinische Hochschule Hannover, Germany

***Correspondence:**
Robert Brenner, Department of Physiology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA
e-mail: brennerr@uthscsa.edu

While most large-conductance, calcium-, and voltage-activated potassium channels (BK or Maxi-K type) are blocked by the scorpion venom iberiotoxin, the so-called "type II" subtype has the property of toxin resistance. This property is uniquely mediated by channel assembly with one member of the BK accessory β subunit family, the neuron-enriched β4 subunit. This review will focus on current understanding of iberiotoxin-resistant, β4-containing BK channel properties and their function in the CNS. Studies have shown that β4 dramatically promotes BK channel opening by shifting voltage sensor activation to more negative voltage ranges, but also slows activation to timescales that theoretically preclude BK ability to shape action potentials (APs). In addition, β4 membrane trafficking is regulated through an endoplasmic retention signal and palmitoylation. More recently, the challenge has been to understand the functional role of the iberiotoxin-resistant BK subtype utilizing computational modeling of neurons and neurophysiological approaches. Utilizing iberiotoxin-resistance as a footprint for these channels, they have been identified in dentate gyrus granule neurons and in purkinje neurons of the cerebellum. In these neurons, the role of these channels is largely consistent with slow-gated channels that reduce excitability either through an interspike conductance, such as in purkinje neurons, or by replacing fast-gating BK channels that otherwise facilitate high frequency AP firing, such as in dentate gyrus neurons. They are also observed in presynaptic mossy fiber terminals of the dentate gyrus and posterior pituitary terminals. More recent studies suggest that β4 subunits may also be expressed in some neurons lacking iberiotoxin-resistant BK channels, such as in CA3 hippocampus neurons. Ongoing research using novel, specific blockers and agonists of BK/β4, and β4 knockout mice, will continue to move the field forward in understanding the function of these channels.

Keywords: BK channel, iberiotoxin, beta4, KCNMB4, type II bk channels, KCNMA1, MaxiK, calcium-activated potassium channel

INTRODUCTION

While BK K^+ channels are often identified using the scorpion venom iberiotoxin, seminal work by Rinehart and Levitan identified an *iberiotoxin-resistant*, slow-gated BK channel subtype from brain synaptosomal membranes (Reinhart et al., 1989; Reinhart and Levitan, 1995). The investigators classified this as the so-called "type II BK channel" which was in contrast to the more conventional iberiotoxin-sensitive type I, fast-gated BK channels. A similar type II toxin-resistant BK channel was observed in posterior pituitary nerve terminals soon after (Bielefeldt et al., 1992; Wang et al., 1992). The molecular basis for type II BK channels was revealed in 1999 when random cDNA sequences began flooding DNA databases and perusing BKologists identified three additional accessory subunit family members (β2, β3, and β4) similar to the previously cloned β1 that modulate the BK pore-forming α subunit. Among these, the neuron-specific β4 subunit was found to confer the slow-gating and iberiotoxin-resistance that likely underlies the type II BK channels seen in synaptosomal membranes (Behrens et al., 2000; Brenner et al., 2000; Meera et al., 2000; Weiger et al., 2000; Lippiat et al., 2003). This was confirmed by gene knockout of the β4 subunit that converted BK channels in neurons from iberiotoxin-resistant to iberiotoxin-sensitive channels (Brenner et al., 2005). While cloned more than 14 years ago, our understanding of the functional role of β4-containing BK potassium channels in neurons is still very limited. This short review will discuss current understanding of BK/β4 biophysical properties, their regulation, and neurophysiological function.

BK CHANNELS ARE COMPOSED OF DIVERSE SUBTYPES IN CENTRAL NEURONS

Large conductance calcium-activated (BK-type) potassium channels are potassium channels uniquely activated by both calcium and depolarization (Kaczorowski et al., 1996; Gribkoff et al., 1997; Calderone, 2002). When open, BK channels have among the largest ion channel conductance (>200 pS) and are very effective in hyperpolarizing the membrane. BK channels are expressed relatively broadly in many excitatory neurons of the

CNS (Wanner et al., 1999). Early studies used scorpion venoms including charybdotoxin, and later the uniquely BK-selective iberiotoxin, to block channels and thereby study BK effects in neurons. Use of these blockers has revealed their key role in shaping action potentials. Depending on the neuron, BK channels affect the repolarization phase and the fast-component of the afterhyperpolarization to various extents (Sah and Faber, 2002).

Although the pore-forming subunit (α subunit) is encoded by a single gene, a family of four tissue-specific accessory subunits, β1 through β4 (Orio et al., 2002), confer BK channels with diverse functional properties affecting steady-state conductance properties, gating kinetics, inactivation, and pharmacology (Behrens et al., 2000; Brenner et al., 2000). Expression studies suggest that β2 and β4 are the principle β subunits expressed in central neurons (**Figure 1A**) (Brenner et al., 2000), and electrophysiology and pharmacology studies (discussed below) suggest that α interactions with these subunits define one of three BK channel subtypes generally observed in central neurons. These are the inactivating BK channels (α + β2), and the non-inactivating type I (α alone) and type II BK channels (α + β4). A simple overview of some key properties that distinguishes these channel subtypes in neurons is shown in **Figure 1B**. In heterologous expression systems, the accessory β2 subunit confers N-type inactivation to BK channels and is sensitive to iberiotoxin block (Wallner et al., 1999; Xia et al., 1999). Inactivating BK channels are observed in CA1 (Cornu Ammonis-1) neurons of the hippocampus (McLarnon, 1995) and adrenal chromaffin cells (Solaro and Lingle, 1992). The effect of these channels in central neurons is to repolarize the first few, but not later, action potentials in a train, resulting in a frequency-dependent spike broadening (Shao et al., 1999; Faber and Sah, 2003). Although inactivating BK channels are likely mediated by the β2 accessory subunit, some splice products of the β3 subunit also confer inactivation. However, this protein has weak expression in the brain (Wallner et al., 1999; Xia et al., 1999; Uebele et al., 2000; Hu et al., 2003).

The non-inactivating type I and type II BK channel subtypes were originally identified from bilayer recordings from synaptosomal membrane preparations from brain (Reinhart et al., 1989; Reinhart and Levitan, 1995). Type I BK channels have relatively fast gating kinetics, are sensitive to iberiotoxin block, and likely represent BK channels lacking accessory β subunits. Type II BK channels have slow gating kinetics and are insensitive to

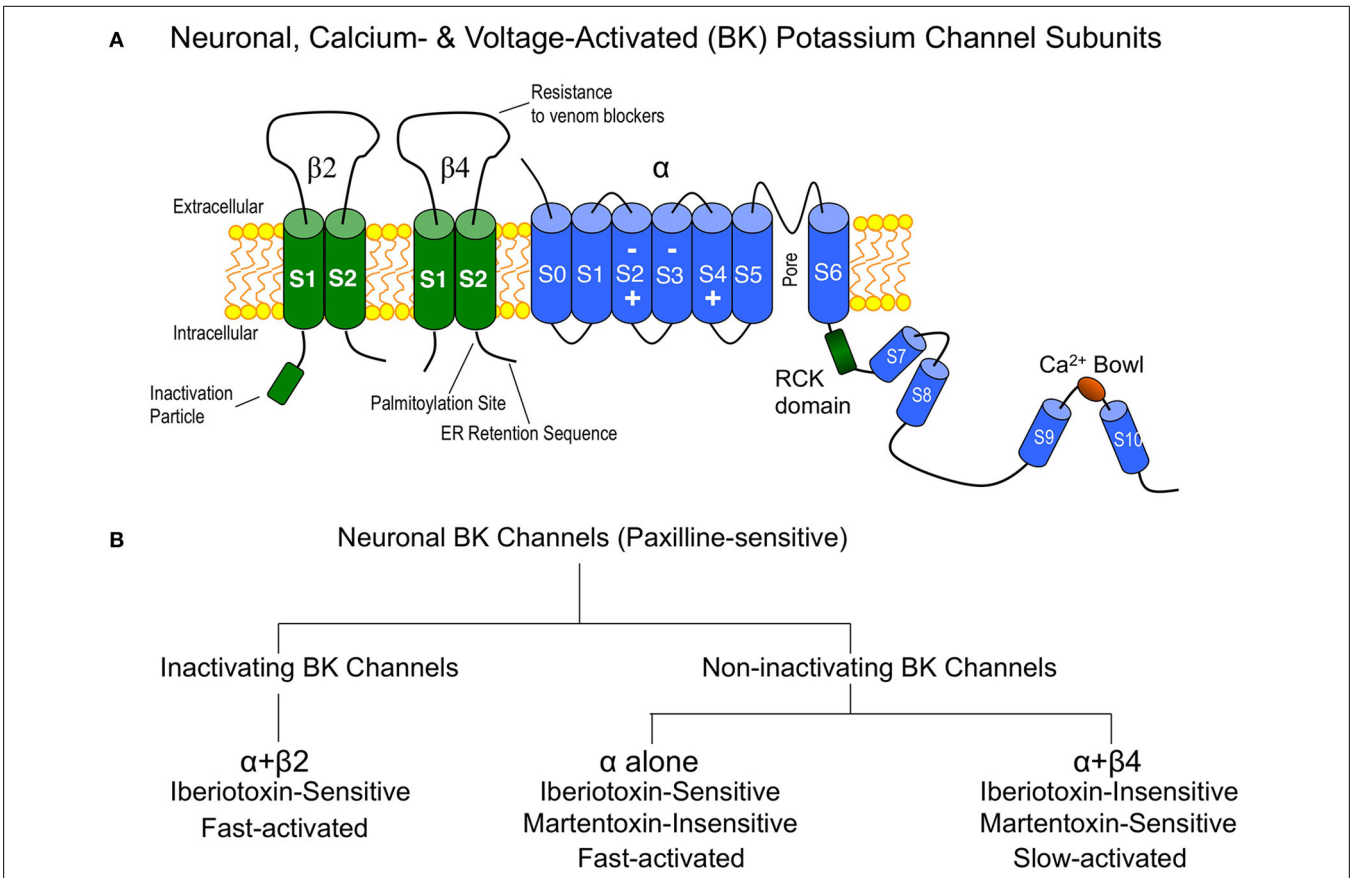


FIGURE 1 | (A) Cartoon of the major BK channel subunits expressed in neurons of the central nervous system and key domains. "Inactivation particle" is β2 amino terminal sequence that confers fast-type inactivation on BK channels (Wallner et al., 1999; Xia et al., 1999). The β4 subunit extracellular domain contains residues that confer BK channel "resistance to venom blockers" including charybdotoxin and iberiotoxin (Meera et al., 2000; Gan et al., 2008). Surface trafficking of β4 is regulated by carboxyl-terminal "palmitoylation" (Chen et al., 2013) and an "ER (endoplasmic reticulum) retention sequence" (Shruti et al., 2012; Cox et al., 2014). The BK channel is depicted with voltage-sensor regions (S2–S4) (Ma et al., 2006) and calcium binding sites (RCK and Ca^{2+} bowl domains) (Bao et al., 2002; Xia et al., 2002). **(B)** Flow chart of neuronal BK channel pharmacology and gating properties.

iberiotoxin block (Reinhart et al., 1989). As discussed below, the slow gating and iberiotoxin-resistance are hallmarks of BK channels containing β4 subunits. In addition, type II BK channels are coupled to protein kinase C and protein phosphatase (Reinhart and Levitan, 1995). Historically the functional role of type II BK channels are less understood perhaps due to their resistance to iberiotoxin block, but also because they are less often observed in neurons. Later, paxilline was identified as a useful blocker for BK channels (Knaus et al., 1994) that indiscriminately blocks both type I and type II BK channels (Hu et al., 2001). Thus, investigators can unambiguously identify BK/β4 channels as those that are resistant to iberiotoxin and sensitive to paxilline. Recently, Martentoxin (Shi et al., 2008; Tao et al., 2012) and Conopeptide Vt3.1 (Li et al., 2014) were identified as more selective blockers of BK/β4 channels than the pore-forming α subunit alone. The neuronal β2 subunit does not alter Conopeptide Vt3.1 block (Li et al., 2014). Whether or not Martentoxin also blocks BK/β2 channels has not been established with certainty. Although Martentoxin blocks a large fraction of BK currents in adrenal chromaffin cells where BK/β2 channels are expressed (Ji et al., 2003), pharmacological studies on pure α + β2 have not yet been conducted.

β4 SUBUNITS SLOW GATING AND INCREASE OPEN PROBABILITY OF BK CHANNELS

BK channels are unique among other potassium channels in being activated by both voltage and calcium. Therefore, these channels are believed to be a negative feedback mechanism regulating voltage-dependent calcium channels and other calcium-modulated voltage-dependent channels (**Figure 2A**). Theoretically, the tissue-specific accessory β subunits tune the response properties of BK channels to the needs of the cell. The dual activation properties of BK channels are most clearly apparent in single channel recordings (**Figure 2B**). In constant calcium (1.7 μM internal calcium, **Figure 2B**, left panel) channel opening increases in a voltage-dependent manner. As well, at constant voltage (+40 mV, **Figure 2B**, right panel) increasing calcium increases channel open probability. The response properties of BK channels to voltage and calcium are well represented in plots of conductance-voltage relationships (G-V relationships, **Figure 2C**). The pore-forming subunit of BK channels (α subunit) binds calcium at affinities that are relatively low (tens of micromole) compared to physiological global calcium concentrations (i.e., bulk calcium rises that occur throughout the cell, ∼ 100–200 nM) (Cox et al., 1997; Cui et al., 1997). At resting calcium concentrations (nanomolar) BK channels in neurons do not show significant channel openings at physiological voltage ranges (Horrigan et al., 1999) (**Figure 2C**). Thus, to obtain high open probabilities, BK channels require coincident depolarization and calcium rises such as might occur during an action potential and in close apposition to voltage-activated calcium channels (Fakler and Adelman, 2008). Experimentally, the dual calcium and voltage sensitivities are seen as shifts of the G-V relations to negative potentials with increasing calcium (**Figure 2C**). Thus,

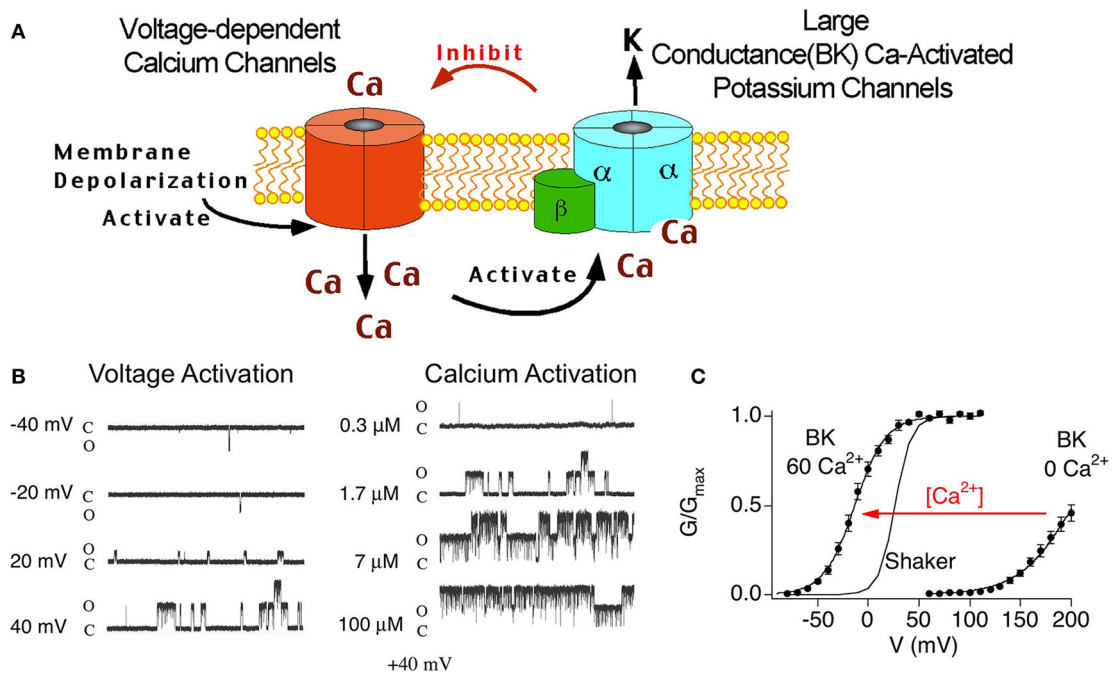


FIGURE 2 | (A) Cartoon of voltage-dependent calcium channel and BK channel negative feedback mechanism. Depolarization opens voltage-dependent calcium channels. The coincident calcium influx and depolarization activate BK channels, that in-turn, repolarize the membrane and deactivate voltage-dependent currents. **(B)** Single channel recording of BK α alone at various voltage (left panel) and calcium (right panel). Note: there are two BK channels in this patch. **(C)** Conductance-voltage relations of BK α alone recorded from inside/out patches containing macroscopic currents. 0 Ca^{2+} is internal solution containing 5 mM EGTA to buffer calcium. 60 μM internal calcium shifts the G-V relations to more negative membrane potentials.

neurons can theoretically convert BK channels from channels that are high voltage-activated channels, to channels activated at threshold or subthreshold potentials by dynamically increasing calcium concentrations. Indeed, although Shaker-type Kv channels are more voltage-sensitive than BK channels, high local calcium can cause BK channels to open in a voltage-dependent fashion at more negative membrane potentials (**Figure 2C**).

Functional studies in heterologous expression systems provided strong evidence that β4 confers properties of type II BK channels. The extracellular domain of β4 has been shown to occlude toxin binding to BK channels, a key property of type II channels (Meera et al., 2000; Gan et al., 2008). The β4 subunits' predominant effect on channel gating is to slow activation and deactivation kinetics, also a property of type II channels (Behrens et al., 2000; Brenner et al., 2000; Ha et al., 2004; Wang et al., 2006). This is quite obvious in the slow activation rise time and slow tail currents, respectively (**Figures 3A,B**). The biophysical mechanisms underlying kinetic changes are not understood. β4 also modulates steady-state properties by causing negative shifts of the conductance-voltage relations in high calcium ($> \sim 10\,\mu M$) but positive shifts in low calcium (Behrens et al., 2000; Brenner et al., 2000; Lippiat et al., 2003; Ha et al., 2004; Wang et al., 2006). These effects on channel opening suggest that β4 regulates BK channels through multiple, opposing mechanisms on channel opening. Several studies have investigated how β4 alters steady-state gating (Wang et al., 2006; Contreras et al., 2012) in the context of an allosteric gating model for BK channels (Horrigan and Aldrich, 1999; Horrigan et al., 1999; Rothberg and Magleby, 1999). The allosteric model describes BK channel opening to be due to relatively independent coupling of voltage-sensors and Ca^{2+} sensors to the channel gate [(Horrigan and Aldrich, 2002); an excellent, short review in Lingle (2002)]. β4 was shown to increase BK channel opening by shifting voltage-sensor activation to more negative potentials (Wang et al., 2006; Contreras et al., 2012). This property has also been reported for the related β1 and β2 subunits (Bao and Cox, 2005; Orio and Latorre, 2005; Contreras et al., 2012). Channel modeling studies of β4 effects suggest that this calcium-independent property contributes to the negative-shift of the conductance-voltage relations that is apparent at high calcium (Wang et al., 2006). How does β4 reduce channel opening (cause a positive-shift of the conductance-voltage relations) at low calcium? Two mechanisms have been ascribed to this effect. β4, like the related β1 and β2, cause an increased energetic barrier for gate opening (Orio and Latorre, 2005; Wang and Brenner, 2006; Wang et al., 2006). As well, β4 was shown to reduce gating charge which also can contribute to reduced channel openings (Contreras et al., 2012).

The slow gating and the depolarization of the G-V relations at low calcium suggested that β4 subunits could be regarded as an inhibitory neuronal subunit of BK channels (Behrens et al., 2000; Weiger et al., 2000; Brenner et al., 2005). However, BK channel biophysicists generally find it convenient to record currents with symmetrical, high potassium concentrations (replacing external sodium with potassium). Surprisingly, recordings conducted at physiological external sodium, low external potassium concentration instead indicate that β4 confers a negative shift of the G-V relationship at all calcium concentrations (Wang et al., 2009; Jaffe et al., 2011) (**Figure 3C**). This casts some doubt into conclusions obtained from previous biophysical studies using non-physiological solutions. The slow activation and deactivation gating kinetics nevertheless is observed, albeit to a lesser extent (**Figure 3D**). Thus, from a steady-state perspective β4 generally promotes BK channel opening. However, during the fast times of an action potential, the slow-activation conferred by

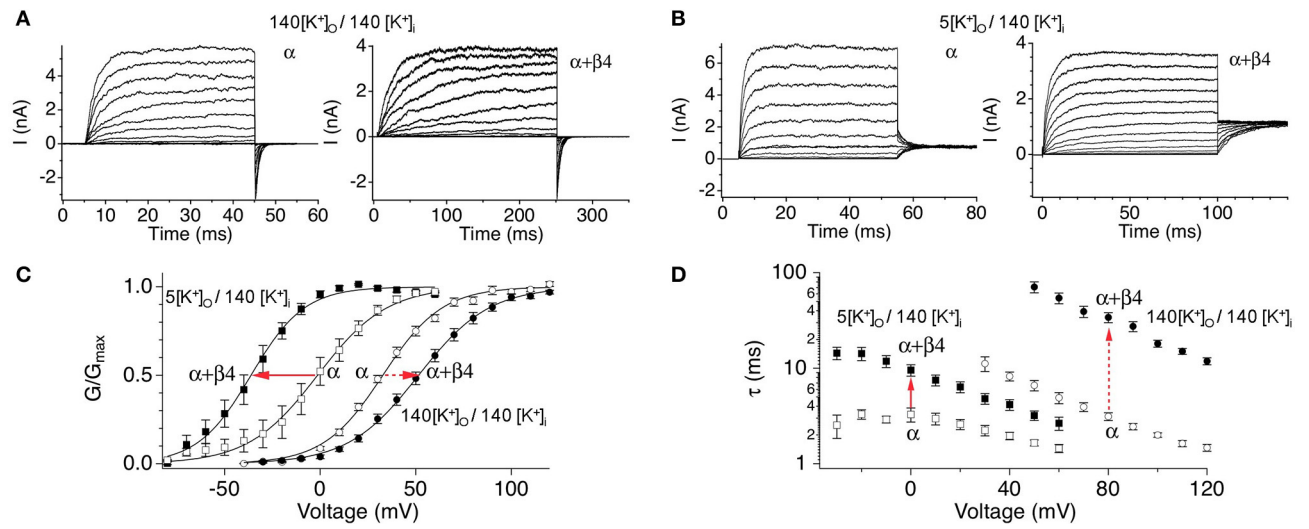


FIGURE 3 | Steady-state and kinetic properties of BK/β4 channels. Macroscopic recording comparing α alone and α + β4 channels in **(A)** symmetrical high external potassium (140 mM outside/140 inside) to recordings in **(B)** asymmetrical 5 mM external/140 mM internal potassium. **(C)** G-V relations. **(D)** Activation kinetics. Currents were acquired at 7 μM buffered internal calcium. Arrows indicate effect of β4 on BK channels in physiological asymmetric potassium (solid arrows) or in non-physiological symmetrical potassium (dashed arrows). Data are replotted from Wang et al. (2009) and Jaffe et al. (2011).

β4 could theoretically inhibit BK channels from contributing to repolarization, whereas their slow-deactivation might sustain open channels following repolarization.

β4 EFFECTS ON BK CHANNELS IN THE CONTEXT OF BK α SUBUNIT ALTERNATIVE SPLICING OR GENE MUTATIONS

The effect of β4 on BK channel biophysical properties is also dependent on splicing isoforms or mutations of the α subunit. One well-studied splice variant is the stress-axis activated exon (STREX) (Xie and McCobb, 1998; Tian et al., 2001) that promotes BK channel opening via decreasing the closing rates of the channels. While β4 and STREX both slow deactivation, the combined effect of STREX and β4 are to speed deactivation (Petrik and Brenner, 2007). In addition, at low calcium, β4 further inhibits channel opening of STREX channels by dramatically slowing channel activation kinetics (Petrik and Brenner, 2007). Studies of the inhibitory "SRKR" exon of BK channels also showed a more dramatic inhibition when coexpressed with β4 (Shelley et al., 2013). Given its expression in the CNS, the effect of β4 on a human mutation (D434G) that causes epilepsy and paroxysmal dyskinesia (Du et al., 2005) has also been studied (Du et al., 2005). The mutation dramatically increases BK channel openings and speeds channel activation. However, β4 effects on steady-state, conductance-voltage relations is largely reduced with the human D434G mutation (Diez-Sampedro et al., 2006; Lee and Cui, 2009; Wang et al., 2009). Paradoxically, the mutation in the murine α (D369G) and β4 channel subunit shows full effects of β4. Why murine and human channels behave differently with regard to β4 effects on the epilepsy D/G mutation is currently unknown.

BIOPHYSICAL NEURONAL MODELS CONTAINING TYPE I AND TYPE II BK CHANNELS

Computer models of excitable cells containing BK channels have been used to understand the differential effects of inactivating and non-inactivating channels on output firing patterns. In adrenal chromaffin cells inactivating BK channels (containing α + β2 subunits) enhance excitability (Sun et al., 2009) by boosting the afterhyperpolarization (AHP) and, in turn, presumably enhancing the recovery of Na^+ channels from inactivation (Erisir et al., 1999). Modeling of inactivating BK channels in simulations of CA1 pyramidal neuron firing reproduces the decreased spike duration seen in the first few action potentials in a train of spikes (Shao et al., 1999). As discussed above, this allows for greater instantaneous firing rates at the beginning of an episode of spike generation.

The role of iberiotoxin-resistant (type II) BK channels on neuronal excitability has also been investigated by us in the modeling of BK channels in dentate gyrus granule neurons (Jaffe et al., 2011). Using physiological-appropriate recordings of type I (α alone) and type II (α + β4) BK channels *in vitro*, the investigators developed analytical equations describing the combined voltage-, calcium-, and time-dependence of both type I and II BK channels (**Figure 4A**). These were incorporated into a model of DG neurons containing either type I or type II channels (Jaffe et al., 2011). The model reproduced *in silico* the relative reduction in spike duration shaped by type I BK channels (**Figure 4B1**), similar to that observed from recordings of DG neurons where the β4 was knocked out (Brenner et al., 2005). In contrast, type II channels achieved less spike reduction; ∼50% of that produced by type I channels (assuming the same BK channel density) because of the relative difference in activated BK channel current (**Figure 4B2**). The difference in BK current between the two channel types was readily explained by the difference in BK channel kinetics. The ratio of the steady-state activation variable plotted against the activation time constant variable (w_∞/τ_w) illustrates a greater likelihood for type I channel activation for the short period of depolarization during peak of the action potential. It was not during the down-stroke of the spike where Ca^{2+} influx is maximal (**Figure 4C**). At the peak of the spike the initial influx of calcium reaches a concentration where it shifts the channels' voltage-dependence into the voltage range of an action potential (see **Figure 4A**). As a result, type II channel activation lags compared to that of type I channels. Subsequently, their differential effects on spike waveform can, in turn, influence neuronal excitability by altering slower Ca^{2+}-dependent conductances (Brenner et al., 2005; Jaffe et al., 2011; Ly et al., 2011), Na^+ influx and Na^+-dependent conductances, as well as voltage-gated potassium conductances (Shao et al., 1999). This model also supports the hypothesis that both type I and II BK channels express a low-affinity Ca^{2+} sensor (Schreiber and Salkoff, 1997; Zhang et al., 2001) and that they are in close physical proximity to voltage-gated Ca^{2+} channels (Marrion and Tavalin, 1998; Grunnet and Kaufmann, 2004; Berkefeld et al., 2006; Muller et al., 2007). In particular, this model is highly consistent with the modeling of Engbers et al. (2013) where Ca^{2+} concentrations of 10 μM or greater were required to observe a significant difference in spike duration (**Figures 4B3,D**).

β4 SURFACE TRAFFICKING IS HIGHLY REGULATED

Considering the very broad and high level of β4 mRNA throughout the brain (Behrens et al., 2000; Brenner et al., 2000; Meera et al., 2000; Uebele et al., 2000; Weiger et al., 2000) it is quite surprising that iberiotoxin-resistant BK channels have been described in only few types of neurons. These are cerebellar purkinje neurons (Benton et al., 2013), dentate gyrus neuronal soma (Brenner et al., 2005) and their mossy fiber terminals (Alle et al., 2011), and posterior pituitary terminals (Wang et al., 1992). One may conclude that BK/β4 channel surface trafficking must be post-transcriptionally regulated. Recent studies indeed suggest that β4 subunits are largely retained in intracellular compartments through an endoplasmic reticulum (ER) retention signal (Bai et al., 2011; Shruti et al., 2012; Cox et al., 2014). The efficiency of BK/β4 channels surface trafficking is controversial. Studies in transfected HEK293 cells by one group indicated that while β4 is retained in the ER, it did not affect BK channel surface trafficking (Cox et al., 2014). While another group indeed showed a dominant negative effect of β4 on BK channel surface trafficking in HEK293 cells and CA3 neurons (Shruti et al., 2012).

In addition to an ER retention motif (Shruti et al., 2012; Cox et al., 2014), the β4 subunit was shown to be palmitoylated at a site in the carboxyl terminal cysteine 193 that is necessary for surface trafficking (Chen et al., 2013). Palmitoylated β4 appears to oppose a BK channel splice variant retention signal

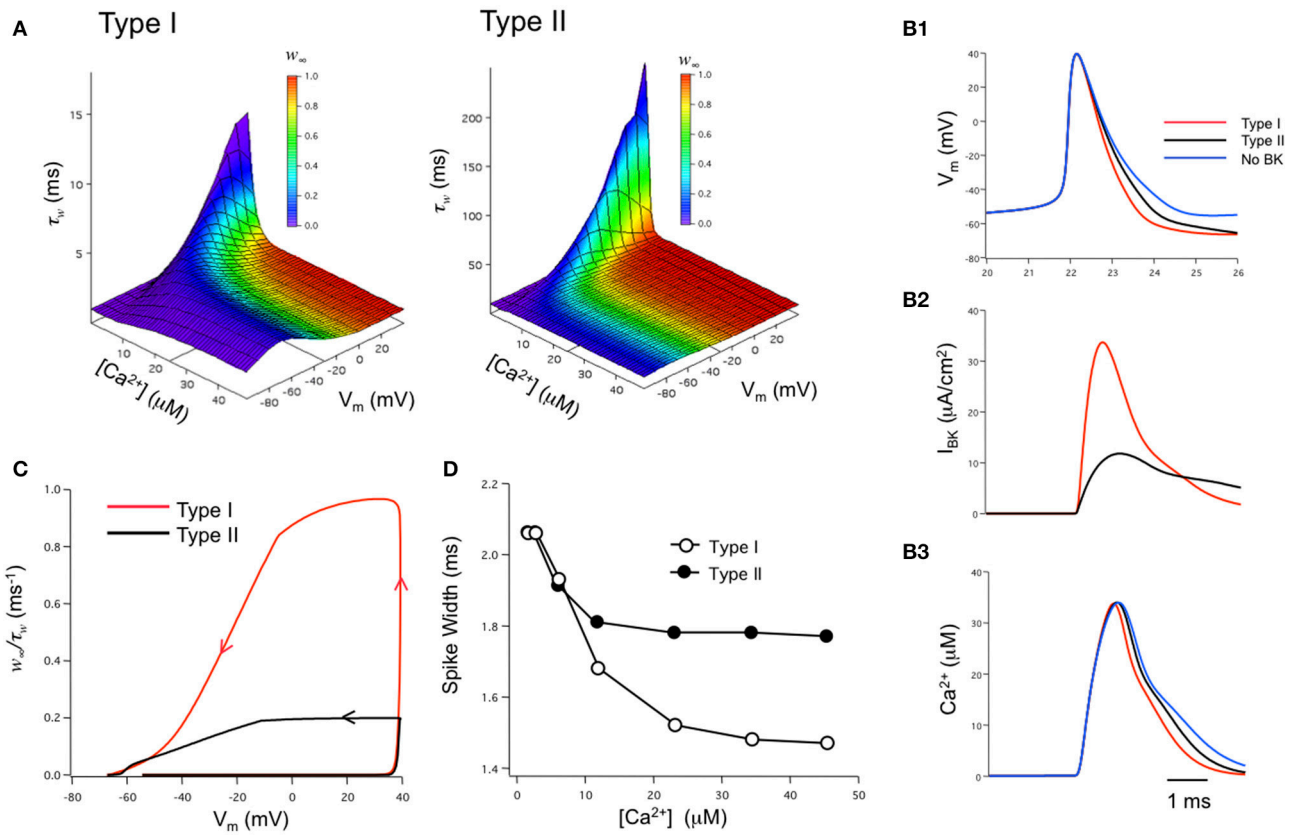


FIGURE 4 | Biophysical modeling: the effects of type I and II BK channels on neuronal function. (A) Voltage-, Ca^{2+}-, and time-dependence of type I and type II BK channels. w_∞ is steady-state activation while τ_w is the time constant for channel activation. The state variable w was integrated over time as $dw/d\tau = (w_\infty - w)/\tau_w$. The w_∞ and τ_w parameter space was described by analytical equations derived from voltage-clamp measurements obtained under physiologically-appropriate conditions (see Jaffe et al., 2011). **(B)** Type I BK channels shorten the spike duration and increase the fAHP relative to type II channels **(B1)**. Type I BK channels are more strongly activated during the down-stroke of the action potential than type II BK channels **(B2)**. As a result, the down-stroke is faster for the model with type I BK channels compared with a model containing type II BK channels or no BK channels **(B1)**. The sharper spike resulting from type I channels reduces the duration, but not amplitude, of the calcium transient sensed by BK channels compared to type II channels or blocked BK channels **(B3)**. Channel density in these simulations was 1 mS/cm^2. **(C)** Phase-plane analysis of the ratio of w_∞/τ_w against membrane potential achieved during the time course of an action potentials. Arrows indicate the direction of time through the course of the spike. **(D)** A difference in action potential duration between type I or type II-containing models occurs when local peak Ca^{2+} concentration exceeds 10 μM. Figures are replotted from equations described in Jaffe et al. (2011).

(VEDEC carboxyl variant) and thereby increase surface expression of this splice variant. For BK channel splice variants that lack this alternative splice site but are not maximally trafficked to the surface, palmitoylated β4 does not promote surface expression but depalmitoylated β4 subunits appear to retain channels in the ER. Thus, differences in neuronal palmitoylation, or BK channel splicing, might explain the paradoxical finding that although β4 mRNA is more abundant in CA3 than dentate gyrus neurons, patch clamp recording show a largely iberiotoxin-sensitive current in CA3 and iberiotoxin-resistant current in DG neurons (Shruti et al., 2012).

β4 SUBUNIT REGULATION BY STEROID HORMONES AND FATTY ACIDS

Steroids have been known to modulate BK channels through non-genomic actions. Particularly the β1 subunit, that is enriched in vascular smooth muscle, has drawn extensive attention given that it can directly interact with estrogen compounds (Valverde et al., 1999; Dick et al., 2001), activate smooth muscle BK channels (Dick and Sanders, 2001) and potentially have beneficial effects on vascular function. BK channels in the CNS (area postrema) have also shown to be activated by estrogen compounds (Li and Hay, 2000). However, it is often unclear whether these are direct effects or indirect effects since estrogen acutely modulates a large number of targets including protein kinase G, which also activates BK channels (Rosenfeld et al., 2000; White et al., 2002; Dimitropoulou et al., 2005). Nevertheless, early cloning and expression of the β4 subunit showed that estrogen could, similar to β1, activate BK/β4 channels (Behrens et al., 2000). A comparative study of different steroids indicated the β4 conferred a greater sensitivity to BK channels for corticosterone than estrogen, and followed by progesterone (King et al., 2006). This is in contrast to the β2 subunit that is much less sensitive to corticosterone but activated by dehydroepiandrosterone (DHEA), a stress-related

adrenal androgen. The differential sensitivities to corticosterone and DHEA provide an interesting opportunity to differentially activate BK/β4 and BK/β2 channels in neurons. The effect of corticosterone (1 μM) on BK/β4 channels was to negative-shift the conductance-voltage relations (−13 mV) and slow deactivation kinetics about 3 fold (at −80 mV, 1 μM internal calcium) (King et al., 2006). In addition to steroid hormones, some β subunits also confer sensitivity to fatty acids. Recent studies showed that β4, like β1 but not β2 confer a sensitivity to BK channels for the long chain omega-3 fatty acid, docosahexaenoic acid (DHA) (Hoshi et al., 2013). This is one of the fatty acid compounds enriched in fish oil that provides beneficial health effects (Harris et al., 2013). DHA (3 μM) causes a robust ∼ −60 mV shift of conductance-voltage relations in BK/β4 channels (measured in absence of calcium), but only ∼ −10 mV shift in BK channels lacking β subunits (Hoshi et al., 2013).

PHYSIOLOGICAL FUNCTION OF IBERIOTOXIN-RESISTANT BK CHANNELS IN NEURONS

β4 DISTRIBUTION IN THE BRAIN

While BK channels expression in many regions of the nervous system is well-established (Wanner et al., 1999), the expression of β4 needs further study. Gene targeting of an EGFP reporter into the KCNMB4 (β4 gene) locus, and *in situ* hybridization studies have provided some information regarding regions of high β4 mRNA expression (Weiger et al., 2000; Brenner et al., 2005; Petrik and Brenner, 2007; Shruti et al., 2012). Using these approaches, β4 shows strong staining in the posterior pituitary (Brenner et al., 2005), pyramidal neurons of the cortex, CA3 pyramidal neurons, and dentate gyrus region of the hippocampus (Weiger et al., 2000; Brenner et al., 2005; Petrik and Brenner, 2007; Shruti et al., 2012), olfactory bulb, and purkinje neurons of the cerebellum (Petrik and Brenner, 2007). To date, expression data is corroborated by functional studies of BK/β4 channels in the posterior pituitary, CA3 pyramidal neurons, dentate gyrus granule neurons, and cerebellar purkinje neurons (will be discussed further below). In contrast to β4 mRNA, reports of immunolocalization of the protein in neurons is limited (Piwonska et al., 2008). In part, this is because antibodies to this protein, although commercially available, often lack sufficient specificity to unambiguously identify β4 (a personal observation that many commercial anti-β4 antibodies detect equivalent signals in our β4 knockout mice).

POSTERIOR PITUITARY TERMINALS

Physiological function of BK/β4 channels was likely first studied in posterior pituitary nerve terminals (Bielefeldt and Jackson, 1993). The investigators described a calcium- and voltage-activated, large conductance potassium current that was resistant to charybdotoxin and apamin in rat posterior pituitary terminals (Bielefeldt et al., 1992). These characteristics strongly suggest BK/β4 channels, and indeed β4 gene expression was later observed in posterior pituitary nerve terminals (Brenner et al., 2005). The investigators found that high frequency firing of action potentials was terminated by increased calcium influx using the L-type calcium channel opener Bay K 8644, presumably through activation of the calcium-activated potassium channels. A key observation was that these calcium-activated potassium channels were particularly slow- activated and deactivated. Thus, the investigators suggested that these channels are tailored for silencing bursts of action potentials that might arise possibly due to calcium accumulation. Later studies showed that while iberiotoxin-resistant BK channels are enriched in the pituitary terminals, BK channels of the hypothalamic somas that connect to these terminals are fast-gated, iberiotoxin-sensitive type I channels (Dopico et al., 1999). These results indicate that neurons may co-express distinct BK channel subtypes in different subcompartments,

CEREBELLAR PURKINJE NEURONS

BK channels likely have an important role in the cerebellum since knockout of the pore-forming subunit causes a profound ataxia in the mice (Meredith et al., 2004; Sausbier et al., 2004). Similar to hypothalamic neurons, a mixture of BK channel subtypes were recently identified in cerebellar purkinje neurons (Benton et al., 2013). However, the different subtypes did not appear to be segregated to different compartments. Whole cell recordings identified a mixture of slow-gated, non-inactivating iberiotoxin-resistant channels, and fast-gated, inactivating iberiotoxin-sensitive BK channels (Benton et al., 2013). The presence of iberiotoxin-resistant BK channels is consistent with expression of β4 in cerebellar purkinje neurons (Petrik and Brenner, 2007). The slow-gating of the iberiotoxin-resistant BK channels sustains opening of these channels during the AHP and contributes to a sustained interspike conductance (Benton et al., 2013). Unfortunately, the investigators did not specifically block these channels and not the inactivating subtype to investigate the consequence of the iberiotoxin-resistant currents on firing frequency. However, they had the novel observation that the SK channel agonist EBIO is an agonist for iberiotoxin-resistant channels, and indeed observed a larger interspike conductance and reduced spike frequency with EBIO. Given that SK current is inconsequential in the age of neurons studied, then the effect of EBIO was attributed to the BK/β4 channels. Teleologically, one can make the observation that BK/β4 are being employed to replace the calcium-activated, SK-type potassium channels that similarly have sustained interspike openings that allows for effective regulation of spike frequency (Sah and Faber, 2002). Despite the fact that knockout of the BK channel pore-forming subunit causes a profound ataxia (Meredith et al., 2004; Sausbier et al., 2004), the role of the β4 accessory subunit in the cerebellum is either more subtle or somewhat different since an ataxic phenotype has not been reported in β4 knockout mice.

CA3 HIPPOCAMPUS NEURONS

In situ hybridization reported by investigators (Shruti et al., 2012) and the Allen Brain Atlas (http://mouse.brain-map.org/gene/show/37365) indicate greatest expression of β4 mRNA in CA3 neurons. Utilizing a gene knockout for the Fragile X Mental Retardation Protein (FMRP), it was recently found that this protein regulates action potential duration through direct interactions with the β4 subunit (Deng et al., 2013). The FMRP gene is essential for cognitive development and strongly linked to mental disabilities and autism (Wijetunge et al., 2013). Knockout of the FMRP gene was found to cause broadening of action potentials and reduced fast-afterhyperpolarizations. Knockout of β4

occludes FMRP protein effects on action potential duration. The authors demonstrated that the effect on action potential duration consequently affects calcium influx and neurotransmitter release at CA3–CA1 synapses. This novel finding suggests that the β4 protein, through interactions with FMRP, might be a direct mediator of BK channel regulation in cognitive function.

The findings of Deng et al. (2013) also pose some interesting questions. Despite a key role of β4 subunits in mediating effects of FMRP on action potentials, iberiotoxin apparently blocked these channels in CA3 neurons (Deng et al., 2013). This was a similar finding as Shruti et al. (2012). It is therefore not clear how BK/β4 might regulate action potential shape, yet be sensitive to iberiotoxin. BK channels are homotetramers and are believed to express a theoretical four or less β subunits (Ding et al., 1998; Wang et al., 2002). As yet we do not know the stoichiometry of BK α and β4 subunits sufficient to confer iberiotoxin resistance. One might speculate that in CA3 neurons, membrane-associated BK channels might have a less than saturating concentration of β4 subunits that perhaps allow access of iberiotoxin to the BK channel pore, whilst still modulating BK channel activity. Given that knockout of the β4 gene appears to allow greater trafficking of channels to the membrane (Shruti et al., 2012), one might also speculate that FMRP may function to retain more β4 subunits in the ER in some neurons such as CA3, to allow BK channels to be fast-gated and more effectively shape action potentials.

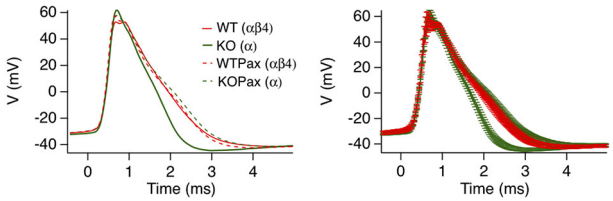


FIGURE 5 | β4 inhibits BK channel activation in mouse hippocampal DG neurons. Averaged action potential waveform from 2nd action potential evoked from a 105-pA current injection. Four traces are wild type or $\beta4^{-/-}$ mice in the presence (WTPax and KOPax) or absence of 5 μM paxilline (WT and KO). The right panel show the same average data with error bars reflecting the s.e.m. Data was derived from Brenner et al. (2005).

DENTATE GYRUS GRANULE NEURONS

Dentate gyrus neurons in mice express predominantly iberiotoxin-resistant, type II channels as evidenced by whole cell current recordings (Shruti et al., 2012) and single channel recordings (Brenner et al., 2005). Presumably due to the slow gating, blocking these channels with paxilline had no significant affect on action potential shape (WT red trace vs. WTPax red dashed trace, **Figure 5**) (Brenner et al., 2005). Knockout of β4 results in a conversion to type I, iberiotoxin-sensitive channels and a BK channel gain-of-function as evidenced by sharper action potentials and a larger fast-afterhyperpolarization (**Figure 5**). The greater contribution to action potential repolarization in the β4 KO is largely consistent with the computational predictions of Jaffe et al. (2011), and discussed above. Evidence that β4 knockout results in a gain-of-function of BK channels is apparent from paxilline block of KO neurons, which broadens the action potential trace to that of wild type (**Figure 5**). It is important to point out that in other studies paxilline shows a greater effect on BK-mediated repolarization in recordings of wild type rat dentate gyrus neurons (Muller et al., 2007). This is perhaps due to warmer recording conditions (35°C) that likely speeds BK/β4 channel gating, compared to the 25°C recording conditions in the mouse KO studies (Brenner et al., 2005). The relative contribution to action potential repolarization of BK channels in wild type vs. β4 KO neurons at near physiological temperatures has yet to be determined.

A surprising observation was that the β4 knockout conversion to type I, fast-gated BK channels increased the action potential frequency in dentate gyrus neurons and resulted in spontaneous temporal lobe seizures in the mice (Brenner et al., 2005). A similar effect has been seen with a human gain-of-function mutation of the pore-forming α subunit that results in epilepsy in family members carrying the mutant allele (Du et al., 2005). As well, pro-excitatory BK channels appear to be acquired in a picrotoxin seizure model (Shruti et al., 2008), and paxilline block of these channels appears to protect against subsequent seizures following a second insult (Sheehan et al., 2009). Thus, one may conclude that fast-gated BK channels can be pro-excitatory and slowing of BK channels with β4 can reduce excitability. The pro-excitatory mechanisms of BK channels need to be further studied. But they may be mediated by secondary effects of fast-gated BK channel: a sharper action potential or larger fAHP that would theoretically remove sodium channel inactivation or reduce activation of other potassium currents such as delayed rectifier currents (Gu et al., 2007) or SK-type calcium activated currents (Brenner et al., 2005), that otherwise reduce spike frequency.

PRESYNAPTIC BK/β4 CHANNELS

Axons of dentate gyrus granule cells, the mossy fibers, have very large presynaptic terminals that provide one of the few opportunities in central neurons (in addition to the calyx of held and posterior pituitary terminals) where voltage clamping of a presynaptic terminal is feasible. Recordings of mossy fiber terminals identified a mixture of iberiotoxin-sensitive and iberiotoxin-resistant BK channels suggesting that the β4 subunit also traffic to presynaptic locations (Alle et al., 2011). Similar to purkinje neurons discussed above, the iberiotoxin-sensitive fraction was fast-gated, and inactivating. The iberiotoxin-resistant fraction were slow-gated, typical of BK/β4 type II channels. Interestingly, even the fast-gated BK channel subtype could not contribute to presynaptic terminal action potential repolarization owing to the faster-gating of Kv3 type channels that dominate the presynaptic repolarization. Similar to studies in Schaffer collateral-commissural fibers (Hu et al., 2001), a role for mossy fiber terminal BK channels was revealed only when Kv channels were first blocked (Alle et al., 2011). By showing that even the fast BK component was sensitive to a slow calcium buffer (EGTA), the investigators concluded that slow-activation of presynaptic BK channels might also be due to a lack of nanodomain calcium source required for fast BK channel activation. In contrast, somatic BK channels were reported to be insensitive to EGTA or even moderate concentrations of fast chelator BAPTA (Muller

et al., 2007) indicating somatic BK channels are distinguished from terminal BK channels, not only in being more homogeneously composed of BK/β4 channels (Brenner et al., 2005; Shruti et al., 2012), but also being more tightly coupled to their calcium source (Muller et al., 2007).

SUMMARY AND FUTURE DIRECTIONS

Our understanding of BK channel subtypes appears to be shifting from ascribing different BK channel subtypes in different neurons, to different BK channel subtypes cohabitating neurons. As well, we are beginning to recognize how different subtypes are employed toward the needs of the cell. BK/β4 may coexist with other subtypes in a single compartment, such as inactivating BK channels and BK/β4 channels in mossy fiber presynaptic terminals (Alle et al., 2011) and pyramidal neuronal soma (Benton et al., 2013). Alternatively, BK/β4 channels may be segregated to different subcompartments, such as fast-gated type I channels in hypothalamic magnocellular neurons in the soma, and type II BK/β4 channels residing only in their posterior pituitary terminals. In general, slow-gating by β4 appears to either reduce BK channel contribution to action potentials or contribute to a more sustained interspike conductance while the fast-gated BK channels having a more conventional role to shape action potentials. Finally, the β4 subunit has roles in addition to modulating BK channel biophysical properties that include regulation of BK channel surface trafficking and as a receptor for other proteins such as FMRP to modulate BK channels.

There is much more that is necessary to understand with regard to the nature of iberiotoxin-resistant BK channels. Studies of β1 and β2 stoichiometry indicate that BK channels can assemble with a less than saturating (four or less subunits per channel) concentration of β subunits (Ding et al., 1998; Wang et al., 2002) resulting in intermediate gating properties than is expected from fully β–saturated channels (Ding et al., 1998; Wang et al., 2002). One might speculate that a less than saturating concentration of β4 might not occlude iberiotoxin access, and therefore β4 might also be assembled with BK channels more broadly in the nervous system than we can infer from the iberiotoxin-resistant pharmacology. This might explain why β4 subunit mRNA is broadly expressed (Brenner et al., 2000), yet iberiotoxin-resistant channels seem to be more restricted, and mainly seen in neurons that have relatively high expression levels of β4. Further, we do not yet know if BK channels can assemble to include mixtures of accessory subunits, such as both β2 and β4 subunits, to further increase BK channel diversity. Certainly, future studies are needed to better understand the pharmacology of BK/β4 channels with subsaturating β4 accessory subunits, or with coexpressed β2 and β4 subunits, so that we can relate this to the molecular underpinnings of these channels in neurons. One should expect that our understanding of the functional role of iberiotoxin-resistant BK channels should improve as utilization of BK/β4-specific toxin Mallotoxin will allow investigation of these channels specifically, without blocking all BK channels. As well, our understanding of BK/β4 channels should increase as the β4 knockout mice are employed to a greater extent.

ACKNOWLEDGMENTS

Robert Brenner was supported by the Research Enhancement Program of the University of Texas Health Science Center at San Antonio, and by NIH grant 1R21AI113724.

REFERENCES

Alle, H., Kubota, H., and Geiger, J. R. (2011). Sparse but highly efficient Kv3 outpace BKCa channels in action potential repolarization at hippocampal mossy fiber boutons. *J. Neurosci.* 31, 8001–8012. doi: 10.1523/JNEUROSCI.0972-11.2011

Bai, J. P., Surguchev, A., and Navaratnam, D. (2011). beta4-subunit increases Slo responsiveness to physiological Ca2+ concentrations and together with beta1 reduces surface expression of Slo in hair cells. *Am. J. Physiol. Cell Physiol.* 300, C435–C446. doi: 10.1152/ajpcell.00449.2010

Bao, L., and Cox, D. H. (2005). Gating and ionic currents reveal how the BKCa channel's Ca2+ sensitivity is enhanced by its {beta}1 subunit. *J. Gen. Physiol.* 126, 393–412. doi: 10.1085/jgp.200509346

Bao, L., Rapin, A. M., Holmstrand, E. C., and Cox, D. H. (2002). Elimination of the BK(Ca) channel's high-affinity Ca(2+) sensitivity. *J. Gen. Physiol.* 120, 173–189. doi: 10.1085/jgp.20028627

Behrens, R., Nolting, A., Reimann, F., Schwarz, M., Waldschutz, R., and Pongs, O. (2000). hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family. *FEBS Lett.* 474, 99–106. doi: 10.1016/S0014-5793(00)01584-2

Benton, M. D., Lewis, A. H., Bant, J. S., and Raman, I. M. (2013). Iberiotoxin-sensitive and -insensitive BK currents in Purkinje neuron somata. *J. Neurophysiol.* 109, 2528–2541. doi: 10.1152/jn.00127.2012

Berkefeld, H., Sailer, C. A., Bildl, W., Rohde, V., Thumfart, J. O., Eble, S., et al. (2006). BKCa-Cav channel complexes mediate rapid and localized Ca^{2+}-activated K+ signaling. *Science* 314, 615–620. doi: 10.1126/science.1132915

Bielefeldt, K., and Jackson, M. B. (1993). A calcium-activated potassium channel causes frequency-dependent action-potential failures in a mammalian nerve terminal. *J. Neurophysiol.* 70, 284–298.

Bielefeldt, K., Rotter, J. L., and Jackson, M. B. (1992). Three potassium channels in rat posterior pituitary nerve terminals. *J. Physiol.* 458, 41–67.

Brenner, R., Chen, Q. H., Vilaythong, A., Toney, G. M., Noebels, J. L., and Aldrich, R. W. (2005). BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat. Neurosci.* 8, 1752–1759. doi: 10.1038/nn1573

Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y., and Aldrich, R. W. (2000). Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. *J. Biol. Chem.* 275, 6453–6461. doi: 10.1074/jbc.275.9.6453

Calderone, V. (2002). Large-conductance, ca(2+)-activated k(+) channels: function, pharmacology and drugs. *Curr. Med. Chem.* 9, 1385–1395. doi: 10.2174/0929867023369871

Chen, L., Bi, D., Tian, L., McClafferty, H., Steeb, F., Ruth, P., et al. (2013). Palmitoylation of the beta4-subunit regulates surface expression of large conductance calcium-activated potassium channel splice variants. *J. Biol. Chem.* 288, 13136–13144. doi: 10.1074/jbc.M113.461830

Contreras, G. F., Neely, A., Alvarez, O., Gonzalez, C., and Latorre, R. (2012). Modulation of BK channel voltage gating by different auxiliary beta subunits. *Proc. Natl. Acad. Sci. U.S.A.* 109, 18991–18996. doi: 10.1073/pnas.1216953109

Cox, D. H., Cui, J., and Aldrich, R. W. (1997). Allosteric gating of a large conductance Ca-activated K+ channel. *J. Gen. Physiol.* 110, 257–281. doi: 10.1085/jgp.110.3.257

Cox, N., Toro, B., Pacheco-Otalora, L. F., Garrido-Sanabria, E. R., and Zarei, M. M. (2014). An endoplasmic reticulum trafficking signal regulates surface expression of beta4 subunit of a voltage- and Ca(2)(+)-activated K(+) channel. *Brain Res.* 1553, 12–23. doi: 10.1016/j.brainres.2014.01.028

Cui, J., Cox, D. H., and Aldrich, R. W. (1997). Intrinsic voltage dependence and Ca2+ regulation of mslo large conductance Ca-activated K+ channels. *J. Gen. Physiol.* 109, 647–673. doi: 10.1085/jgp.109.5.647

Deng, P. Y., Rotman, Z., Blundon, J. A., Cho, Y., Cui, J., Cavalli, V., et al. (2013). FMRP regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels. *Neuron* 77, 696–711. doi: 10.1016/j.neuron.2012.12.018

Dick, G. M., Rossow, C. F., Smirnov, S., Horowitz, B., and Sanders, K. M. (2001). Tamoxifen activates smooth muscle BK channels through the regulatory beta 1 subunit. *J. Biol. Chem.* 276, 34594–34599. doi: 10.1074/jbc.M104689200

Dick, G. M., and Sanders, K. M. (2001). (Xeno)estrogen sensitivity of smooth muscle BK channels conferred by the regulatory beta1 subunit: a study of beta1 knockout mice. *J. Biol. Chem.* 276, 44835–44840. doi: 10.1074/jbc.M106851200

Diez-Sampedro, A., Silverman, W. R., Bautista, J. F., and Richerson, G. B. (2006). Mechanism of increased open probability by a mutation of the BK channel. *J. Neurophysiol.* 96, 1507–1516. doi: 10.1152/jn.00461.2006

Dimitropoulou, C., White, R. E., Ownby, D. R., and Catravas, J. D. (2005). Estrogen reduces carbachol-induced constriction of asthmatic airways by stimulating large-conductance voltage and calcium-dependent potassium channels. *Am. J. Respir. Cell Mol. Biol.* 32, 239–247. doi: 10.1165/rcmb.2004-0331OC

Ding, J. P., Li, Z. W., and Lingle, C. J. (1998). Inactivating BK channels in rat chromaffin cells may arise from heteromultimeric assembly of distinct inactivation-competent and noninactivating subunits. *Biophys. J.* 74, 268–289. doi: 10.1016/S0006-3495(98)77785-9

Dopico, A. M., Widmer, H., Wang, G., Lemos, J. R., and Treistman, S. N. (1999). Rat supraoptic magnocellular neurones show distinct large conductance, Ca2+-activated K+ channel subtypes in cell bodies versus nerve endings. *J. Physiol.* 519 Pt 1, 101–114. doi: 10.1111/j.1469-7793.1999.0101o.x

Du, W., Bautista, J. F., Yang, H., Diez-Sampedro, A., You, S. A., Wang, L., et al. (2005). Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat. Genet.* 37, 733–738. doi: 10.1038/ng1585

Engbers, J. D., Zamponi, G. W., and Turner, R. W. (2013). Modeling interactions between voltage-gated Ca (2+) channels and KCa1.1 channels. *Channels (Austin)* 7, 524–529. doi: 10.4161/chan.25867

Erisir, A., Lau, D., Rudy, B., and Leonard, C. S. (1999). Function of specific K(+) channels in sustained high-frequency firing of fast-spiking neocortical interneurons. *J. Neurophysiol.* 82, 2476–2489.

Faber, E. S., and Sah, P. (2003). Ca2+-activated K+ (BK) channel inactivation contributes to spike broadening during repetitive firing in the rat lateral amygdala. *J. Physiol.* 552, 483–497. doi: 10.1113/jphysiol.2003.050120

Fakler, B., and Adelman, J. P. (2008). Control of K(Ca) channels by calcium nano/microdomains. *Neuron* 59, 873–881. doi: 10.1016/j.neuron.2008.09.001

Gan, G., Yi, H., Chen, M., Sun, L., Li, W., Wu, Y., et al. (2008). Structural basis for toxin resistance of beta4-associated calcium-activated potassium (BK) channels. *J. Biol. Chem.* 283, 24177–24184. doi: 10.1074/jbc.M800179200

Gribkoff, V. K., Starrett, J. E. Jr., and Dworetzky, S. I. (1997). The pharmacology and molecular biology of large-conductance calcium- activated (BK) potassium channels. *Adv. Pharmacol.* 37, 319–348. doi: 10.1016/S1054-3589(08)60954-0

Grunnet, M., and Kaufmann, W. A. (2004). Coassembly of big conductance Ca^{2+}-activated K+ channels and L-type voltage-gated Ca^{2+} channels in rat brain. *J. Biol. Chem.* 279, 36445–36453. doi: 10.1074/jbc.M402254200

Gu, N., Vervaeke, K., and Storm, J. F. (2007). BK potassium channels facilitate high-frequency firing and cause early spike frequency adaptation in rat CA1 hippocampal pyramidal cells. *J. Physiol.* 580, 859–882. doi: 10.1113/jphysiol.2006.126367

Ha, T. S., Heo, M. S., and Park, C. S. (2004). Functional effects of auxiliary beta4-subunit on rat large-conductance Ca(2+)-activated K(+) channel. *Biophys. J.* 86, 2871–2882. doi: 10.1016/S0006-3495(04)74339-8

Harris, W. S., Dayspring, T. D., and Moran, T. J. (2013). Omega-3 fatty acids and cardiovascular disease: new developments and applications. *Postgrad. Med.* 125, 100–113. doi: 10.3810/pgm.2013.11.2717

Horrigan, F. T., and Aldrich, R. W. (1999). Allosteric voltage gating of potassium channels II. Mslo channel gating charge movement in the absence of Ca(2+). *J. Gen. Physiol.* 114, 305–336. doi: 10.1085/jgp.114.2.305

Horrigan, F. T., and Aldrich, R. W. (2002). Coupling between voltage sensor activation, Ca2+ binding and channel opening in large conductance (BK) potassium channels. *J. Gen. Physiol.* 120, 267–305. doi: 10.1085/jgp.20028605

Horrigan, F. T., Cui, J., and Aldrich, R. W. (1999). Allosteric voltage gating of potassium channels I. Mslo ionic currents in the absence of Ca(2+). *J. Gen. Physiol.* 114, 277–304. doi: 10.1085/jgp.114.2.277

Hoshi, T., Tian, Y., Xu, R., Heinemann, S. H., and Hou, S. (2013). Mechanism of the modulation of BK potassium channel complexes with different auxiliary subunit compositions by the omega-3 fatty acid DHA. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4822–4827. doi: 10.1073/pnas.1222003110

Hu, H., Shao, L. R., Chavoshy, S., Gu, N., Trieb, M., Behrens, R., et al. (2001). Presynaptic Ca2+-activated K+ channels in glutamatergic hippocampal terminals and their role in spike repolarization and regulation of transmitter release. *J. Neurosci.* 21, 9585–9597.

Hu, S., Labuda, M. Z., Pandolfo, M., Goss, G. G., McDermid, H. E., and Ali, D. W. (2003). Variants of the KCNMB3 regulatory subunit of maxi BK channels affect channel inactivation. *Physiol. Genomics* 15, 191–198. doi: 10.1152/physiolgenomics.00110.2003

Jaffe, D. B., Wang, B., and Brenner, R. (2011). Shaping of action potentials by type I and type II large-conductance Ca(2)+-activated K+ channels. *Neuroscience* 192, 205–218. doi: 10.1016/j.neuroscience.2011.06.028

Ji, Y. H., Wang, W. X., Ye, J. G., He, L. L., Li, Y. J., Yan, Y. P., et al. (2003). Martentoxin, a novel K+-channel-blocking peptide: purification, cDNA and genomic cloning, and electrophysiological and pharmacological characterization. *J. Neurochem.* 84, 325–335. doi: 10.1046/j.1471-4159.2003.01516.x

Kaczorowski, G. J., Knaus, H. G., Leonard, R. J., McManus, O. B., and Garcia, M. L. (1996). High-conductance calcium-activated potassium channels; structure, pharmacology, and function. *J. Bioenerg. Biomembr.* 28, 255–267. doi: 10.1007/BF02110699

King, J. T., Lovell, P. V., Rishniw, M., Kotlikoff, M. I., Zeeman, M. L., and McCobb, D. P. (2006). Beta2 and beta4 subunits of BK channels confer differential sensitivity to acute modulation by steroid hormones. *J. Neurophysiol.* 95, 2878–2888. doi: 10.1152/jn.01352.2005

Knaus, H. G., McManus, O. B., Lee, S. H., Schmalhofer, W. A., Garcia-Calvo, M., Helms, L. M., et al. (1994). Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated potassium channels. *Biochemistry* 33, 5819–5828. doi: 10.1021/bi00185a021

Lee, U. S., and Cui, J. (2009). {beta} subunit-specific modulations of BK channel function by a mutation associated with epilepsy and dyskinesia. *J. Physiol.* 587, 1481–1498. doi: 10.1113/jphysiol.2009.169243

Li, M., Chang, S., Yang, L., Shi, J., McFarland, K., Yang, X., et al. (2014). Conopeptide Vt3.1 preferentially inhibits BK potassium channels containing beta4 subunits via electrostatic interactions. *J. Biol. Chem.* 289, 4735–4742. doi: 10.1074/jbc.M113.535898

Li, Z., and Hay, M. (2000). 17-beta-estradiol modulation of area postrema potassium currents. *J. Neurophysiol.* 84, 1385–1391.

Lingle, C. J. (2002). Setting the stage for molecular dissection of the regulatory components of BK channels. *J. Gen. Physiol.* 120, 261–265. doi: 10.1085/jgp.20028689

Lippiat, J. D., Standen, N. B., Harrow, I. D., Phillips, S. C., and Davies, N. W. (2003). Properties of BK(Ca) channels formed by bicistronic expression of hSloalpha and beta1-4 subunits in HEK293 cells. *J. Membr. Biol.* 192, 141–148. doi: 10.1007/s00232-002-1070-0

Ly, C., Melman, T., Barth, A. L., and Ermentrout, G. B. (2011). Phase-resetting curve determines how BK currents affect neuronal firing. *J. Comput. Neurosci.* 30, 211–223. doi: 10.1007/s10827-010-0246-3

Ma, Z., Lou, X. J., and Horrigan, F. T. (2006). Role of charged residues in the S1-S4 voltage sensor of BK channels. *J. Gen. Physiol.* 127, 309–328. doi: 10.1085/jgp.200509421

Marrion, N. V., and Tavalin, S. J. (1998). Selective activation of Ca^{2+}-activated K+ channels by co-localized Ca2+ channels in hippocampal neurons. *Nature* 395, 900–905. doi: 10.1038/27674

McLarnon, J. G. (1995). Inactivation of a high conductance calcium dependent potassium current in rat hippocampal neurons. *Neurosci. Lett.* 193, 5–8. doi: 10.1016/0304-3940(95)11651-C

Meera, P., Wallner, M., and Toro, L. (2000). A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca2+-activated K+ channel resistant to charybdotoxin and iberiotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5562–5567. doi: 10.1073/pnas.100118597

Meredith, A. L., Thorneloe, K. S., Werner, M. E., Nelson, M. T., and Aldrich, R. W. (2004). Overactive bladder and incontinence in the absence of the BK large conductance Ca2+-activated K+ channel. *J. Biol. Chem.* 279, 36746–36752. doi: 10.1074/jbc.M405621200

Muller, A., Kukley, M., Uebachs, M., Beck, H., and Dietrich, D. (2007). Nanodomains of single Ca2+ channels contribute to action potential repolarization in cortical neurons. *J. Neurosci.* 27, 483–495. doi: 10.1523/JNEUROSCI.3816-06.2007

Orio, P., and Latorre, R. (2005). Differential effects of beta 1 and beta 2 subunits on BK channel activity. *J. Gen. Physiol.* 125, 395–411. doi: 10.1085/jgp.200409236

Orio, P., Rojas, P., Ferreira, G., and Latorre, R. (2002). New disguises for an old channel: MaxiK channel beta-subunits. *News Physiol. Sci.* 17, 156–161. doi: 10.1152/nips.01387.2002

Petrik, D., and Brenner, R. (2007). Regulation of STREX exon large conductance, calcium-activated potassium channels by the beta4 accessory subunit. *Neuroscience* 149, 789–803. doi: 10.1016/j.neuroscience.2007.07.066

Piwonska, M., Wilczek, E., Szewczyk, A., and Wilczynski, G. M. (2008). Differential distribution of Ca2+-activated potassium channel beta4 subunit in rat brain: immunolocalization in neuronal mitochondria. *Neuroscience* 153, 446–460. doi: 10.1016/j.neuroscience.2008.01.050

Reinhart, P. H., Chung, S., and Levitan, I. B. (1989). A family of calcium-dependent potassium channels from rat brain. *Neuron* 2, 1031–1041. doi: 10.1016/0896-6273(89)90227-4

Reinhart, P. H., and Levitan, I. B. (1995). Kinase and phosphatase activities intimately associated with a reconstituted calcium-dependent potassium channel. *J. Neurosci.* 15, 4572–4579.

Rosenfeld, C. R., White, R. E., Roy, T., and Cox, B. E. (2000). Calcium-activated potassium channels and nitric oxide coregulate estrogen-induced vasodilation. *Am. J. Physiol. Heart Circ. Physiol.* 279, H319–H328.

Rothberg, B. S., and Magleby, K. L. (1999). Gating kinetics of single large-conductance Ca2+-activated K+ channels in high Ca2+ suggest a two-tiered allosteric gating mechanism [In Process Citation]. *J. Gen. Physiol.* 114, 93–124. doi: 10.1085/jgp.114.1.93

Sah, P., and Faber, E. S. (2002). Channels underlying neuronal calcium-activated potassium currents. *Prog. Neurobiol.* 66, 345–353. doi: 10.1016/S0301-0082(02)00004-7

Sausbier, M., Hu, H., Arntz, C., Feil, S., Kamm, S., Adelsberger, H., et al. (2004). Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+-activated K+ channel deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9474–9478. doi: 10.1073/pnas.0401702101

Schreiber, M., and Salkoff, L. (1997). A novel calcium-sensing domain in the BK channel. *Biophys. J.* 73, 1355–1363. doi: 10.1016/S0006-3495(97)78168-2

Shao, L. R., Halvorsrud, R., Borg-Graham, L., and Storm, J. F. (1999). The role of BK-type Ca2+-dependent K+ channels in spike broadening during repetitive firing in rat hippocampal pyramidal cells. *J. Physiol.* 521 Pt 1, 135–146. doi: 10.1111/j.1469-7793.1999.00135.x

Sheehan, J. J., Benedetti, B. L., and Barth, A. L. (2009). Anticonvulsant effects of the BK-channel antagonist paxilline. *Epilepsia* 50, 711–720. doi: 10.1111/j.1528-1167.2008.01888.x

Shelley, C., Whitt, J. P., Montgomery, J. R., and Meredith, A. L. (2013). Phosphorylation of a constitutive serine inhibits BK channel variants containing the alternate exon "SRKR." *J. Gen. Physiol.* 142, 585–598. doi: 10.1085/jgp.201311072

Shi, J., He, H. Q., Zhao, R., Duan, Y. H., Chen, J., Chen, Y., et al. (2008). Inhibition of martentoxin on neuronal BK channel subtype (alpha+beta4): implications for a novel interaction model. *Biophys. J.* 94, 3706–3713. doi: 10.1529/biophysj.107.122150

Shruti, S., Clem, R. L., and Barth, A. L. (2008). A seizure-induced gain-of-function in BK channels is associated with elevated firing activity in neocortical pyramidal neurons. *Neurobiol. Dis.* 30, 323–330. doi: 10.1016/j.nbd.2008.02.002

Shruti, S., Urban-Ciecko, J., Fitzpatrick, J. A., Brenner, R., Bruchez, M. P., and Barth, A. L. (2012). The brain-specific Beta4 subunit downregulates BK channel cell surface expression. *PLoS ONE* 7:e33429. doi: 10.1371/journal.pone.0033429

Solaro, C. R., and Lingle, C. J. (1992). Trypsin-sensitive, rapid inactivation of a calcium-activated potassium channel. *Science* 257, 1694–1698. doi: 10.1126/science.1529355

Sun, L., Xiong, Y., Zeng, X., Wu, Y., Pan, N., Lingle, C. J., et al. (2009). Differential regulation of action potentials by inactivating and noninactivating BK channels in rat adrenal chromaffin cells. *Biophys. J.* 97, 1832–1842. doi: 10.1016/j.bpj.2009.06.042

Tao, J., Shi, J., Liu, Z. R., and Ji, Y. H. (2012). Martentoxin: a unique ligand of BK channels. *Sheng Li Xue Bao* 64, 355–364.

Tian, L., Duncan, R. R., Hammond, M. S., Coghill, L. S., Wen, H., Rusinova, R., et al. (2001). Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J. Biol. Chem.* 276, 7717–7720. doi: 10.1074/jbc.C000741200

Uebele, V. N., Lagrutta, A., Wade, T., Figueroa, D. J., Liu, Y., McKenna, E., et al. (2000). Cloning and functional expression of two families of beta-subunits of the large conductance calcium-activated K+ channel. *J. Biol. Chem.* 275, 23211–23218. doi: 10.1074/jbc.M910187199

Valverde, M. A., Rojas, P., Amigo, J., Cosmelli, D., Orio, P., Bahamonde, M. I., et al. (1999). Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit. *Science* 285, 1929–1931. doi: 10.1126/science.285.5435.1929

Wallner, M., Meera, P., and Toro, L. (1999). Molecular basis of fast inactivation in voltage and Ca2+-activated K+ channels: a transmembrane beta-subunit homolog. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4137–4142. doi: 10.1073/pnas.96.7.4137

Wang, B., and Brenner, R. (2006). An S6 mutation in BK channels reveals beta1 subunit effects on intrinsic and voltage-dependent gating. *J. Gen. Physiol.* 128, 731–744. doi: 10.1085/jgp.200609596

Wang, B., Rothberg, B. S., and Brenner, R. (2006). Mechanism of ß4 Subunit Modulation of BK Channels. *J. Gen. Physiol.* 127, 449–465. doi: 10.1085/jgp.200509436

Wang, B., Rothberg, B. S., and Brenner, R. (2009). Mechanism of increased BK channel activation from a channel mutation that causes epilepsy. *J. Gen. Physiol.* 133, 283–294. doi: 10.1085/jgp.200810141

Wang, G., Thorn, P., and Lemos, J. R. (1992). A novel large-conductance Ca(2+)-activated potassium channel and current in nerve terminals of the rat neurohypophysis. *J. Physiol.* 457, 47–74.

Wang, Y. W., Ding, J. P., Xia, X. M., and Lingle, C. J. (2002). Consequences of the stoichiometry of Slo1 alpha and auxiliary beta subunits on functional properties of large-conductance Ca2+-activated K+ channels. *J. Neurosci.* 22, 1550–1561.

Wanner, S. G., Koch, R. O., Koschak, A., Trieb, M., Garcia, M. L., Kaczorowski, G. J., et al. (1999). High-conductance calcium-activated potassium channels in rat brain: pharmacology, distribution, and subunit composition. *Biochemistry* 38, 5392–5400. doi: 10.1021/bi983040c

Weiger, T. M., Holmqvist, M. H., Levitan, I. B., Clark, F. T., Sprague, S., Huang, W. J., et al. (2000). A novel nervous system beta subunit that downregulates human large conductance calcium-dependent potassium channels. *J. Neurosci.* 20, 3563–3570.

White, R. E., Han, G., Maunz, M., Dimitropoulou, C., El-Mowafy, A. M., Barlow, R. S., et al. (2002). Endothelium-independent effect of estrogen on Ca(2+)-activated K(+) channels in human coronary artery smooth muscle cells. *Cardiovasc. Res.* 53, 650–661. doi: 10.1016/S0008-6363(01)00428-X

Wijetunge, L. S., Chattarji, S., Wyllie, D. J., and Kind, P. C. (2013). Fragile X syndrome: from targets to treatments. *Neuropharmacology* 68, 83–96. doi: 10.1016/j.neuropharm.2012.11.028

Xia, X. M., Ding, J. P., and Lingle, C. J. (1999). Molecular basis for the inactivation of Ca2+- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. *J. Neurosci.* 19, 5255–5264.

Xia, X. M., Zeng, X., and Lingle, C. J. (2002). Multiple regulatory sites in large-conductance calcium-activated potassium channels. *Nature* 418, 880–884. doi: 10.1038/nature00956

Xie, J., and McCobb, D. P. (1998). Control of alternative splicing of potassium channels by stress hormones. *Science* 280, 443–446. doi: 10.1126/science.280.5362.443

Zhang, X., Solaro, C. R., and Lingle, C. J. (2001). Allosteric regulation of BK channel gating by Ca(2+) and Mg(2+) through a nonselective, low affinity divalent cation site. *J. Gen. Physiol.* 118, 607–636. doi: 10.1085/jgp.118.5.607

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 July 2014; accepted: 15 September 2014; published online: 09 October 2014.

*Citation: Wang B, Jaffe DB and Brenner R (2014) Current understanding of iberiotoxin-resistant BK channels in the nervous system. Front. Physiol. **5**:382. doi: 10.3389/fphys.2014.00382*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

MINI REVIEW ARTICLE
published: 22 August 2014
doi: 10.3389/fphys.2014.00312

Lipid regulation of BK channel function

Alex M. Dopico* and Anna N. Bukiya

Department of Pharmacology, The University of Tennessee Health Science Center, Memphis, TN, USA

Edited by:
Thomas M. Weiger, University of Salzburg, Austria

Reviewed by:
Irena Levitan, University of Illinois at Chicago, USA
Rebecca Lam, Max Planck Institute of Biophysics, Germany

****Correspondence:***
Alex M. Dopico, Department of Pharmacology, The University of Tennessee Health Science Center, 874 Union Ave., #115, Memphis, TN 38163, USA
e-mail: adopico@uthsc.edu

This mini-review focuses on lipid modulation of BK (MaxiK, BK_{Ca}) current by a direct interaction between lipid and the BK subunits and/or their immediate lipid environment. Direct lipid-BK protein interactions have been proposed for fatty and epoxyeicosatrienoic acids, phosphoinositides and cholesterol, evidence for such action being less clear for other lipids. BK α (slo1) subunits are sufficient to support current perturbation by fatty and epoxyeicosatrienoic acids, glycerophospholipids and cholesterol, while distinct BK β subunits seem necessary for current modulation by most steroids. Subunit domains or amino acids that participate in lipid action have been identified in a few cases: hslo1 Y318, cerebral artery smooth muscle (cbv1) R334,K335,K336, cbv1 seven cytosolic CRAC domains, slo1 STREX and β1 T169,L172,L173 for docosahexaenoic acid, PIP_2, cholesterol, sulfatides, and cholane steroids, respectively. Whether these protein motifs directly bind lipids or rather transmit the energy of lipid binding to other areas and trigger protein conformation change remains unresolved. The impact of direct lipid-BK interaction on physiology is briefly discussed.

Keywords: MaxiK channel, protein receptor site, protein-lipid interaction, lipids, electrophysiology

Large conductance, Ca^{2+}/voltage-gated K^+ (BK, maxiK, slo1) channels result from tetrameric association of α (slo1) subunits (**Figure 1**). In most tissues, slo1 channels are associated with small accessory proteins termed β subunits. Four types of β subunits have been identified, their expression being tissue-specific (Orio et al., 2002). This mini-review focuses on lipid modulation of BK current observed in cell-free systems and thus, studies supporting direct interactions between lipid and BK proteins and/or their immediate proteo-lipid environment.

FATTY ACIDS

Increase in BK channel activity by low μM FA has been reported in VSM (Kirber et al., 1992; Ahn et al., 1994; Dopico et al., 1994; Clarke et al., 2002, 2003; Martín et al., 2013) and GH3 cells (Denson et al., 2000), and following channel expression in HEK293 cells (Hoshi et al., 2013a-c) and *Xenopus* oocytes (Sun et al., 2007). FA-induced BK activation occurs at a wide range of $[Ca^{2+}]_i$, and $[Mg^{2+}]_i$ (Ahn et al., 1994), and in virtual absence of Ca_i^{2+} (Clarke et al., 2002; Hoshi et al., 2013b). Moreover, FAs neither require voltage-sensor activation (Hoshi et al., 2013b) nor alter the slope of the activity-voltage relationship (Denson et al., 2000). In particular, DHA favors channel dwelling in conducting states by destabilizing the closed conformation of the pore (Hoshi et al., 2013a,b).

FA-induced BK activation does not correlate with changes in membrane fluidity or production of free radicals and oxygen metabolites (Denson et al., 2000). In addition, FA action persists in cell-free, membrane patches (Denson et al., 2000; Clarke et al., 2002; Hoshi et al., 2013b). The membrane-impermeable arachidonoyl-CoA potentiates current only when applied to the cytosolic side of the membrane patch (Denson et al., 2000; Sun et al., 2008), suggesting that the FA-recognition site(s) is accessible from the inner membrane leaflet.

Presence of a negatively charged head-group seems critical for FA "direct" action on BK channels. This action, however, persists after screening membrane surface charge with high-ionic strength solution (Clarke et al., 2002). Structure-activity studies reveal that unsaturated FAs (DHA, arachidonic, and oleic acids) enhance steady-state amplitude and slow inactivation of hslo1+β2 currents whereas saturated FAs fail to do so (Sun et al., 2007). *Cis*-unsaturated FAs increase GH3 cell BK current whereas saturated or *trans*-unsaturated FAs have no effect (Denson et al., 2000). On the other hand, long-chain FAs are more effective than short-chain counterparts in activating VSM BK channels (Ahn et al., 1994; Clarke et al., 2002). The mechanisms and targets underlying differential modulation of BK currents by FA of variant structure remain unidentified.

Channel subunit composition plays a critical role in the final effect of FA on BK current. In the same expression system, DHA potentiates and fails to alter hslo1-mediated and dslo1-mediated current, respectively (Hoshi et al., 2013c). These findings are consistent with the existence of specific, DHA-recognizing sites in slo1 proteins, with hslo1 Y318 playing a

Abbreviations: AA, arachidonic acid; BK, calcium- and voltage-gated, large conductance potassium; cAMP, cyclic adenosine monophosphate; Ca_i^{2+}, intracellular calcium; CB, cannabinoid; CHO, Chinese hamster ovary cells; CLR, cholesterol; CRAC, cholesterol recognition amino acid consensus; CTD, cytosolic tail domain; DHA, docosahexaenoic acid; DHET, 11,12-dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; EETe, epoxyeicosatetraenoic acid; FA, fatty acid; HEK, human embryonic kidney; GH3, rat pituitary tumor epithelial-like cells; HCN-1A, human cortical neuronal cells; HENA, 3-hydroxyolean-12-en-30-oate; I/O, inside-out patch; LPI, lysophosphatidylinositol; LT, leukotriene; PC, phosphatidylcholine; PG, prostaglandins; PI, phosphoinositide; PIP_2, phosphatidylinositol 4,5-bisphosphate; PK, protein kinase; Po, open probability; RCK, regulator of conductance for potassium; SM, smooth muscle; SPL, sphingolipid; TM, transmembrane; TxA2, thromboxane A2; VSM, vascular smooth muscle.

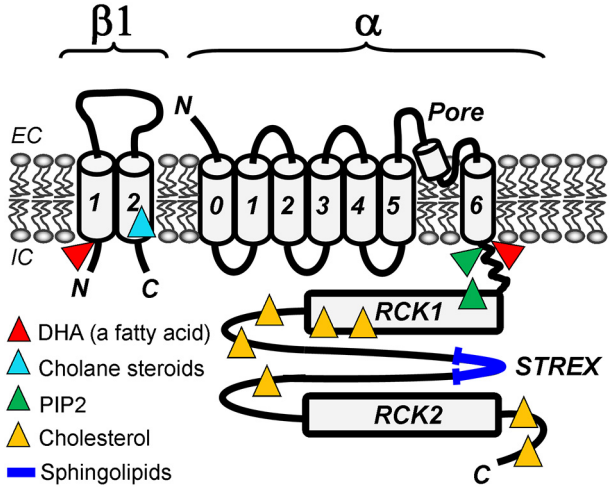


FIGURE 1 | Lipid-sensing areas in a BK heterodimer made of α (slo1) and β1 subunits. The cartoon highlights protein regions in which mutations ablate lipid sensitivity of BK channels in cell-free systems, such as excised membrane patches or planar lipid bilayers. Whether these regions directly bind lipids or allosterically modulate BK channel function upon lipid binding to other BK areas remains unresolved.

critical role in DHA-sensing (Hoshi et al., 2013c). In addition, DHA potentiation of hslo1 current is amplified by BK β1 and β4 subunits (Hoshi et al., 2013a). On the other hand, AA slows inactivation and potentiates current mediated by β2- or β3-containing BK channels. In contrast, long-chain acyl-CoAs facilitate hslo2+β2 inactivation and thus, inhibit overall current (Sun et al., 2008). The presence of opposite charge on residues at positions 11 (N-terminus) and 18 in BK β transmembrane domain-1 (TM1) is crucial for DHA action in presence of β1 and β4 (Hoshi et al., 2013a). It remains unclear whether these residues represent an FA binding site or transduce FA-docking energy into gating modification. Consistent with modulatory or "allosteric" mechanisms, AA inactivates α+β2-mediated currents but fails to affect inactivation of current by the β2-inactivating ball peptide alone, suggesting that AA does not interact with the ball peptide itself (Sun et al., 2007).

The physiological and pathophysiological consequences of BK channel modulation by FAs are under investigation. AA eliminates a transient K^+ current in neocortical neurons (Sun et al., 2007), which should drastically alter excitability. BK currents mediate AA-induced relaxation of pulmonary artery, yet the exact contribution of a direct FA-BK interaction to this AA action remains unclear (Guerard et al., 2004). However, omega-3 FAs lower blood pressure by directly activating BK channels in VSM (Hoshi et al., 2013b).

PROSTANOIDS

PGI_2, PGE_2, unoprostone and AH13205 activate BK currents in retinal pericytes (Burnette and White, 2006), coronary artery SM (Zhu et al., 2002), HCN-1A (Cuppoletti et al., 2007) and trabecular meshwork cells (Stumpff et al., 2005), respectively. In contrast, U46619 inhibits BK current when the channel is co-expressed with thromboxane A_2 (TxA_2) receptors in HEK293 cells (Li et al., 2013). PGI_2- and PGE_2-induced BK activation require cAMP-stimulated cross-activation of PKG, but not PKA (Hata et al., 2000; Zhu et al., 2002). In VSM, however, PGI_2 activation of BK current involves a cAMP-independent, Gs protein-dependent component (Tanaka et al., 2004). In turn, U46619 inhibits channel activity in cell-free patches, an action that involves independent associations between channel-forming subunits, BK β1, and TxA_2 receptors. A direct interaction between prostanoids and BK subunits, however, remains unclear.

Prostanoid-induced BK activation may contribute to the relaxant activity of PGE_2 in trabecular meshworks (Wang et al., 1998; Stumpff et al., 2005), and PGI_2-induced VSM relaxation (Tanaka et al., 2004) with consequent retinal vasodilation and blood flow augmentation (Hata et al., 2000). Prostanoid-BK interactions may provide a basis for using PGI_2-mimetics against pulmonary hypertension (Benyahia et al., 2013). Such interactions may also underlie unoprostone-induced hyperpolarization and consequent protection of cortical neurons against glutamate-induced Ca_i^{2+} dysregulation (Cuppoletti et al., 2007).

EPOXYEICOSATRIENOIC ACIDS AND LEUKOTRIENES

EET and derivatives activate BK channels in VSM (Wu et al., 2000; Zhang et al., 2001; Lauterbach et al., 2002; Archer et al., 2003; Dimitropoulou et al., 2007; Loot et al., 2012) and non-vascular SM (Benoit et al., 2001), cortical collecting duct (Sun et al., 2009), pituitary GH3 (Wu et al., 2000), HEK293 (Fukao et al., 2001), and adrenal chromaffin cells (Twitchell et al., 1997), and crude airway SM microsomes reconstituted into lipid bilayers (Benoit et al., 2001). EET-related epoxyeicosatetraenoic acids (EETe) and 5-oxo-eicosatetraenoic acid potentiate BK current in human pulmonary artery and distal bronchi (Morin et al., 2007, 2009), and cerebral and mesenteric VSM (Hercule et al., 2007). EET and EETe effective concentrations range from nM to low μM (Wu et al., 2000; Benoit et al., 2001; Lauterbach et al., 2002; Hercule et al., 2007; Morin et al., 2009).

EET increases BK current without affecting unitary conductance (Wu et al., 2000; Benoit et al., 2001; Fukao et al., 2001). Rather, EET and 11,12-dihydroxyeicosatrienoic acid (DHET) increase channel open probability (Po) by lengthening open and shortening closed times (Wu et al., 2000; Lu et al., 2001). Modification of gating by EET and EETe is observed across a wide voltage range (Wu et al., 2000; Lauterbach et al., 2002; Hercule et al., 2007) and unaffected by strong buffering of Ca_i^{2+} (Benoit et al., 2001; Hercule et al., 2007). However, DHET fails to activate BK channels in absence of Ca_i^{2+} (Lu et al., 2001). EET-induced BK channel activation is suppressed by anti-Gαs antibody (Fukao et al., 2001), and by protein phosphatase 2A inhibitor (Dimitropoulou et al., 2007). However, EET-induced BK activation could be observed in cell-free patches (Wu et al., 2000; Dimitropoulou et al., 2007) and following channel reconstitution into artificial lipid bilayers (Benoit et al., 2001). EET-induced activation of recombinant channels expressed in HEK293 cells does not require β1 subunits (Fukao et al., 2001). Consistently, EETe action on BK channels is preserved in cerebral and mesenteric VSM lacking BK β1 subunits (Hercule et al., 2007). Collectively, these findings point at the BK β1 subunit and its lipid

microenvironment as the primary target of EETs and related compounds.

It is noteworthy that 11,12-EET but neither 8,9- nor 14,15-EET, activates BK channels in cortical collecting duct cells (Sun et al., 2009). However, 14,15-EET activates BK channels in inside-out patches from GH3 cells (Wu et al., 2000). In addition, while equipotent in activating coronary artery SM BK channels, several DHETs show a reduced efficacy when compared to 11,12-EET (Lu et al., 2001). Structural specificity in EET action on BK channels is consistent with involvement of distinct EET-recognizing protein sites. In contrast, data from coronary microvessel SM cell-free membrane patches demonstrate a low structural specificity for EET action, as several EET regioisomers and enantiomers, epoxyeicosaquatraenoic, and epoxydocosatetraenoic acids activate BK channels with similar potencies and efficacies (Zhang et al., 2001).

In airway SM, 20-hydroxyeicosatetraenoic acid (20-HETE) and EETs cause membrane hyperpolarization and relaxation of human distal bronchi (Morin et al., 2007, 2009). Likewise, EET-induced BK activation leads to hyperpolarization and dilation of internal mammary (Archer et al., 2003), pulmonary (Morin et al., 2007) and mesenteric arteries (Dimitropoulou et al., 2007). However, EET-mediated SM dilation may be counteracted by EET-stimulated physical association of BK α and β1 subunits in mitochondria: this association enhances mitochondria BK function, leading to loss of mitochondrial membrane potential and thus, depolarization, as reported in pulmonary VSM (Loot et al., 2012). Consistently, EETs fail to hyperpolarize the membrane and relax isolated internal carotid artery (Chataigneau et al., 1998). Finally, BK activation by EET plays an important role in flow-stimulated K^+ secretion in the cortical collecting duct (Sun et al., 2009), and possibly in regulating adreno-chromaffin cell secretion (Twitchell et al., 1997).

LTA4, LTB4, LTC4, LTD4, and LTE4 (nM-μM) have been tested on β1 subunit-containing recombinant BK channels in *Xenopus* oocyte I/O patches, with only LTB4 significantly increasing channel activity (Bukiya and Dopico, 2013a). This finding raises the hypothesis that BK activation *via* LTB4-BK interaction reduces LT receptor-mediated, SM contraction by LTB4 (Rosenblum, 1985; Lawson et al., 1986; Peters-Golden and Henderson, 2007).

CANNABINOIDS

BK channel activation by cannabinoids was detected in myometrial strips (Houlihan et al., 2010), trabecular meshwork cells (Stumpff et al., 2005), ophthalmic artery (Romano and Lograno, 2006), coronary (White et al., 2001) and aortic SM (Sade et al., 2006), and HEK293 cells expressing BK α, α+β1 or α+β4 subunits (Sade et al., 2006; Godlewski et al., 2009). In contrast, μM methanandamide decreases BK activity in mesenteric and aortic SM (Bol et al., 2012). Likewise, virodhamine and synthetic analogs inhibit slo1 channels expressed in HEK293 cells (Godlewski et al., 2009).

The differential effects of cannabinoids on BK activity raised speculation on involvement of several mechanisms and molecular entities in cannabinoid action on BK channels. However, cannabinoid activation of SM BK channels involves neither CB1 or CB2 receptors (White et al., 2001; Romano and Lograno, 2006) nor cannabinoid metabolites (White et al., 2001). Moreover, studies in HEK293 cells rule out involvement of G-proteins and protein kinases (Sade et al., 2006), leading to the hypothesis that a direct cannabinoid-BK channel interaction mediates cannabinoid-induced channel activation (Godlewski et al., 2009). However, methanandamide fails to activate BK channels in cell-free medium (Sade et al., 2006; Godlewski et al., 2009), suggesting that cannabinoid action requires cellular signaling. This signal(s) would likely interact on the slo1 protein, as cannabinoid-induced BK activation is observed in homomeric slo1 (Sade et al., 2006; Godlewski et al., 2009). Interestingly, cannabinoid-induced potentiation of slo1 current is lost after membrane CLR depletion and restored upon CLR repletion (Godlewski et al., 2009), with the slo1 CTD providing several CLR-recognition domains that mediate CLR modulation of slo1 activity (Singh et al., 2012) (see below).

Cannabinoid-induced BK activation seems to play a role in endothelium-dependent vasodilation (White et al., 2001; Romano and Lograno, 2006; Godlewski et al., 2009), modulation of ocular outflow (Stumpff et al., 2005), and myometrial quiescence (Houlihan et al., 2010). In addition, BK activation might contribute to cannabinoid-induced neuroprotection; in particular, to cannabidiol-induced protections against pentylenetetrazol-induced seizure (Shirazi-zand et al., 2013).

GLYCEROPHOSPHOLIPIDS

Glycerophospholipid actions on BK function have been extensively studied in artificial lipid bilayers. Glycerophospholipid-induced changes in unitary conductance (Crowley et al., 2005) and Po (Chang et al., 1995; Crowley et al., 2005; Yuan et al., 2007) have been reported. Increase in slo1 conductance is linked to net negative charge in the glycerophospholipid headgroup (Crowley et al., 2005). In turn, data from bilayers made of variant PCs show that Po decreases with increase in bilayer thickness from PC14:1 to PC 22:1 while increasing from PC22:1 to PC24:1 (Yuan et al., 2007). While this dual profile of Po change is paralleled by changes in mean closed times, BK mean open time increases monotonically with bilayer thickness (Yuan et al., 2007). Moreover, increased open times have been linked to an increase in the glycerophospholipid headgroup cross-sectional area (Chang et al., 1995).

The mechanisms underlying glycerophospholipid-induced modification of BK open and closed times and thus, Po, remain unknown. Putative mechanisms include modification in the physical properties of the lipid microenvironment of the slo1 protein (Chang et al., 1995; Crowley et al., 2005; Yuan et al., 2007); changes in lateral stress imposed by the increasing headgroup size (Chang et al., 1995), perturbation of surface charge density and distribution by negatively charged headgroups (Moczydlowski et al., 1985), and hydrophobic mismatch between protein and bilayer thickness (Yuan et al., 2007). Specific glycerophospholipid-slo1 protein binding cannot be ruled out (Crowley et al., 2005), and gains increasing acceptance as evidence documenting direct binding of membrane lipids to transmembrane proteins keeps growing (Yeagle, 2014).

PHOSPHOINOSITIDES

PI-induced BK activation has been reported in cerebral artery and skeletal muscle myocytes (Vaithianathan et al., 2008), and with recombinant channels expressed in *Xenopus* oocytes (Vaithianathan et al., 2008; Tang et al., 2014). Phosphatidylinositol 4,5-bisphosphate (PIP_2)-induced BK activation is independent of PIP_2 metabolites, and occurs in absence of changes in unitary conductance or voltage-gating. However, this PIP_2 action requires Ca_i^{2+}. Moreover, PIP_2 facilitates Ca_i^{2+}-driven gating (Vaithianathan et al., 2008). Very recent work points at the KDRDD loop in the slo1 RCK1 domain as mediator of functional coupling between PIP_2- and Ca_i^{2+}-regulation of channel activity (Tang et al., 2014): in absence of Ca_i^{2+}, the slo1 RCK1 KDRDD loop decreases the channel's affinity for PIP_2 whereas in presence of Ca_i^{2+} the inhibitory modulation of such loop on PIP_2 affinity is relieved by Ca^{2+}-D367 coordination (Tang et al., 2014).

PI-induced BK activation increases with increase in negative charge within the PI headgroup. On the other hand, the more water-soluble analogues diC4 and diC8 are ∼10-fold less effective than PIP_2 in increasing BK activity, a difference that can be explained by their lower affinity to a site(s) and/or by their poor partitioning in the lipid membrane. If membrane partitioning is required for PI to access its site of action, this site should be located in the TM or the intracellular region of the protein, as lipids were more effective when applied to the intracellular side of the membrane. Indeed, the triplet R334,K335,K336 located after S6 in the BK channel-forming cbv1 subunit CTD has been identified as the PI-sensor (Vaithianathan et al., 2008). PIP_2-induced BK activation is observed in homomeric cbv1 channels and drastically amplified by β1 subunits. Whether this amplification involves PIP_2-recognition sites in β1 or distinct coupling between β1 and PIP_2-bound cbv1 is under investigation.

Manipulation of endogenous PIP_2 levels leads to endothelium-independent, BK-mediated cerebral artery dilation, which suggests that VSM PIP_2 regulates myogenic tone *via* BK activation (Vaithianathan et al., 2008).

LYSOPHOSPHOLIPIDS

In I/O patches from an umbilical vein-derived, endothelial cell line, LPI increases BK Po at sub-μM Ca_i^{2+} and following low basal (pre-LPI) activity while decreasing Po at μM Ca_i^{2+} and following high basal activity. LPI has no effect in the absence of Ca_i^{2+} (Bondarenko et al., 2011). The structural bases of LPI-BK interaction and its dependence on Ca_i^{2+} remain unknown. The gating modifications, however, seem complex, as LPI effect results from changes in both open and closed time distributions. Modulation of BK current by endogenous LPI could play a role in the potentiation of endothelial cell hyperpolarization by low histamine concentrations (Bondarenko et al., 2011).

SPHINGOLIPIDS (SPLs)

Sub-μM to low μM SPLs and their metabolites modulate BK activity in pinealocytes (Chik et al., 2001), CHO cells (Chi and Qi, 2006) and an endothelial cell line (Kim et al., 2006). Sulphatides, cerebroside termitomycesphin-A and sphingosine-1-phosphate increase BK current (Chik et al., 2001; Chi and Qi, 2006; Kim et al., 2006; Xu et al., 2011) while ceramides reduce current *via* a PKC-dependent pathway (Chik et al., 2001).

SPL-induced BK current potentiation is dose-dependent, reversible (Kim et al., 2006), and occurs in absence of unitary conductance modification (Xu et al., 2011). SPL action is independent of Ca_i^{2+} and G protein-coupled receptors (Chi and Qi, 2006; Kim et al., 2006; Xu et al., 2011). Moreover, deletion of the STREX insert in the slo1 CTD reduces channel activation by sulphatides (Chi and Qi, 2006) and totally suppresses the channel's sensitivity to termitomycesphin-A (Xu et al., 2011).

SPL modulation of BK activity could play a role in Ca^{2+} mobilization in endothelial cells (Kim et al., 2006), circadian regulation (Chik et al., 2001), and neuroprotection (Chi and Qi, 2006; Xu et al., 2011).

CHOLESTEROL, OTHER STEROIDS, AND VITAMIN D

A comprehensive and recent review on modulation of BK channels by CLR and related cholestanes is provided elsewhere (Dopico et al., 2012a,b). In brief, excessive membrane CLR usually decreases BK current, which has been attributed to direct and indirect mechanisms. For decades, CLR action on BK activity has been primarily linked to modification in membrane physical properties by CLR insertion (Chang et al., 1995; Crowley et al., 2003; Lundbaek, 2008). Direct CLR-BK interactions *via* seven CLR-recognition amino acid consensus (CRAC) motifs in the slo1 CTD were proposed (Singh et al., 2012).

In most cases, bile acids and related cholanes, pregnanes, androstanes, and estranes increase BK current, with eventual modification of physiology (reviewed in Dopico et al., 2012a). Later work identified a cholane-recognition site in the BK β1 TM2 where cholane docks *via* hydrogen bonding between its hydroxyl and T169, as well as *via* van der Waals interactions between the steroidal rings and L172,L173 (Bukiya et al., 2011). This site accommodates non-steroidal compounds, such as sodium 3-hydroxyolean-12-en-30-oate (HENA). Cholane and HENA recognition results in endothelium-independent, cerebral artery dilation *via* BK activation (Bukiya et al., 2013b). Because the identified site is found in the SM-abundant β1 and not in other BK βs (2-4), such a site represents an attractive target for rationale design of agents to counteract SM enhanced contraction, as found in asthma, cerebral vasospasm, systemic hypertension, erectile, bladder and uterine dysfunction (Patil et al., 2008; Bukiya et al., 2012).

Considering: 1-the critical roles of both vitamin D and BK channel function in maintaining healthy blood pressure levels (Holtzclaw et al., 2011; Basit, 2013), and 2-the structural similarity of vitamin D with the cholane lithocholic acid, which activates BK channels (see above), it was hypothesized that vitamin D increased BK activity. Indeed, μM vitamin D3 and 25-OH vitamin D3 increase β1-containing, BK-mediated currents after expression in *Xenopus* oocytes (Bukiya et al., unpublished). The consequences of vitamin D action on BK currents are under investigation.

CONCLUSIONS

Modulation of BK current by direct (e.g., independent of cell integrity, signaling or lipid metabolism) interaction between lipid

ligand and BK subunits has been reported for a wide variety of lipid species. For some lipids (e.g., cholesterol), lipid-BK channel-forming (slo1) subunit interaction accounts for most of the lipid effect. The majority of lipid-sensing regions in slo1 have been mapped to its intracellular tail domain. Whether these regions directly bind lipids or modulate BK channel function following lipid binding to other slo1 areas remains to be determined. For other lipids (e.g., cholanes), accessory β subunits are necessary for lipid action. Still for others (e.g., PIP_2), slo1 subunits suffice for lipid action, yet β subunits drastically modify the lipid's final effect. In most cases, the impact of direct BK channel-lipid interaction on organ function is under investigation.

ACKNOWLEDGMENT

Supported by HL104631 and AA11560 (Alex M. Dopico).

REFERENCES

Ahn, D. S., Kim, Y. B., Lee, Y. H., Kang, B. S., and Kang, D. H. (1994). Fatty acids directly increase the activity of $Ca^{(2+)}$-activated K^+ channels in rabbit coronary smooth muscle cells. *Yonsei Med. J.* 35, 10–24.

Archer, S. L., Gragasin, F. S., Wu, X., Wang, S., McMurtry, S., Kim, D. H., et al. (2003). Endothelium-derived hyperpolarizing factor in human internal mammary artery is 11,12-epoxyeicosatrienoic acid and causes relaxation by activating smooth muscle $BK_{(Ca)}$ channels. *Circulation* 107, 769–776. doi: 10.1161/01.CIR.0000047278.28407.C2

Basit, S. (2013). Vitamin D in health and disease: a literature review. *Br. J. Biomed. Sci.* 70, 161–172.

Benoit, C., Renaudon, B., Salvail, D., and Rousseau, E. (2001). EETs relax airway smooth muscle *via* an EpDHF effect: $BK_{(Ca)}$ channel activation and hyperpolarization. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 280, L965–L973.

Benyahia, C., Boukais, K., Gomez, I., Silverstein, A., Clapp, L., Fabre, A., et al. (2013). A comparative study of PGI2 mimetics used clinically on the vasorelaxation of human pulmonary arteries and veins, role of the DP-receptor. *Prostaglandins Other Lipid Mediat.* 107, 48–55. doi: 10.1016/j.prostaglandins.2013.07.001

Bol, M., Leybaert, L., and Vanheel, B. (2012). Influence of methanandamide and CGRP on potassium currents in smooth muscle cells of small mesenteric arteries. *Pflugers Arch.* 463, 669–677. doi: 10.1007/s00424-012-1083-1

Bondarenko, A. I., Malli, R., and Graier, W. F. (2011). The GPR55 agonist lysophosphatidylinositol acts as an intracellular messenger and bidirectionally modulates Ca^{2+}-activated large-conductance K^+ channels in endothelial cells. *Pflugers Arch.* 461, 177–189. doi: 10.1007/s00424-010-0898-x

Bukiya, A., and Dopico, A. (2013a). "Leukotriene B4 activation of arterial smooth muscle BK channels," in *Biophysical Society 57th Annual Meeting.* (Philadelphia, PA).

Bukiya, A. N., McMillan, J. E., Fedinec, A. L., Patil, S. A., Miller, D. D., Leffler, C. W., et al. (2013b). Cerebrovascular dilation *via* selective targeting of the cholane steroid-recognition site in the BK channel β1-subunit by a novel nonsteroidal agent. *Mol. Pharmacol.* 83, 1030–1044. doi: 10.1124/mol.112.083519

Bukiya, A. N., Patil, S. A., Li, W., Miller, D. D., and Dopico, A. M. (2012). Calcium- and voltage-gated potassium (BK) channel activators in the 5β-cholanic acid-3α-ol analogue series with modifications in the lateral chain. *Chem. Med. Chem.* 7, 1784–1792. doi: 10.1002/cmdc.201200290

Bukiya, A. N., Singh, A. K., Parrill, A. L., and Dopico, A. M. (2011). The steroid interaction site in transmembrane domain 2 of the large conductance, voltage- and calcium-gated potassium (BK) channel accessory β1 subunit. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20207–20212. doi: 10.1073/pnas.1112901108

Burnette, J. O., and White, R. E. (2006). PGI2 opens potassium channels in retinal pericytes by cyclic AMP-stimulated, cross-activation of PKG. *Exp. Eye Res.* 83, 1359–1365. doi: 10.1016/j.exer.2006.07.011

Chang, H. M., Reitstetter, R., and Gruener, R. (1995). Lipid-ion channel interactions: increasing phospholipid headgroup size but not ordering acyl chains alters reconstituted channel behavior. *J. Membr. Biol.* 145, 13–19. doi: 10.1007/BF00233303

Chataigneau, T., Félétou, M., Duhault, J., and Vanhoutte, P. M. (1998). Epoxyeicosatrienoic acids, potassium channel blockers and endothelium-dependent hyperpolarization in the guinea-pig carotid artery. *Br. J. Pharmacol.* 123, 574–580. doi: 10.1038/sj.bjp.0701629

Chi, S., and Qi, Z. (2006). Regulatory effect of sulphatides on BK_{Ca} channels. *Br. J. Pharmacol.* 149, 1031–1038. doi: 10.1038/sj.bjp.0706947

Chik, C. L., Li, B., Karpinski, E., and Ho, A. K. (2001). Ceramide inhibits the outward potassium current in rat pinealocytes. *J. Neurochem.* 79, 339–348. doi: 10.1046/j.1471-4159.2001.00566.x

Clarke, A. L., Petrou, S., Walsh, J. V. Jr., and Singer, J. J. (2002). Modulation of $BK_{(Ca)}$ channel activity by fatty acids: structural requirements and mechanism of action. *Am. J. Physiol. Cell. Physiol.* 283, C1441–C1453. doi: 10.1152/ajpcell.00035.2002

Clarke, A. L., Petrou, S., Walsh, J. V. Jr., and Singer, J. J. (2003). Site of action of fatty acids and other charged lipids on BKCa channels from arterial smooth muscle cells. *Am. J. Physiol. Cell. Physiol.* 284, C607–619. doi: 10.1152/ajpcell.00364.2002

Crowley, J. J., Treistman, S. N., and Dopico, A. M. (2003). Cholesterol antagonizes ethanol potentiation of human brain BK_{Ca} channels reconstituted into phospholipid bilayers. *Mol. Pharmacol.* 64, 365–372. doi: 10.1124/mol.64.2.365

Crowley, J. J., Treistman, S. N., and Dopico, A. M. (2005). Distinct structural features of phospholipids differentially determine ethanol sensitivity and basal function of BK channels. *Mol. Pharmacol.* 68, 4–10.

Cuppoletti, J., Malinowska, D. H., Tewari, K. P., Chakrabarti, J., and Ueno, R. (2007). Cellular and molecular effects of unoprostone as a BK channel activator. *Biochim. Biophys. Acta.* 1768, 1083–1092. doi: 10.1016/j.bbamem.2006.12.015

Denson, D. D., Wang, X., Worrell, R. T., and Eaton, D. C. (2000). Effects of fatty acids on BK channels in GH(3) cells. *Am. J. Physiol. Cell. Physiol.* 279, C1211–C1219.

Dimitropoulou, C., West, L., Field, M. B., White, R. E., Reddy, L. M., Falck, J. R., et al. (2007). Protein phosphatase 2A and Ca^{2+}-activated K^+ channels contribute to 11,12-epoxyeicosatrienoic acid analog mediated mesenteric arterial relaxation. *Prostaglandins Other Lipid Mediat.* 83, 50–61. doi: 10.1016/j.prostaglandins.2006.09.008

Dopico, A., Bukiya, A., and Singh, A. (2012a). "Differential contribution of BK subunits to nongenomic regulation of channel function by steroids," in *Cholesterol Regulation of Ion Channels and Receptors,* eds I. Levitan and F. Barrantes (Hoboken, NJ: J. Wiley and sons), 109–134. doi: 10.1002/9781118342312.ch6

Dopico, A. M., Bukiya, A. N., and Singh, A. K. (2012b). Large conductance, calcium- and voltage-gated potassium (BK) channels: regulation by cholesterol. *Pharmacol. Ther.* 135, 133–150. doi: 10.1016/j.pharmthera.2012.05.002

Dopico, A. M., Kirber, M. T., Singer, J. J., and Walsh, J. V. Jr. (1994). Membrane stretch directly activates large conductance $Ca^{(2+)}$-activated K^+ channels in mesenteric artery smooth muscle cells. *Am. J. Hypertens.* 7, 82–89.

Fukao, M., Mason, H. S., Kenyon, J. L., Horowitz, B., and Keef, K. D. (2001). Regulation of $BK_{(Ca)}$ channels expressed in human embryonic kidney 293 cells by epoxyeicosatrienoic acid. *Mol. Pharmacol.* 59, 16–23.

Godlewski, G., Offertáler, L., Osei-Hyiaman, D., Mo, F. M., Harvey-White, J., Liu, J., et al. (2009). The endogenous brain constituent N-arachidonoyl L-serine is an activator of large conductance Ca^{2+}-activated K^+ channels. *J. Pharmacol. Exp. Ther.* 328, 351–361. doi: 10.1124/jpet.108.144717

Guerard, P., Goirand, F., Fichet, N., Bernard, A., Rochette, L., Morcillo, E. J., et al. (2004). Arachidonic acid relaxes human pulmonary arteries through K+ channels and nitric oxide pathways. *Eur. J. Pharmacol.* 501, 127–135. doi: 10.1016/j.ejphar.2004.08.007

Hata, Y., Clermont, A., Yamauchi, T., Pierce, E. A., Suzuma, I., Kagokawa, H., et al. (2000). Retinal expression, regulation, and functional bioactivity of prostacyclin-stimulating factor. *J. Clin. Invest.* 106, 541–550. doi: 10.1172/JCI8338

Hercule, H. C., Salanova, B., Essin, K., Honeck, H., Falck, J. R., Sausbier, M., et al. (2007). The vasodilator 17,18-epoxyeicosatetraenoic acid targets the pore-forming BK alpha channel subunit in rodents. *Exp. Physiol.* 92, 1067–1076. doi: 10.1113/expphysiol.2007.038166

Holtzclaw, J. D., Grimm, P. R., and Sansom, S. C. (2011). Role of BK channels in hypertension and potassium secretion. *Curr. Opin. Nephrol. Hypertens.* 20, 512–517. doi: 10.1097/MNH.0b013e3283488889

Hoshi, T., Tian, Y., Xu, R., Heinemann, S. H., and Hou, S. (2013a). Mechanism of the modulation of BK potassium channel complexes with different auxiliary subunit compositions by the omega-3 fatty acid DHA. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4822–4827. doi: 10.1073/pnas.1222003110

Hoshi, T., Wissuwa, B., Tian, Y., Tajima, N., Xu, R., Bauer, M., et al. (2013b). Omega-3 fatty acids lower blood pressure by directly activating large-conductance Ca^{2+}-dependent K^+ channels. *Proc. Natl. Acad. Sci. U S A.* 110, 4816–4821. doi: 10.1073/pnas.1221997110

Hoshi, T., Xu, R., Hou, S., Heinemann, S. H., and Tian, Y. (2013c). A point mutation in the human Slo1 channel that impairs its sensitivity to omega-3 docosahexaenoic acid. *J. Gen. Physiol.* 142, 507–522. doi: 10.1085/jgp.201311061

Houlihan, D. D., Dennedy, M. C., and Morrison, J. J. (2010). Effects of abnormal cannabidiol on oxytocin-induced myometrial contractility. *Reproduction* 139, 783–788. doi: 10.1530/REP-09-0496

Kim, M. Y., Liang, G. H., Kim, J. A., Kim, Y. J., Oh, S., and Suh, S. H. (2006). Sphingosine-1-phosphate activates BK_{Ca} channels independently of G protein-coupled receptor in human endothelial cells. *Am. J. Physiol. Cell. Physiol.* 290, C1000–C1008. doi: 10.1152/ajpcell.00353.2005

Kirber, M. T., Ordway, R. W., Clapp, L. H., Walsh, J. V. Jr., and Singer, J. J. (1992). Both membrane stretch and fatty acids directly activate large conductance $Ca^{(2+)}$-activated K^+ channels in vascular smooth muscle cells. *FEBS Lett.* 297, 24–28. doi: 10.1016/0014-5793(92)80319-C

Lauterbach, B., Barbosa-Sicard, E., Wang, M. H., Honeck, H., Kärgel, E., Theuer, J., et al. (2002). Cytochrome P450-dependent eicosapentaenoic acid metabolites are novel BK channel activators. *Hypertension* 39, 609–613. doi: 10.1161/hy0202.103293

Lawson, C., Bunting, S., Holzgrefe, H., and Fitzpatrick, F. (1986). Leukotriene B4 and 20-hydroxyleukotriene B4 contract guinea-pig trachea strips *in vitro*. *J. Pharmacol. Exp. Ther.* 237, 888–892.

Li, M., Zhang, Z., Koh, H., Lu, R., Jiang, Z., Alioua, A., et al. (2013). The β1-subunit of the MaxiK channel associates with the thromboxane A2 receptor and reduces thromboxane A2 functional effects. *J. Biol. Chem.* 288, 3668–3677. doi: 10.1074/jbc.M112.426585

Loot, A. E., Moneke, I., Keserü, B., Oelze, M., Syzonenko, T., Daiber, A., et al. (2012). 11,12-EET stimulates the association of BK channel α and β(1) subunits in mitochondria to induce pulmonary vasoconstriction. *PLoS ONE* 7:e46065. doi: 10.1371/journal.pone.0046065

Lu, T., Katakam, P. V., VanRollins, M., Weintraub, N. L., Spector, A. A., and Lee, H. C. (2001). Dihydroxyeicosatrienoic acids are potent activators of $Ca^{(2+)}$-activated $K^{(+)}$ channels in isolated rat coronary arterial myocytes. *J. Physiol.* 534, 651–667. doi: 10.1111/j.1469-7793.2001.t01-1-00651.x

Lundbaek, J. A. (2008). Lipid bilayer-mediated regulation of ion channel function by amphiphilic drugs. *J. Gen. Physiol.* 131, 421–429. doi: 10.1085/jgp.200709948

Martín, P., Moncada, M., Enrique, N., Asuaje, A., Valdez Capuccino, J. M., Gonzalez, C., et al. (2013). Arachidonic acid activation of BKCa (Slo1) channels associated to the β1-subunit in human vascular smooth muscle cells. *Pflugers Arch.* doi: 10.1007/s00424-013-1422-x. [Epub ahead of print].

Moczydlowski, E., Alvarez, O., Vergara, C., and Latorre, R. (1985). Effect of phospholipid surface charge on the conductance and gating of a Ca^{2+}-activated K^+ channel in planar lipid bilayers. *J. Membr. Biol.* 83, 273–282. doi: 10.1007/BF01868701

Morin, C., Sirois, M., Echave, V., Gomes, M. M., and Rousseau, E. (2007). Relaxing effects of 5-oxo-ETE on human bronchi involve BK_{Ca} channel activation. *Prostaglandins Other Lipid Mediat.* 83, 311–319. doi: 10.1016/j.prostaglandins.2007.03.001

Morin, C., Sirois, M., Echave, V., Rizcallah, E., and Rousseau, E. (2009). Relaxing effects of 17(18)-EpETE on arterial and airway smooth muscles in human lung. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 296, L130–L139. doi: 10.1152/ajplung.90436.2008

Orio, P., Rojas, P., Ferreira, G., and Latorre, R. (2002). New disguises for an old channel: MaxiK channel beta-subunits. *News Physiol. Sci.* 17, 156–161.

Patil, S., Bukiya, A. N., Li, W., Dopico, A. M., and Miller, D. (2008). Design and synthesis of hydroxy-alkynoic acids and their methyl esters as novel activators of BK channels. *Bioorg. Med. Chem. Lett.* 18, 3427–3430. doi: 10.1016/j.bmcl.2008.03.080

Peters-Golden, M., and Henderson, W. R. Jr. (2007). Leukotrienes. *N. Engl. J. Med.* 357, 1841–1854. doi: 10.1056/NEJMra071371

Romano, M. R., and Lograno, M. D. (2006). Cannabinoid agonists induce relaxation in the bovine ophthalmic artery: evidences for CB1 receptors, nitric oxide and potassium channels. *Br. J. Pharmacol.* 147, 917–925. doi: 10.1038/sj.bjp.0706687

Rosenblum, W. I. (1985). Constricting effect of leukotrienes on cerebral arterioles of mice. *Stroke.* 16, 262–263. doi: 10.1161/01.STR.16.2.262

Sade, H., Muraki, K., Ohya, S., Hatano, N., and Imaizumi, Y. (2006). Activation of large-conductance, Ca^{2+}-activated K^+ channels by cannabinoids. *Am. J. Physiol. Cell. Physiol.* 290, C77–C86. doi: 10.1152/ajpcell.00482.2004

Shirazi-zand, Z., Ahmad-Molaei, L., Motamedi, F., and Naderi, N. (2013). The role of potassium BK channels in anticonvulsant effect of cannabidiol in pentylenetetrazole and maximal electroshock models of seizure in mice. *Epilepsy Behav.* 28, 1–7. doi: 10.1016/j.yebeh.2013.03.009

Singh, A. K., McMillan, J., Bukiya, A. N., Burton, B., Parrill, A. L., and Dopico, A. M. (2012). Multiple cholesterol recognition/interaction amino acid consensus (CRAC) motifs in cytosolic C tail of Slo1 subunit determine cholesterol sensitivity of Ca^{2+}- and voltage-gated K^+ (BK) channels. *J. Biol. Chem.* 287, 20509–20521. doi: 10.1074/jbc.M112.356261

Stumpff, F., Boxberger, M., Krauss, A., Rosenthal, R., Meissner, S., Choritz, L., et al. (2005). Stimulation of cannabinoid (CB1) and prostanoid (EP2) receptors opens BK_{Ca} channels and relaxes ocular trabecular meshwork. *Exp. Eye Res.* 80, 697–708. doi: 10.1016/j.exer.2004.12.003

Sun, P., Liu, W., Lin, D. H., Yue, P., Kemp, R., Satlin, L. M., et al. (2009). Epoxyeicosatrienoic acid activates BK channels in the cortical collecting duct. *J. Am. Soc. Nephrol.* 20, 513–523. doi: 10.1681/ASN.2008040427

Sun, X., Yao, H., Zhou, D., Gu, X., and Haddad, G. G. (2008). Modulation of hSlo BK current inactivation by fatty acid esters of CoA. *J. Neurochem.* 104, 1394–1403. doi: 10.1111/j.1471-4159.2007.05083.x

Sun, X., Zhou, D., Zhang, P., Moczydlowski, E. G., and Haddad, G. G. (2007). Beta-subunit-dependent modulation of hSlo BK current by arachidonic acid. *J. Neurophysiol.* 97, 62–69. doi: 10.1152/jn.00700.2006

Tanaka, Y., Yamaki, F., Koike, K., and Toro, L. (2004). New insights into the intracellular mechanisms by which PGI2 analogues elicit vascular relaxation: cyclic AMP-independent, Gs-protein mediated-activation of MaxiK channel. *Curr. Med. Chem. Cardiovasc. Hematol. Agents.* 2, 257–265. doi: 10.2174/1568016043356273

Tang, Q. Y., Zhang, Z., Meng, X. Y., Cui, M., and Logothetis, D. E. (2014). Structural determinants of PIP2 regulation of BK channel activity through the RCK1 Ca2+ coordination site. *J. Biol. Chem.* 289, 18860–18872. doi: 10.1074/jbc.M113.538033

Twitchell, W. A., Peña, T. L., and Rane, S. G. (1997). Ca^{2+}-dependent K^+ channels in bovine adrenal chromaffin cells are modulated by lipoxygenase metabolites of arachidonic acid. *J. Membr. Biol.* 158, 69–75.

Vaithianathan, T., Bukiya, A., Liu, J., Liu, P., Asuncion-Chin, M., Fan, Z., et al. (2008). Direct regulation of BK channels by phosphatidylinositol 4,5-bisphosphate as a novel signaling pathway. *J. Gen. Physiol.* 132, 13–28. doi: 10.1085/jgp.200709913

Wang, R. F., Lee, P. Y., Mittag, T. W., Podos, S. M., Serle, J. B., and Becker, B. (1998). Effect of 8-iso prostaglandin E2 on aqueous humor dynamics in monkeys. *Arch. Ophthalmol.* 116, 1213–1216. doi: 10.1001/archopht.116.9.1213

White, R., Ho, W. S., Bottrill, F. E., Ford, W. R., and Hiley, C. R. (2001). Mechanisms of anandamide-induced vasorelaxation in rat isolated coronary arteries. *Br. J. Pharmacol.* 134, 921–929. doi: 10.1038/sj.bjp.0704333

Wu, S. N., Li, H. F., and Chiang, H. T. (2000). Actions of epoxyeicosatrienoic acid on large-conductance $Ca^{(2+)}$-activated $K^{(+)}$ channels in pituitary GH(3) cells. *Biochem. Pharmacol.* 60, 251–262. doi: 10.1016/S0006-2952(00)00317-8

Xu, H., Qi, J., Wang, G., Deng, H., and Qi, Z. (2011). The effect of single cerebroside compounds on activation of BK_{Ca} channels. *Mol. Membr. Biol.* 28, 145–154. doi: 10.3109/09687688.2010.538731

Yeagle, P. L. (2014). Non-covalent binding of membrane lipids to membrane proteins. *Biochim. Biophys. Acta.* 1838, 1548–1559. doi: 10.1016/j.bbamem.2013.11.009

Yuan, C., O'Connell, R. J., Jacob, R. F., Mason, R. P., and Treistman, S. N. (2007). Regulation of the gating of BK_{Ca} channel by lipid bilayer thickness. *J. Biol. Chem.* 282, 7276–7286. doi: 10.1074/jbc.M607593200

Zhang, Y., Oltman, C. L., Lu, T., Lee, H. C., Dellsperger, K. C., and VanRollins, M. (2001). EET homologs potently dilate coronary microvessels and activate $BK_{(Ca)}$ channels. *Am. J. Physiol. Heart. Circ. Physiol.* 280, H2430–H2440.

Zhu, S., Han, G., and White, R. E. (2002). PGE2 action in human coronary artery smooth muscle: role of potassium channels and signaling cross-talk. *J. Vasc. Res.* 39, 477–488. doi: 10.1159/000067201

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 May 2014; accepted: 31 July 2014; published online: 22 August 2014.
Citation: Dopico AM and Bukiya AN (2014) Lipid regulation of BK channel function. Front. Physiol. ***5****:312. doi: 10.3389/fphys.2014.00312*
This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 05 August 2014
doi: 10.3389/fphys.2014.00281

S-acylation dependent post-translational cross-talk regulates large conductance calcium- and voltage-activated potassium (BK) channels

Michael J. Shipston*

Centre for Integrative Physiology, College of Medicine and Veterinary Medicine, University of Edinburgh, Edinburgh, UK

Edited by:
Thomas M. Weiger, University of Salzburg, Austria

Reviewed by:
Stuart E. Dryer, University of Houston, USA
Ita O'Kelly, University of Southampton, UK

****Correspondence:***
Michael J. Shipston, Centre for Integrative Physiology, College of Medicine and Veterinary Medicine, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH89XD, UK
e-mail: mike.shipston@ed.ac.uk; Skype: mshipsto

Mechanisms that control surface expression and/or activity of large conductance calcium-activated potassium (BK) channels are important determinants of their (patho)physiological function. Indeed, BK channel dysfunction is associated with major human disorders ranging from epilepsy to hypertension and obesity. S-acylation (S-palmitoylation) represents a major reversible, post-translational modification controlling the properties and function of many proteins including ion channels. Recent evidence reveals that both pore-forming and regulatory subunits of BK channels are S-acylated and control channel trafficking and regulation by AGC-family protein kinases. The pore-forming α-subunit is S-acylated at two distinct sites within the N- and C-terminus, each site being regulated by different palmitoyl acyl transferases (zDHHCs) and acyl thioesterases (APTs). S-acylation of the N-terminus controls channel trafficking and surface expression whereas S-acylation of the C-terminal domain determines regulation of channel activity by AGC-family protein kinases. S-acylation of the regulatory β4-subunit controls ER exit and surface expression of BK channels but does not affect ion channel kinetics at the plasma membrane. Furthermore, a significant number of previously identified BK-channel interacting proteins have been shown, or are predicted to be, S-acylated. Thus, the BK channel multi-molecular signaling complex may be dynamically regulated by this fundamental post-translational modification and thus S-acylation likely represents an important determinant of BK channel physiology in health and disease.

Keywords: acylation, palmitoylation, phosphorylation, trafficking, *KCNMA1*, *KCNMB4*, MaxiK channel, Slo1

INTRODUCTION

The pore-forming α-subunits of large conductance calcium- and voltage- activated potassium (BK) channels are encoded by only a single gene, *KCNMA1*, yet these channels display considerable functional diversity to control an eclectic array of physiological processes in distinct cells and systems of the body (Salkoff et al., 2006; Contreras et al., 2013). Multiple mechanisms exist and work combinatorially to expand this physiological diversity including alternative pre-mRNA splicing of the α-subunit (Fodor and Aldrich, 2009), assembly with regulatory and accessory β- and γ- subunits (Orio et al., 2002; Yan and Aldrich, 2012) and post-translational modification via a diverse array of signaling pathways (Schubert and Nelson, 2001; Hou et al., 2009; Toro et al., 2013). These mechanisms ultimately control either the number of BK channels that are resident at a plasma membrane or affect the intrinsic properties or regulation of the channel at the membrane. For BK channels, due to their large conductance, small changes in either the number or activity of the channel can dramatically modify potassium flux across the membrane with subsequent impact on cellular physiology. Indeed, disruption of BK channel function is linked to a variety of disorders of the vascular, nervous, endocrine and renal systems including hypertension, obesity, epilepsy, autism, incontinence and stress-related disorders (Brenner et al., 2000, 2005; Meredith et al., 2004; Sausbier et al., 2004, 2005; Du et al., 2005; Werner et al., 2005; Jiao et al., 2011; Deng et al., 2013).

Thus, post-translational modifications that control BK channel surface expression and/or activity represent powerful mechanisms to regulate both the normal physiological function of BK channels as well as serve as potential nodes of channel disruption in disease. In the past few years S-acylation, the only true fully reversible post-translational lipid modification of proteins (**Figure 1A**), has emerged as a fundamental mechanism controlling the surface expression and activity of a diverse array of ion channels, including BK channels (Shipston, 2011, 2014). Although S-acylation was first described more than 30 years ago, at the same time as tyrosine kinase phosphorylation, only relatively recently have the enzymes that control S-acylation been identified (Fukata et al., 2004; Linder and Deschenes, 2007; Fukata and Fukata, 2010; Greaves and Chamberlain, 2011; Resh, 2012) and an array of proteomic and imaging tools developed (Drisdel and Green, 2004; Forrester et al., 2009; Hannoush and Sun, 2010; Martin et al., 2012) to allow rigorous examination of the functional role of protein S-acylation.

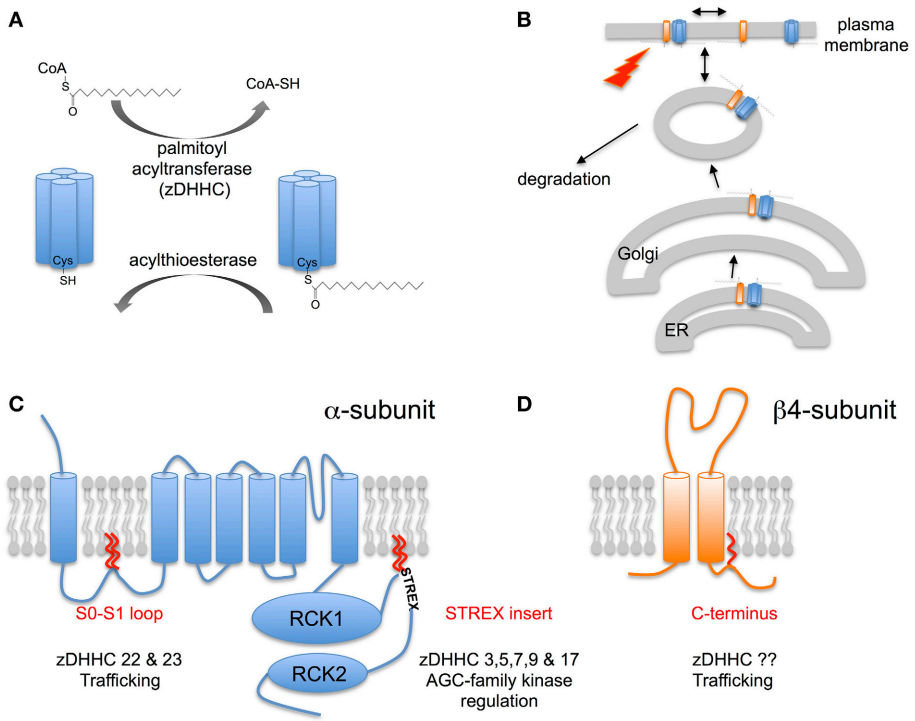


FIGURE 1 | S-acylation of BK channels. (A) Schematic of reversible enzymatic regulation S-acylation of proteins. Addition of lipid (typically palmitate) to cysteine residues in target proteins via a thioester bond is catalyzed by a family of palmitoyl acyltransferases (zDHHCs). Removal of lipid results from the action of acylthioesterases. **(B)** S-acylation controls multiple steps in the lifecycle of BK channels including control of forward trafficking, surface expression and intrinsic channel properties and modulation by other signaling pathways. **(C)** Schematic of the pore-forming α-subunit of the BK channel encoded by the single *KCNMA1* gene. α-subunits are S-acylated at two distinct sites by distinct acyl transferase (zDHHCs): the conserved intracellular S0-S1 loop and the alternatively spliced STREX insert in the C-terminal linker between the two regulator of potassium conductance (RCK) domains. S-acylation of the S0-S1 loop controls surface trafficking of the channel whereas S-acylation of the STREX insert determines channel activity and regulation by AGC-family protein kinases. **(D)** Schematic of the regulatory β4-subunit encoded by the *KCNMB4* gene. The β4-subunit is S-acylated at a single cysteine juxtaposed to the second transmembrane domain in the intracellular C-terminus. S-acylation of the β4-subunit controls surface expression of distinct BK channel α-subunit splice variants.

As well as providing new insights into BK channel regulation and physiology these studies are also revealing important mechanisms, properties, and function of protein S-acylation.

S-ACYLATION: A REVERSIBLE LIPID POST-TRANSLATIONAL MODIFICATION

Protein S-acylation involves the post-translational addition of a lipid (typically, but not exclusively, palmitate) via a labile thioester bond to intracellular cysteine residues. Thus, S-acylation, unlike other lipid modifications such as myristoylation or prenylation, is dynamically reversible (**Figure 1A**). Indeed, S-acylation of most proteins is enzymatically driven by a large family (23 in mammals) of transmembrane zinc-finger containing protein acyltransferases (zDHHC family). zDHHCs have a highly conserved Asp-His-His-Cys (DHHC) signature sequence within a cysteine rich stretch of ∼50 amino acids critical for catalytic activity. zDHHCs display distinct tissue expression as well as subcellular localization with zDHHCs expressed typically at the ER, Golgi or plasma membrane (Fukata et al., 2004; Linder and Deschenes, 2007; Fukata and Fukata, 2010; Greaves and Chamberlain, 2011; Resh, 2012). Conversely, de-acylation is determined by acylthioesterases belonging to the large serine hydrolase superfamily including the cytosolic LYPLA1, LYPLA2 enzymes, and the lysozomal PPT1 (Zeidman et al., 2009; Bachovchin et al., 2010; Martin et al., 2012). For most proteins, let alone ion channels, the zDHHCs or thioesterases that control S-acylation are poorly characterized.

Over the last few years developments in both characterization of the enzymes that control S-acylation as well as improved biochemical methods to interrogate native protein S-acylation have begun to reveal the a major role for S-acylation in controlling both trafficking and regulation of many different types of ion channel, incuding BK channels (**Figure 1B**).

S-ACYLATION OF BK CHANNEL PORE-FORMING α-SUBUNITS

BK CHANNEL α-SUBUNITS ARE S-ACYLATED AT TWO DISTINCT SITES

In native tissues, such as brain, BK channels pore-forming α-subunits are robustly S-acylated as revealed using biotin-exchange or acyl-resin assisted capture (acyl-RAC) methodologies (Kang et al., 2008; Tian et al., 2008; Alioua et al., 2011). Although, these approaches did not reveal cysteine residues that are S-acylated freely available S-acylation prediction algorithms [e.g., CSS-palm (http://csspalm.biocuckoo.org) (Ren et al., 2008)] reveal that

cysteine residues in both the intracellular S0-S1 loop as well as the alternatively spliced stress-regulated exon (STREX) insert, in the C-terminal linker between the two regulator of K conductance (RCK) domains, are likely S-acylated (**Figure 1C**). Using a combination of site directed mutagenesis, acyl-RAC and ^{3}H-palmitate labeling of recombinant murine ZERO variant BK channels (that lack the STREX insert) expressed in HEK293 cells revealed that cysteine residues C53 and 56 in the S0-S1 loop were endogenously S-acylated in HEK293 cells (Jeffries et al., 2010; Tian et al., 2012). Site directed mutagenesis of C53 and C56 abolished S-acylation of the full-length ZERO variant suggesting these are the only cysteines S-acylated in the entire ZERO channel. S-acylation of the S0-S1 loop allows the isolated S0-S1 loop (assayed as a –GFP fusion protein) to associate with the plasma membrane in the absence of transmembrane domains. This suggests that S-acylation acts as an additional membrane anchor in this otherwise largely unstructured domain. Using an siRNA based screen to knock-down the expression of individual zDHHCs expressed endogenously in HEK293 cells revealed that zDHHC 22 and zDHHC 23 are the major acyltransferases that control S-acylation of the S0-S1 loop (Jeffries et al., 2010; Tian et al., 2012). Whether, these distinct zDHHCs differential control S-acylation of C53 or C56, respectively is unknown. However, the zDHHCs may also control S-acylation of the S0-S1 loop at different stages during the lifecycle of the channel as it traffics from the ER to plasma membrane (**Figure 1B**). Furthermore, overexpression of zDHHC23, but not the catalytically inactive zDHHS23 mutant, increased S0-S1 S-acylation.

Similar approaches have also revealed that the alternatively spliced STREX insert located within the unstructured linker between RCK1 and RCK2 in the large intracellular C-terminus is S-acylated at two tandem cysteine residues: C645 and C646 (Tian et al., 2008, 2010; Jeffries et al., 2012). For example, using an imaging screen exploiting a –GFP fusion of the entire C-terminus of the ZERO or STREX variant BK channel (i.e., in the absence of transmembrane domains) revealed that the STREX C-terminus, but not the ZERO C-terminus, was robustly associated with the plasma membrane. Site directed mutagenesis of the two cysteines, C645 and C646 in STREX predicted to be S-acylated by the CSS-palm algorithm, abolished S-acylation and membrane association of the fusion protein (Tian et al., 2008; Jeffries et al., 2012). Similar data were also obtained using a –GFP fusion of just the 59 amino acid STREX insert alone. S-acylation of the STREX insert at these residues was confirmed in full-length STREX channels in which the S0-S1 S-acylated cysteines were mutated to alanine (Tian et al., 2008; Jeffries et al., 2012). Using the siRNA screen to knock-down individual zDHHCs revealed several zDHHCs (zDHHCs 3, 5, 7, 9, and 17) as potential S-acylating enzymes of the STREX insert (Tian et al., 2010). Moreover, S-acylation of the STREX domain was enhanced by over expression of the cognate zDHHCs with zDHHC17 showing the greater selectivity for the dicysteine C645:646 motif.

S-acylation is a reversible post-translational modification catalyzed by members of the serine hydrolase superfamily. The lysosomal acylthioesterase, PPT1 that typically de-acylates proteins undergoing lysosomal degradation, appears to have little role in controlling S0-S1 loop S-acylation. In contrast, over expression of the cytosolic thioesterase LYPLA1 and a splice variant of the related LYPLAL1, but not LYPLA2, depalmitoylated BK channels at the S0-S1 loop (Tian et al., 2012). In both cases, catalytically "dead" mutants of the thioesterases were ineffective. However, steady-state S-acylation of the S0-S1 loop was not significantly affected by knockdown of LYPLA1 suggesting that either deacylation is not rate limiting or that S-acylation of the S0-S1 loop has a long half life in the lifecycle of the channel. To date, the acyltransferases controlling STREX insert S-acylation, and the dynamics of S-acylation of either site are not fully elucidated.

Taken together, these data reveal that the pore-forming α-subunits can be S-acylated at distinct intracellular domains of the channel. Remarkably, each site is differentially regulated by distinct zDHHC enzymes suggesting that each site can be controlled independently and supporting the hypothesis that distinct zDHHCs can display substrate specificity (Greaves and Chamberlain, 2011). This also raises a challenge for interrogating BK S-acylation in native tissues. For example, to interrogate how S-acylation of each domain may be differentially controlled by distinct physiological challenges will require adaptation of current biochemical assays. As an example, using the acyl-RAC approach would require approaches such as on-bead tryptic digests followed by elution of S-acylated peptides for analysis by mass spectrometry. The zDHHCs that control S0-S1 loop or STREX insert S-acylation are expressed at either the ER, Golgi or plasma membrane suggesting that the pore-forming α-subunits may be regulated at multiple sites in the trafficking pathways to the plasma membrane. Clearly, a challenge for the future is also to establish the tissue and cellular distribution of these zDHHCs and thioesterase and their regulation of BK channels in native tissues.

Importantly, as S-acylation controls two functionally distinct domains on the pore-forming α-subunits this suggests that differential S-acylation may control distinct channel properties as discussed below.

S0-S1 LOOP S-ACYLATION CONTROLS CELL SURFACE TRAFFICKING

The functional role of the BK channel S0-S1 loop is poorly understood although it is thought to be largely unstructured and includes residue(s) important for magnesium ion coordination with the C-terminal RCK domains (Yang et al., 2007; Cui, 2010; Shi et al., 2013). Furthermore, in some species it is also a site for both post-translational modification by phosphorylation as well as sites of alternative splicing (c.f. Liu et al., 2006). Moreover, the transmembrane S0 domain is important for functional coupling with regulatory β-subunits (Orio et al., 2002). Thus, potentially S-acylation of the S0-S1 loop may have multiple functional consequences.

However, the major role of S-acylation of the S0-S1 loop appears to be in the control of cell surface expression of α-subunits (Jeffries et al., 2010; Tian et al., 2012) (**Figures 1B**, **2**). Depalmitoylation of the S0-S1 loop, using several approaches including inhibition of global S-acylation with the broad spectrum zDHHC inhibitor 2-bromopalmitate (2-BP), siRNA mediated knockdown of zDHHCs 22 or 23, or site directed mutagenesis of the S-acylated cysteine residues to alanine, results in a suppression of BK channel steady-state surface expression by more than 50%. This effect appears to be independent of the

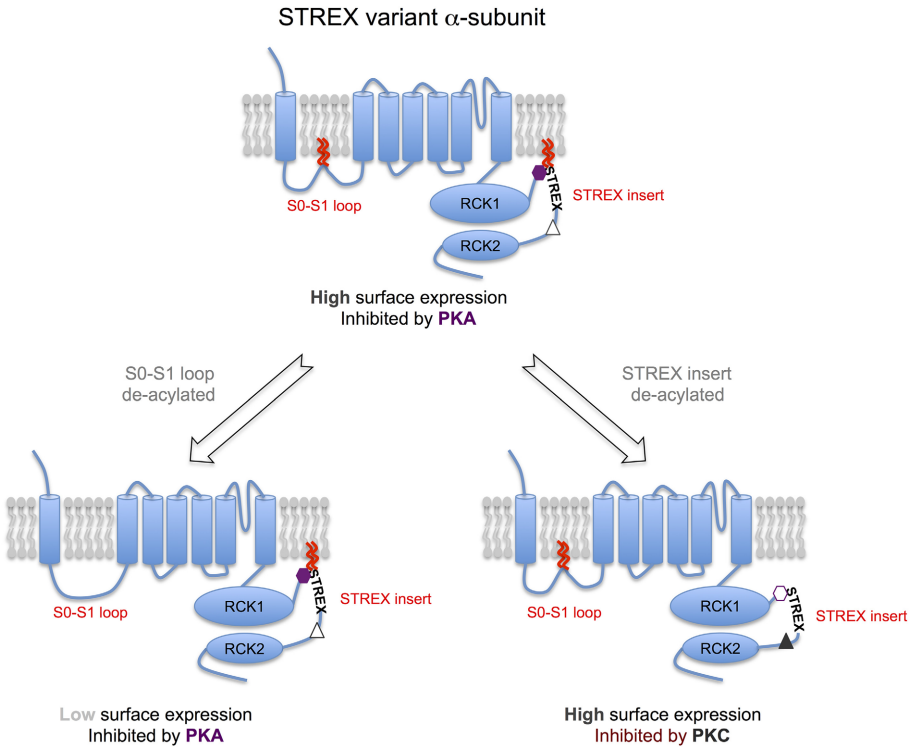


FIGURE 2 | S-acylation of the α-subunit controls distinct properties of BK channels. Model schema for regulation of STREX variant α-subunits by S-acylation and AGC-family kinases. The STREX variant of the BK channel can be S-acylated at two distinct sites: (i) the S0-S1 loop allowing the loop to associate with the plasma membrane and is important in controlling surface delivery of the BK channel; (ii) in the alternatively spliced STREX insert that allows the cytosolic STREX domain to interact with the plasma membrane. S-acylation of the STREX insert determines STREX channel regulation by AGC-family protein kinase dependent phosphorylation. Protein kinase A (PKA)-dependent phosphorylation of S^{636} in the STREX insert (purple hexagon), that is immediately upstream of the S-acylated cysteine residues, results in dissociation of the STREX domain from the plasma membrane and inhibition of STREX channel activity. In contrast, when STREX is S-acylated, protein kinase C (PKC) has no effect on channel activity even though phosphorylation of the PKC-consensus sites (S^{700} and S^{1156}), that are downstream of the STREX insert, result in channel inhibition in channels lacking the STREX insert (e.g., ZERO variant). Thus, the S-acylated STREX insert prevents PKC-mediated inhibition in STREX channels. However, deacylation of the STREX insert, or PKA mediated dissociation of the STREX domain from the plasma membrane, now allows phosphorylation of the S^{700} PKC-site (S^{700}, gray triangle) that, in conjunction with the C-terminal PKC site S^{1156}, confers PKC-dependent inhibition of STREX channels. Thus, the S-acylation of STREX serves as a switch to determine STREX BK channel regulation by either PKA or PKC. PKG- mediated activation of STREX channels, dependent on phosphorylation of other C-terminal serine residues, is not controlled by STREX insert S-acylation. In combination with control of S0-S1 loop S-acylation channels with distinct surface expression and regulation by AGC-kinases can be generated thus expanding BK channel physiological diversity. For example, channels that are S-acylated at both the S0-S1 loop and STREX insert (center top) would be predicted to have high surface expression and inhibited by PKA. Channels de-acylated at only the S0-S1 loop would have low surface expression but inhibited by PKA (bottom left) whereas channels S-acylated at only the STREX insert would have high surface expression and now inhibited by PKC, but not PKA (bottom right).

splice variant of the α-subunit under investigation, including whether the C-terminal STREX insert is S-acylated (Jeffries et al., 2010; Tian et al., 2012; Chen et al., 2013b). Although, the suppression of surface expression can be partially rescued by expression with some regulator subunits (see Section S-acylation of β4-Subunits Controls Surface Delivery and Chen et al., 2013b) depalmitoylated α-subunits surface expression is still compromised compared to expression of S-acylated subunit and regulatory subunits. Thus, S-acylation of S0-S1 plays a dominant role in surface expression. BK channel α-subunits that can never be S-acylated at the S0-S1 loop (e.g., site directed cysteine to alanine mutants) show enhanced trapping at the Endoplasmic reticulum suggesting that ER exit may be a key regulatory step, although S-acylation is not essential as mutant α-subunits can still reach the cell surface. However, a key regulatory site for BK channel trafficking controlled by the S0-S1 loop is also at the level of exit from the trans-golgi network (TGN) (Tian et al., 2012). Overexpression of the acylthioesterase LYPLA1, but not LYPLA2, also decreased steady-state cell surface expression by approximately half in accordance with the studies above. Channels that were de-acylated by LYPLA1 were largely retained in the TGN and this TGN accumulation was associated with a reduction in channel co-localization in recycling endosomes (Tian et al., 2012). Clearly, a major goal will be to understand the spatiotemporal dynamics of S0-S1 S-acylation during both forward trafficking to the cell surface as well as routes for channel recycling.

As indicated above, channels that are de-acylated can still reach the plasma membrane. Recent studies that S0-S1 loop S-acylation (using site directed alanine mutants) controls the lateral mobility of single BK channels in the plasma membrane (Kim

et al., 2014). De-acylated channels display a faster and more random lateral diffusion compared to wild-type channels suggesting S-acylation constrains the movement of channels in the plasma membrane (Kim et al., 2014). Whether this reduced mobility is a function of the ability of the S-acylated S0-S1 loop to act as an additional membrane anchor or allows BK channels to assemble with the cytoskeleton, or other membrane domain organizing components, remains to be determined.

S0-S1 loop S-acylation does not have any significant effect on the intrinsic calcium/voltage sensitivity of the α-subunit as site directed mutation of the S-acylated cysteine residues has no effect on the voltage for half maximal activation over a range of calcium concentrations (Jeffries et al., 2010; Kim et al., 2014). This also suggests that S-acylation is not central to the role of the S0-S1 loop in coordinating magnesium binding with the RCK domains. However, whether S0-S1 loop S-acylation controls physical and/or functional coupling to regulatory β- or γ- subunits remains to be determined.

S-ACYLATION OF A RCK1-RCK2 LINKER SPLICE VARIANT (STREX) DETERMINES REGULATION BY AGC FAMILY PROTEIN KINASES

BK channels are subject to post-translational regulation by an array of distinct protein kinases and phosphatases including members of the classical AGC-family of protein kinases: cAMP dependent protein kinase (PKA); protein kinase C (PKC) and cGMP-dependent protein kinase (PKG). Importantly, the effect of AGC-family kinase mediated phosphorylation on the activity of BK channels is determined by the pore-forming α-subunit splice variant (e.g., Tian et al., 2001, 2004; Zhou, 2001). In this regard, inclusion of the alternatively spliced STREX insert introduces an additional consensus PKA-phosphorylation that switches channel regulation by PKA from channels that are activated (when the STREX insert is absent) to channels that are inhibited (when the STREX insert is included) (Tian et al., 2001, 2004). The STREX insert is a cysteine rich domain that is located in the structurally disordered intracellular linker between the RCK1 and RCK2 domains in the C-terminus (**Figure 1C**). As well as controlling regulation by PKA, the STREX insert also shifts the voltage for half-maximal activation to the left such that STREX BK channels are more sensitive to calcium and voltages in the physiological range and also changes both activation and deactivation kinetics (Xie and McCobb, 1998; Tian et al., 2001). Intriguingly, the PKA consensus site required for PKA-mediated inhibition within STREX (S636) is located immediately upstream of the di-cysteine cluster (C645:646) that is S-acylated, and within a highly polybasic sequence of amino acids (**Figure 1C**). As outlined above (section BK Channel α-Subunits are S-acylated at Two Distinct Sites) the S-acylated STREX domain associates with the plasma membrane. PKA-mediated phosphorylation of S636, or phosphomimetic mutation to S636E or S636D, all of which introduce negative charge into the otherwise polybasic region upstream of the S-acylated dicysteines S645:646 attenuate STREX S-acylation and results in dissociation of the STREX domain from the plasma membrane (Tian et al., 2008; Jeffries et al., 2012). This data also suggest that both the polybasic domain and S-acylated di-cysteine motif are required for association of the STREX domain with the plasma membrane. Indeed, mutation of residues of the polybasic domain *per se* control S645:646 S-acylation and membrane association of STREX. As S-acylation is mediated at membrane interfaces this may suggest that the polybasic domain is required for an initial transient association with the membrane to allow S-acylation of C645:646 by cognate zDHHCs, as reported for other proteins with such polybasic domains (Linder and Deschenes, 2007; Greaves and Chamberlain, 2011). In addition, destabilization of the polybasic domain by PKA-phosphorylation may also allow cytosolic acylthioesterases access to the di-cysteine cysteines to promote de-acylation. In this way, palmitoylation and phosphorylation may interact and allow temporal control of STREX channel function.

Thus, this data supports a model in which introduction of negative charge disrupts interaction of the polybasic region upstream of the site of S-acylation, leading to conformational rearrangements in the channel and subsequent inhibition of channel properties (**Figures 1C**, **2**). In support of this, de-acylation of STREX results in channels with kinetic and conductance/voltage relationships similar to channels inhibited by PKA, or lacking the STREX insert. Thus, pharmacological inhibition of STREX S-acylation, or knockdown of the cognate zDHHCs (as in section BK Channel α-Subunits are S-acylated at Two Distinct Sites), prevents PKA-mediated inhibition and converts STREX BK channels to a phenotype closer to that of channels lacking the STREX insert. This suggests that STREX insert S-acylation is critical for conferring core properties, as well as PKA-mediated inhibition, of STREX variant BK channels. Importantly, other modes of regulation conferred by the STREX insert, such as regulation by low oxygen tension (hypoxia) (McCartney et al., 2005) are not dependent upon S-acylation of the STREX domain. Furthermore, at least in recombinant systems, STREX insert S-acylation has no effect on plasma membrane surface expression of BK channels illustrating that S-acylation of the STREX and S0-S1 domains have distinct functions, controlled by different zDHHCs. Taken together this suggests an important level of cross-talk between the PKA and S-acylation signaling pathways via mutual control of an S-acylated domain of proteins (**Figures 1C**, **2**)—such cross-talk is an emerging concept in a range of other ion channels and signaling proteins (Shipston, 2014).

The studies discussed above reveal a level of interaction between a phosphorylation (PKA) and lipid post-translational modification through mutual regulation of a protein domain at the plasma membrane with the phosphorylation and S-acylation sites close together (in linear sequence ∼10 amino acids). However, recent studies from Thomas Wieland's laboratory examining PKC-mediated regulation of BK channel activity have also revealed a role for STREX S-acylation in controlling PKC phosphorylation mediated regulation via consensus phosphorylation sites outwith the STREX domain (Zhou et al., 2012). PKC has been shown to inhibit BK channels, in splice variants lacking the STREX insert, through phosphorylation of two distinct PKC consensus sites located in the BK channel C-terminus (Zhou et al., 2010). One PKC site is located toward the very C-terminus of the channel (S1156) with an additional site (S700) located downstream of the STREX insert site of splicing. Importantly, for PKC-mediated inhibition the S700 site is only phosphorylated once S1156 is phosphorylated and both sites must be

phosphorylated for PKC to inhibit channel activity. However, in channels in which the STREX insert is included, PKC has no effect on channel activity even though both the S700 and S1156 sites are present in the C-terminus (Zhou et al., 2012). Strikingly, in STREX channels in which S-acylation of the STREX di-cysteine S-acylation motif was prevented, either by global zDHHC inhibition by 2-BP, or site directed mutagenesis of C645:646, PKC could now inhibit channel activity. Furthermore, in S-acylated STREX channels in which the S700 PKC consensus site was mutated to the phosphomimetic S700E PKC could now inhibit channel activity (Zhou et al., 2012). This suggests a model in which the S-acylated STREX domain normally occludes phosphorylation of S700 thus preventing PKC mediated inhibition. Dissociation of the STREX domain from the plasma membrane via either de-acylation *per se* or prior PKA-mediated phosphorylation of the STREX insert at the PKA site S636, would thus gate the ability of STREX variant channels to be inhibited by PKC (**Figure 2**). Indeed, this appears to be an important mode of regulation in pituitary endocrine cells that express STREX variant channels (Zhou et al., 2012).

In contrast, PKG mediated regulation of STREX containing, or STREX-less channels, is independent of the S-acylation status of the STREX insert *per se* (Zhou et al., 2012), most likely as the major consensus site for PKG-mediated regulation is toward the very C-terminus of the channel α-subunit. Thus, these data reveal an important role in S-acylation in acting as a switch to specify STREX variant regulation by PKA and PKC (**Figure 2**). Furthermore, it may also provide a mechanism to provide robust regulation by both PKA and PKC in STREX-expressing cells in which BK channels are targets for signaling pathways activating the PKC and PKA pathways such as in some endocrine cells.

S-ACYLATION OF BK CHANNEL REGULATORY SUBUNITS AND ASSOCIATED PROTEINS

S-ACYLATION OF β4-SUBUNITS CONTROLS SURFACE DELIVERY

The assembly of BK channel pore-forming α-subunits with a diverse family of transmembrane β- and γ- subunits provides additional mechanisms to determine physiological diversity of the BK channel in different cells and tissues. Regulatory subunits play diverse roles in modifying both the intrinsic properties of the channel (kinetics and calcium/voltage sensitivity) as well as trafficking of the channel to the cell surface (Orio et al., 2002; Yan and Aldrich, 2012). Recent evidence reveals that S-acylation of the regulatory β4-subunit may play an important role in the latter. The β4-subunit is highly expressed in the nervous system, as well as endocrine tissues, and confers complex effects on channel activity dependent upon the local calcium concentration and also modifies BK channel pharmacology by making the channel largely resistant to the toxins Iberiotoxin and Charybdotoxin (Meera et al., 2000; Brenner et al., 2005; Wang et al., 2006). The β4-subunit, as for other β-subunits, has two transmembrane domains with a large extracellular loop and short intracellular N- and C-termini (**Figure 1D**). Previous data have revealed that the β4 C-terminus contains a basic ER-retention trafficking motif that controls β4-subunit surface expression (Shruti et al., 2012). β4-subunits are S-acylated in mouse brain and when expressed in recombinant systems at a single cysteine residue (C193) juxtaposed to the second transmembrane domain immediately upstream of the C-terminal trafficking motif (Chen et al., 2013b) (**Figure 1D**). β4-subunits alone are typically trafficking incompetent largely residing in the ER and S-acylation has no effect on β4-subunit localization. However, mutation of the ER retention motif allows β4-subunts to traffic to the cell surface and the exit from the ER is dependent upon S-acylation of the β4-subunit at C193. Importantly, assembly of β4-subunits with α-subunits controls surface expression of the channel complex. Rather surprisingly, β4-subunits can up- or down- regulate α-subunit surface expression depending on the specific splice variant of the pore forming subunit (Shruti et al., 2012; Chen et al., 2013a). Surface expression of BK channel α-subunits that include the C-terminal splice variant ..REVEDEC motif is upregulated by β4-subunits (Chen et al., 2013b). Upregulation of α-subunit surface expression is dependent upon S-acylation of the β4-subunit at C193. β4-subunits that are de-acylated at C193 do not promote surface expression of the ..REVEDEC α-subunit variants. The REVEDEC heptapeptide has been reported to suppress α-subunit surface expression and thus hypothesized to act as a trafficking motif (Kim et al., 2007; Chiu et al., 2010). Transplanting the REVEDEC heptapeptide onto the C-terminus of α-subunits whose surface expression was not normally enhanced upon co-expression with β4-subunits, resulted in a β4-subunit S-acylation dependent up regulation of surface expression. This suggests that ..REVEDEC acts as a trafficking motif and that S-acylated β4-subunits may mask this motif to allow enhanced surface expression. The mechanistic basis for this effect remains to be resolved however a possible explanation is that S-acylation of β4-subunits is required for the correct structural interaction with α-subunits at the ER. Chemical crosslinking experiments reveal that the extracellular aspect of the second transmembrane domain of the β4-subunit is in close apposition to S0 of the α-subunit (Wu et al., 2009). In other systems, S-acylated cysteines juxtaposed to transmembrane domains promote tilting of the transmembrane domain and this may be important at the thinner ER membrane to reduce hydrophobic mismatch as well as confer restraints on the β4-peptide (Nyholm et al., 2007; Abrami et al., 2008; Baekkeskov and Kanaani, 2009; Charollais and der Goot, 2009).

However, although such a model may explain how β4-subunits control surface expression of the α-subunit ..REVEDEC splice variant, β4-subunit control of BK channel surface expression is clearly more complex than the model outlined above. Indeed, surface expression of another distinct α-subunit splice variant was in fact suppressed upon co-expression with *de*-acylated β4-subunits. This is in accordance with other data supporting a role for β4-subunits to suppress BK channel expression in some neurons, although how selectivity of action between different α-subunit splice variants is conferred is not known. Moreover, although β4-subunits can also enhance surface expression of de-acylated α-subunit ..REVEDEC splice variants at the S0-S1 loop, an effect that is β4-subunit S-acylation dependent, β4-subunit co-expression cannot rescue to the levels achieved by expression of α- and β4-subunits that can both be S-acylated. Thus, in BK channels, S-acylation of the S0-S1 loop of the pore-forming subunit controls global BK channel surface expression

and β4-subunit S-acylation additionally controls surface expression of specific pore-forming subunit splice variants.

CONTROL OF BK CHANNELS VIA S-ACYLATION OF OTHER COMPONENTS OF THE CHANNEL COMPLEX?

BK channels, as for other ion channels, do not exist in "isolation" in membranes but assemble into functional complexes with an array of adapter, structural and signaling proteins (e.g., see Lu et al., 2006; Kathiresan et al., 2009; Berkefeld et al., 2010; Toro et al., 2013). As S-acylation can control a wide variety of proteins, from G-protein coupled receptors, to tyrosine kinases and multifunctional adapter proteins such as PSD-95 (Fukata et al., 2004; Linder and Deschenes, 2007; Fukata and Fukata, 2010; Greaves and Chamberlain, 2011; Shipston, 2011, 2013; Resh, 2012), S-acylation may also exert effects on BK channel trafficking and function through modulation of the channel multi-molecular complex *per se* or signaling pathways that converge on the complex. As assembly of BK channels with different interacting proteins is likely to be cell specific S-acylation may control BK channel function differentially through modulation of components of the larger multi-molecular BK channel signaling complex.

As outlined in section S-acylation of β4-subunits Controls Surface Delivery β4-subunits are S-acylated and control trafficking however, although other regulatotry subunits are also predicted to be S-acylated (**Figure 3**) the functional consequence is as yet unknown. Furthermore, a significant number of BK channel interacting proteins previously reported in proteomic screens (e.g., see Lu et al., 2006; Kathiresan et al., 2009; Berkefeld et al., 2010; Toro et al., 2013) are also predicted, or have been shown, to be S-acylated (**Figure 3**). A clear challenge will be to define S-acylated components of the BK channel complex in defined cell types and understand both how S-acylation of these proteins is controlled as well as the functional consequence of S-acylation on BK channel physiology.

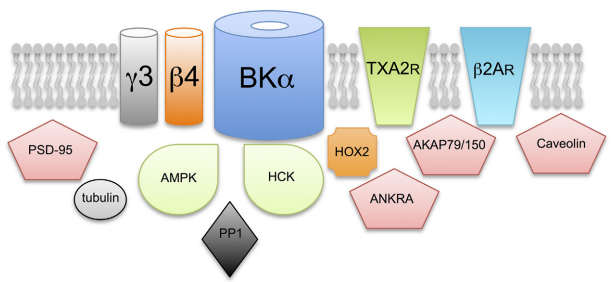


FIGURE 3 | Does S-acylation control multiple components of the BK channel signaling complex? Schematic illustrating exemplar proteins that have previously been reported to assembly within a multimolecular complex with the BK channel pore forming α-subunit and are strongly predicted, or have been previously shown, to be S-acylated in cells. The functional consequence of S-acylation, except for the β4-regulatory subunit, of these components on BK channel function is not yet known. Proteins include: additional accessory subunits of the BK channels such as the γ3 subunit LRRC55; G-protein coupled receptors such as the thromboxane A2 (TXA2R) and β2-adrenergic (β2AR) receptors; adapter and scaffolding proteins such as PSD-95, AKAP79/150, caveolin and ANKRA; structural and cytoskeletal proteins such as tubulin and signaling proteins such as the AMP-activated serine/threonine protein kinase (AMPK), the tyrosine kinase, HCK and the serine/threonine protein phosphatase PP1.

CONCLUSIONS

Multiple mechanisms, including alternative splicing, assembly with accessory subunits and post-translational modifications allow considerable functional diversity of BK channels to be generated from a single gene, *KCNMA1*, encoding for the pore-forming α-subunits. The work described above implicates S-acylation, a reversible post-translational lipid modification of proteins, as a major mechanism to control both the number of BK channels at the cell surface as well as their activity and regulation at the membrane. Importantly, S-acylation can control multiple sites within the BK channel complex and this post-translational modification works combinatorially with other mechanisms that specify functional diversity to fine tune BK channel properties and regulation.

Clearly to understand the contribution of this important post-translational modification in BK channel physiology several major challenges need to be addressed. Firstly, although we are beginning to define the enzymes that control BK channel S-acylation the temporal and spatial dynamics of BK channel S-acylation is very poorly understood. Furthermore, although distinct enzymes can control different aspects of BK channel physiology how these enzymes themselves are regulated is largely unknown. As S-acylation works in combination with other mechanisms, including controlling cysteine reactivity *per se* (Hess et al., 1993; Sen and Snyder, 2010; Ho et al., 2011), a clear challenge is to define how such interactions control specific cellular functions. For example, defining cellular physiological processes and specific cell types in which S-acylation controls: AGC-family kinase regulation of the STREX variant, β4-subunit mediated regulation of α-subunit splice variant surface expression, or regulation conferred by assembly with other S-acylated components of the BK channel complex. Ultimately the major challenge will be to understand how S-acylation controls BK channel function at the systems and whole animal level and understanding how palmitoylation status may be controlled and/or dysregulated in disease. For example, several of the zDHHC enzymes implicated in BK channel control, such as zDHHCs 5, 9, and 17, are also implicated in a variety of disorders including endocrine dysfunction and neurological deficits. In contrast, the physiology of other zDHHC enzymes that control BK channel function, such as zDHHC23, is completely unexplored.

With the continued development of new tools to interrogate and manipulate S-acylation we are now at the foothills of being able to define and understand the role of S-acylation in the physiological function of BK channels as well as providing an opportunity to gain greater insights into the mechanisms and function of S-acylation itself.

ACKNOWLEDGMENTS

Work in the author's laboratory is generously supported by The Wellcome Trust and British Heart Foundation. I am grateful to all members of the Shipston lab who have contributed to work discussed in this review and to Charlie Donnelly for his contribution to **Figure 3**.

REFERENCES

Abrami, L., Kunz, B., Iacovache, I., and van der Goot, F. G. (2008). Palmitoylation and ubiquitination regulate exit of the Wnt signaling protein LRP6 from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5384–5389. doi: 10.1073/pnas.0710389105

Alioua, A., Li, M., Wu, Y., Stefani, E., and Toro, L. (2011). Unconventional myristoylation of large-conductance Ca2+-activated K+ channel (Slo1) via serine/threonine residues regulates channel surface expression. *Proc. Natl. Acad. Sci. U.S.A.* 108, 10744–10749. doi: 10.1073/pnas.1008863108

Bachovchin, D. A., Ji, T., Li, W., Simon, G. M., Blankman, J. L., Adibekian, A., et al. (2010). Superfamily-wide portrait of serine hydrolase inhibition achieved by library-versus-library screening. *Proc. Natl. Acad. Sci. U.S.A.* 107, 20941–20946. doi: 10.1073/pnas.1011663107

Baekkeskov, S., and Kanaani, J. (2009). Palmitoylation cycles and regulation of protein function. *Mol. Membr. Biol.* 26, 42–54. doi: 10.1080/09687680802680108

Berkefeld, H., Fakler, B., and Schulte, U. (2010). Ca2+-activated k+ channels: from protein complexes to function. *Physiol. Rev.* 90, 1437–1459. doi: 10.1152/physrev.00049.2009

Brenner, R., Chen, Q. H., Vilaythong, A., Toney, G. M., Noebels, J. L., and Aldrich, R. W. (2005). BK channel β4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat. Neurosci.* 8, 1752–1759. doi: 10.1038/nn1573

Brenner, R., Peréz, G. J., Bonev, A. D., Eckman, D. M., Kosek, J. C., Wiler, S. W., et al. (2000). Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature* 407, 870–876. doi: 10.1038/35038011

Charollais, J., and der Goot, V. F. G. (2009). Palmitoylation of membrane proteins. *Mol. Membr. Biol.* 26, 55–66. doi: 10.1080/09687680802620369

Chen, C., Xu, Y., Zhang, J., Zhu, J., Zhang, J., Hu, N., et al. (2013a). Altered Expression of nNOS/NIDD in the retina of a glaucoma model of DBA/2J mice and the intervention by nNOS inhibition. *J. Mol. Neurosci.* 51, 47–56. doi: 10.1007/s12031-012-9943-x

Chen, L., Bi, D., Tian, L., Mcclafferty, H., Steeb, F., Ruth, P., et al. (2013b). Palmitoylation of the β4-subunit regulates surface expression of large conductance calcium-activated potassium channel splice variants. *J. Biol. Chem.* 288, 13136–13144. doi: 10.1074/jbc.M113.461830

Chiu, Y.-H., Alvarez-Baron, C., Kim, E. Y., and Dryer, S. E. (2010). Dominant-negative regulation of cell surface expression by a pentapeptide motif at the extreme COOH terminus of an Slo1 calcium-activated potassium channel splice variant. *Mol. Pharmacol.* 77, 497–507. doi: 10.1124/mol.109.061929

Contreras, G. F., Castillo, K., Enrique, N., Carrasquel-Ursulaez, W., Castillo, J. P., Milesi, V., et al. (2013). A BK (Slo1) channel journey from molecule to physiology. *Channels* 7, 442–458. doi: 10.4161/chan.26242

Cui, J. (2010). BK-type calcium-activated potassium channels: coupling of metal ions and voltage sensing. *J. Physiol.* 588, 4651–4658. doi: 10.1113/jphysiol.2010.194514

Deng, P.-Y., Rotman, Z., Blundon, J. A., Cho, Y., Cui, J., Cavalli, V., et al. (2013). FMRP regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels. *Neuron* 77, 696–711. doi: 10.1016/j.neuron.2012.12.018

Drisdel, R. C., and Green, W. N. (2004). Labeling and quantifying sites of protein palmitoylation. *Biotechniques* 36, 276–285. doi: 10.2144/3602A0276

Du, W., Bautista, J. F., Yang, H., Diez-Sampedro, A., You, S.-A., Wang, L., et al. (2005). Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat. Genet.* 37, 733–738. doi: 10.1038/ng1585

Fodor, A. A., and Aldrich, R. W. (2009). Convergent evolution of alternative splices at domain boundaries of the BK channel. *Annu. Rev. Physiol.* 71, 19–36. doi: 10.1146/annurev.physiol.010908.163124

Forrester, M. T., Foster, M. W., Benhar, M., and Stamler, J. S. (2009). Detection of protein S-nitrosylation with the biotin-switch technique. *Free Radic. Biol. Med.* 46, 119–126. doi: 10.1016/j.freeradbiomed.2008.09.034

Fukata, M., Fukata, Y., Adesnik, H., Nicoll, R. A., and Bredt, D. S. (2004). Identification of PSD-95 palmitoylating enzymes. *Neuron* 44, 987–996. doi: 10.1016/j.neuron.2004.12.005

Fukata, Y., and Fukata, M. (2010). Protein palmitoylation in neuronal development and synaptic plasticity. *Nat. Rev. Neurosci.* 11, 161–175. doi: 10.1038/nrn2788

Greaves, J., and Chamberlain, L. H. (2011). DHHC palmitoyl transferases: substrate interactions and (patho)physiology. *Trends Biochem. Sci.* 36, 245–253. doi: 10.1016/j.tibs.2011.01.003

Hannoush, R. N., and Sun, J. (2010). The chemical toolbox for monitoring protein fatty acylation and prenylation. *Nat. Chem. Biol.* 6, 498–506. doi: 10.1038/nchembio.388

Hess, D. T., Patterson, S. I., Smith, D. S., and Skene, J. H. (1993). Neuronal growth cone collapse and inhibition of protein fatty acylation by nitric oxide. *Nature* 366, 562–565. doi: 10.1038/366562a0

Ho, G. P. H., Selvakumar, B., Mukai, J., Hester, L. D., Wang, Y., Gogos, J. A., et al. (2011). S-Nitrosylation and S-Palmitoylation reciprocally regulate synaptic targeting of PSD-95. *Neuron* 71, 131–141. doi: 10.1016/j.neuron.2011.05.033

Hou, S., Heinemann, S. H., and Hoshi, T. (2009). Modulation of BKCa channel gating by endogenous signaling molecules. *Physiology* 24, 26–35. doi: 10.1152/physiol.00032.2008

Jeffries, O., Geiger, N., Rowe, I. C. M., Tian, L., McClafferty, H., Chen, L., et al. (2010). Palmitoylation of the S0-S1 linker regulates cell surface expression of voltage- and calcium-activated potassium (BK) channels. *J. Biol. Chem.* 285, 33307–33314. doi: 10.1074/jbc.M110.153940

Jeffries, O., Tian, L., McClafferty, H., and Shipston, M. J. (2012). An electrostatic switch controls palmitoylation of the large conductance voltage- and calcium-activated potassium (BK) channel. *J. Biol. Chem.* 287, 1468–1477. doi: 10.1074/jbc.M111.224840

Jiao, H., Arner, P., Hoffstedt, J., Brodin, D., Dubern, B., Czernichow, S., et al. (2011). Genome wide association study identifies KCNMA1 contributing to human obesity. *BMC Med. Genomics* 4:51. doi: 10.1186/1755-8794-4-51

Kang, R., Wan, J., Arstikaitis, P., Takahashi, H., Huang, K., Bailey, A. O., et al. (2008). Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. *Nature* 456, 904–909. doi: 10.1038/nature07605

Kathiresan, T., Harvey, M., Orchard, S., Sakai, Y., and Sokolowski, B. (2009). A protein interaction network for the large conductance Ca(2+)-activated K(+) channel in the mouse cochlea. *Mol. Cell. Proteomics* 8, 1972–1987. doi: 10.1074/mcp.M800495-MCP200

Kim, E. Y., Ridgway, L. D., Zou, S., Chiu, Y.-H., and Dryer, S. E. (2007). Alternatively spliced C-terminal domains regulate the surface expression of large conductance calcium-activated potassium channels. *Neuroscience* 146, 1652–1661. doi: 10.1016/j.neuroscience.2007.03.038

Kim, S., Lee, B.-C., Lee, A.-R., Won, S., and Park, C.-S. (2014). Effects of palmitoylation on the diffusional movement of BKCa channels in live cells. *FEBS Lett.* 588, 713–719. doi: 10.1016/j.febslet.2014.01.014

Linder, M. E., and Deschenes, R. J. (2007). Palmitoylation: policing protein stability and traffic. *Nat. Rev. Mol. Cell Biol.* 8, 74–84. doi: 10.1038/nrm2084

Liu, J., Asuncion-Chin, M., Liu, P., and Dopico, A. M. (2006). CaM kinase II phosphorylation of slo Thr107 regulates activity and ethanol responses of BK channels. *Nat. Neurosci.* 9, 41–49. doi: 10.1038/nn1602

Lu, R., Alioua, A., Kumar, Y., Eghbali, M., Stefani, E., and Toro, L. (2006). MaxiK channel partners: physiological impact. *J. Physiol.* 570, 65–72. doi: 10.1113/jphysiol.2005.098913

Martin, B. R., Wang, C., Adibekian, A., Tully, S. E., and Cravatt, B. F. (2012). Global profiling of dynamic protein palmitoylation. *Nat. Methods* 9, 84–89. doi: 10.1038/nmeth.1769

McCartney, C. E., McClafferty, H., Huibant, J.-M., Rowan, E. G., Shipston, M. J., and Rowe, I. C. M. (2005). A cysteine-rich motif confers hypoxia sensitivity to mammalian large conductance voltage- and Ca-activated K (BK) channel alpha-subunits. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17870–17876. doi: 10.1073/pnas.0505270102

Meera, P., Wallner, M., and Toro, L. (2000). A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca2+-activated K+ channel resistant to charybdotoxin and iberiotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5562–5567. doi: 10.1073/pnas.100118597

Meredith, A. L., Thorneloe, K. S., Werner, M. E., Nelson, M. T., and Aldrich, R. W. (2004). Overactive bladder and incontinence in the absence of the BK large conductance Ca2+-activated K+ channel. *J. Biol. Chem.* 279, 36746–36752. doi: 10.1074/jbc.M405621200

Nyholm, T. K. M., Ozdirekcan, S., and Killian, J. A. (2007). How protein transmembrane segments sense the lipid environment. *Biochemistry* 46, 1457–1465. doi: 10.1021/bi061941c

Orio, P., Rojas, P., Ferreira, G., and Latorre, R. (2002). New disguises for an old channel: MaxiK channel beta-subunits. *News Physiol. Sci.* 17, 156–161. doi: 10.1152/nips.01387.2002

Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y., and Yao, X. (2008). CSS-Palm 2.0: an updated software for palmitoylation sites prediction. *Protein Eng. Des. Sel.* 21, 639–644. doi: 10.1093/protein/gzn039

Resh, M. D. (2012). Targeting protein lipidation in disease. *Trends Mol. Med.* 18, 206–214. doi: 10.1016/j.molmed.2012.01.007

Salkoff, L., Butler, A., Ferreira, G., Santi, C., and Wei, A. (2006). High-conductance potassium channels of the SLO family. *Nat. Rev. Neurosci.* 7, 921–931. doi: 10.1038/nrn1992

Sausbier, M., Arntz, C., Bucurenciu, I., Zhao, H., Zhou, X.-B., Sausbier, U., et al. (2005). Elevated blood pressure linked to primary hyperaldosteronism and impaired vasodilation in BK channel-deficient mice. *Circulation* 112, 60–68. doi: 10.1161/01.CIR.0000156448.74296.FE

Sausbier, M., Hu, H., Arntz, C., Feil, S., Kamm, S., Adelsberger, H., et al. (2004). Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+-activated K+ channel deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9474–9478. doi: 10.1073/pnas.0401702101

Schubert, R., and Nelson, M. T. (2001). Protein kinases: tuners of the BKCa channel in smooth muscle. *Trends Pharmacol. Sci.* 22, 505–512. doi: 10.1016/S0165-6147(00)01775-2

Sen, N., and Snyder, S. H. (2010). Protein modifications involved in neurotransmitter and gasotransmitter signaling. *Trends Neurosci.* 33, 493–502. doi: 10.1016/j.tins.2010.07.004

Shi, P., Li, D., Lai, C., Zhang, L., and Tian, C. (2013). Intracellular segment between transmembrane helices S0 and S1 of BK channel α subunit contains two amphipathic helices connected by a flexible loop. *Biochem. Biophys. Res. Comm.* 437, 408–412. doi: 10.1016/j.bbrc.2013.06.091

Shipston, M. J. (2011). Ion channel regulation by protein palmitoylation. *J. Biol. Chem.* 286, 8709–8716. doi: 10.1074/jbc.R110.210005

Shipston, M. J. (2013). Regulation of large conductance calcium- and voltage-activated potassium (BK) channels by S-palmitoylation. *Biochem. Soc. Trans.* 41, 67–71. doi: 10.1042/BST20120226

Shipston, M. J. (2014). Ion channel regulation by protein S-acylation. *J. Gen. Physiol.* 143, 659–678. doi: 10.1085/jgp.201411176

Shruti, S., Urban-Ciecko, J., Fitzpatrick, J. A., Brenner, R., Bruchez, M. P., and Barth, A. L. (2012). The brain-specific Beta4 subunit downregulates BK channel cell surface expression. *PLoS ONE* 7:e33429. doi: 10.1371/journal.pone.0033429

Tian, L., Coghill, L. S., McClafferty, H., MacDonald, S. H.-F., Antoni, F. A., Ruth, P., et al. (2004). Distinct stoichiometry of BKCa channel tetramer phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11897–11902. doi: 10.1073/pnas.0402590101

Tian, L., Duncan, R. R., Hammond, M. S., Coghill, L. S., Wen, H., Rusinova, R., et al. (2001). Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J. Biol. Chem.* 276, 7717–7720. doi: 10.1074/jbc.C000741200

Tian, L., Jeffries, O., McClafferty, H., Molyvdas, A., Rowe, I. C. M., Saleem, F., et al. (2008). Palmitoylation gates phosphorylation-dependent regulation of BK potassium channels. *Proc. Natl. Acad. Sci. U.S.A.* 105, 21006–21011. doi: 10.1073/pnas.0806700106

Tian, L., McClafferty, H., Jeffries, O., and Shipston, M. J. (2010). Multiple palmitoyltransferases are required for palmitoylation-dependent regulation of large conductance calcium- and voltage-activated potassium channels. *J. Biol. Chem.* 285, 23954–23962. doi: 10.1074/jbc.M110.137802

Tian, L., McClafferty, H., Knaus, H.-G., Ruth, P., and Shipston, M. J. (2012). Distinct acyl protein transferases and thioesterases control surface expression of calcium-activated potassium channels. *J. Biol. Chem.* 287, 14718–14725. doi: 10.1074/jbc.M111.335547

Toro, L., Li, M., Zhang, Z., Singh, H., Wu, Y., and Stefani, E. (2013). MaxiK channel and cell signalling. *Pflugers Arch*. 466, 875–886. doi: 10.1007/s00424-013-1359-0

Wang, B., Rothberg, B. S., and Brenner, R. (2006). Mechanism of beta4 subunit modulation of BK channels. *J. Gen. Physiol.* 127, 449–465. doi: 10.1085/jgp.200509436

Werner, M. E., Zvara, P., Meredith, A. L., Aldrich, R. W., and Nelson, M. T. (2005). Erectile dysfunction in mice lacking the large-conductance calcium-activated potassium (BK) channel. *J. Physiol. (Lond.)* 567, 545–556. doi: 10.1113/jphysiol.2005.093823

Wu, R. S., Chudasama, N., Zakharov, S. I., Doshi, D., Motoike, H., Liu, G., et al. (2009). Location of the beta 4 transmembrane helices in the BK potassium channel. *J. Neurosci.* 29, 8321–8328. doi: 10.1523/JNEUROSCI.6191-08.2009

Xie, J., and McCobb, D. P. (1998). Control of alternative splicing of potassium channels by stress hormones. *Science* 280, 443–446.

Yan, J., and Aldrich, R. W. (2012). BK potassium channel modulation by leucine-rich repeat-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7917–7922. doi: 10.1073/pnas.1205435109

Yang, H., Hu, L., Shi, J., Delaloye, K., Horrigan, F. T., and Cui, J. (2007). Mg2+ mediates interaction between the voltage sensor and cytosolic domain to activate BK channels. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18270–18275. doi: 10.1073/pnas.0705873104

Zeidman, R., Jackson, C. S., and Magee, A. I. (2009). Protein acyl thioesterases (Review). *Mol. Membr. Biol.* 26, 32–41. doi: 10.1080/09687680802629329

Zhou, X., Wulfsen, I., Korth, M., McClafferty, H., Lukowski, R., Shipston, M. J., et al. (2012). Palmitoylation and membrane association of the stress axis regulated insert (STREX) controls BK channel regulation by protein kinase C. *J. Biol. Chem.* 287, 32161–32171. doi: 10.1074/jbc.M112.386359

Zhou, X.-B. (2001). A molecular switch for specific stimulation of the BKCa channel by cGMP and cAMP kinase. *J. Biol. Chem.* 276, 43239–43245. doi: 10.1074/jbc.M104202200

Zhou, X.-B., Wulfsen, I., Utku, E., Sausbier, U., Sausbier, M., Wieland, T., et al. (2010). Dual role of protein kinase C on BK channel regulation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8005–8010. doi: 10.1073/pnas.0912029107

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 June 2014; accepted: 09 July 2014; published online: 05 August 2014.
*Citation: Shipston MJ (2014) S-acylation dependent post-translational cross-talk regulates large conductance calcium- and voltage- activated potassium (BK) channels. Front. Physiol. **5**:281. doi: 10.3389/fphys.2014.00281*
This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 15 October 2014
doi: 10.3389/fphys.2014.00401

Regulation of BK channels by auxiliary γ subunits

Jiyuan Zhang and Jiusheng Yan*

Department of Anesthesiology and Perioperative Medicine, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Edited by:
Anton Hermann, University of Salzburg, Austria

Reviewed by:
Jonathan H. Jaggar, University of Tennessee Health Science Center, USA
Christopher J. Lingle, Washington University in St. Louis School of Medicine, USA
Antonios Pantazis, University of California, Los Angeles, USA

***Correspondence:**
Jiusheng Yan, Department of Anesthesiology and Perioperative Medicine, University of Texas MD Anderson Cancer Center, Unit 110, 1515 Holcombe Blvd, Houston TX 77030, USA
e-mail: jyan1@mdanderson.org

The large-conductance, calcium- and voltage-activated potassium (BK) channel has the largest single-channel conductance among potassium channels and can be activated by both membrane depolarization and increases in intracellular calcium concentration. BK channels consist of pore-forming, voltage- and calcium-sensing α subunits, either alone or in association with regulatory subunits. BK channels are widely expressed in various tissues and cells including both excitable and non-excitable cells and display diverse biophysical and pharmacological characteristics. This diversity can be explained in part by posttranslational modifications and alternative splicing of the α subunit, which is encoded by a single gene, *KCNMA1*, as well as by tissue-specific β subunit modulation. Recently, a leucine-rich repeat-containing membrane protein, LRRC26, was found to interact with BK channels and cause an unprecedented large negative shift (∼-140 mV) in the voltage dependence of the BK channel activation. LRRC26 allows BK channels to open even at near-physiological calcium concentration and membrane voltage in non-excitable cells. Three LRRC26-related proteins, LRRC52, LRRC55, and LRRC38, were subsequently identified as BK channel modulators. These LRRC proteins are structurally and functionally distinct from the BK channel β subunits and were designated as γ subunits. The discovery of the γ subunits adds a new dimension to BK channel regulation and improves our understanding of the physiological functions of BK channels in various tissues and cell types. Unlike BK channel β subunits, which have been intensively investigated both mechanistically and physiologically, our understanding of the γ subunits is very limited at this stage. This article reviews the structure, modulatory mechanisms, physiological relevance, and potential therapeutic implications of γ subunits as they are currently understood.

Keywords: BK channels, KCNMA1, Slo1, $K_{Ca1.1}$, auxiliary subunit, accessory protein, regulation, modulation

DISCOVERY OF γ SUBUNITS

Among the numerous K^+ channels, the large-conductance Ca^{2+}- and voltage-activated K^+ channel (termed BK, Slo1, $K_{Ca1.1}$, or KCNMA1; hereafter BK) is considered unique, characterized by its large single-channel conductance and dual activation by membrane depolarization and elevation in intracellular free calcium ($[Ca^{2+}]_i$) (Marty, 1981; Latorre and Miller, 1983; Golowasch et al., 1986; Latorre et al., 1989). The structure, function, and regulatory mechanisms of BK channels have been investigated over the past 3 decades. BK channels are considered channel complexes composed of either homotetramers of the pore-forming and calcium- and voltage-sensing α subunit (BKα) alone or BKα together with tissue-specific auxiliary subunits. BKα is structurally distinct from most other K^+ channels because it possesses an extra *N*-terminal transmembrane segment (S0) (Wallner et al., 1996; Meera et al., 1997) and a large Ca^{2+}-sensing cytosolic *C*-terminus composed of two RCK (regulating conductance of K^+) domains (Wu et al., 2010; Yuan et al., 2012) (**Figure 1**). BKα is also distinct from most other voltage-gated K^+ (Kv) channels in the pore-forming transmembrane S1–S6 domains by a very low amino acid sequence similarity with them.

BK channels occur in many different tissues and cells and display diverse biophysical or pharmacological characteristics. This diversity can be explained in part by posttranslational modifications and alternative splicing of the large BKα subunit (∼130 kDa), which is encoded by a single gene, *KCNMA1*, as well as by tissue-specific β subunit modulation. Four different β subunits (β1–β4) have been cloned and identified in mammals. The first β subunit was identified as a binding partner of BKα in the BK channel complexes purified from bovine tracheal smooth muscle by extensive conventional chromatography together with sucrose gradient centrifugation or by immunoprecipitation (Knaus et al., 1994a,b,c). In these early experiments, charybdotoxin (ChTx), which is a specific peptide blocker of BK channels, was radiolabeled as a tool for BK channel complex or protein detection and ChTx was found to be attached to a β subunit upon cross-linking. This smooth muscle–specific auxiliary protein was later named the β1 subunit, and the other 3 family members were discovered thereafter (Wallner et al., 1999; Xia et al., 1999; Behrens et al., 2000; Brenner et al., 2000; Meera et al., 2000; Uebele et al., 2000).

Each β subunit has 2 transmembrane segments connected by a large loop on the extracellular side (**Figure 1**). The 4β subunits

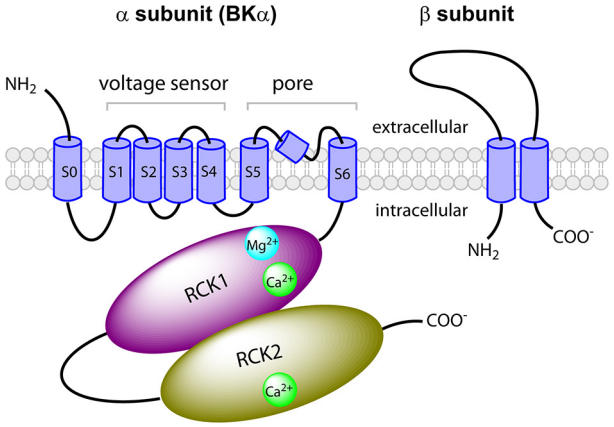


FIGURE 1 | Schematic structure and membrane topology of BK channel α and β subunits.

display different and complex effects on apparent calcium and voltage sensitivities, macroscopic current kinetics, and pharmacological sensitivities, which involve multiple distinct mechanisms. For example, the β1 and β2 subunits overall induce slowing of the macroscopic kinetics and an increase in apparent calcium and voltage sensitivity (Behrens et al., 2000; Brenner et al., 2000; Savalli et al., 2007; Contreras et al., 2012). The β2 and some splice variants of β3 subunits also cause rapid inactivation through their intracellular *N*-termini (Wallner et al., 1999; Xia et al., 1999, 2000; Uebele et al., 2000). The β3 subunits generate rectifying outward currents regulated by their extracellular loops (Xia et al., 2000; Zeng et al., 2003). The brain-specific β4 subunit, in addition to greatly slowing activation and deactivation kinetics, reduces apparent calcium sensitivity in low $[Ca^{2+}]_i$ conditions but increases apparent sensitivity in high $[Ca^{2+}]_i$ conditions (Behrens et al., 2000; Brenner et al., 2000).

The wide distribution of BKα suggests that BK channels have a potentially important function in various physiological processes (Nelson et al., 1995; Hu et al., 2001; Gu et al., 2007). The activation mechanisms and functions of BK channels in excitable neuronal and smooth muscle cells have been intensively studied and are relatively well understood in principle, although more remains to be examined, such as channel heterogeneity caused by different auxiliary subunit composition in different cells. Compared with other voltage-gated or Ca^{2+}-activated K^+ channels, BK channels have much higher thresholds for channel activation by either voltage or $[Ca^{2+}]_i$ alone; these thresholds are generally outside of physiological ranges. Thus, in excitable cells, activation of BK channels typically requires coincident membrane depolarization and elevation in $[Ca^{2+}]_i$ levels (Brenner et al., 2000; Salkoff et al., 2006), and BK channels are physically coupled to voltage-gated Ca^{2+} channels in order to sense locally enriched Ca^{2+} (Berkefeld et al., 2006; Fakler and Adelman, 2008). However, non-excitable cells generally have a relatively constant low resting membrane potential and a marginal amount of $[Ca^{2+}]_i$. BK channel function and mechanism of activation has been largely unexplored in non-excitable cells.

In 2005, an unusal type of K^+ current was reported in lymph node carcinoma of the prostate (LNCaP) cells, which showed a Kv-like low half-activation voltage ($V_{1/2}$) of ∼30 mV in the absence of $[Ca^{2+}]_i$ but had many characteristics of BK chanenls (Gessner et al., 2005). These BK-like features included large single-channel conductance (220 pS at 140 mM symmetric K^+), activation by $[Ca^{2+}]_i$ in the μM range and $[Mg^{2+}]_i$ in the mM range, and sensitivity to specific activator NS1619 and blockers ChTx, IbTx, paxilline, and penitrem A (Gessner et al., 2005). The voltage dependence of channel activation for this endogenous BK-like channel in LNCaP cells was shifted to the hyperpolarization direction by more than 120 mV compared with human BKα channels expressed in HEK cells. The researchers therefore concluded that they had observed a special BK channel or at least a BK-like channel in LNCaP cells, which they designated BK_L. However, it remained unknown how the channel's voltage dependence was unprecedentedly shifted to such a great extent, and this could not be readily explained by any previously known modulatory mechanisms, such as alternative splicing, phosphorylation, or β subunits.

Five years later, it was demonstrated that the LNCaP cells did express BKα at the protein level, as detected by the BKα antibody, but the BKα existed in a normal splicing form, according to reverse transcriptase PCR and sequencing of mRNA (Yan and Aldrich, 2010). The authors then took a proteomic approach to immunopurify the channel complex and used mass spectrometry to identify potential novel interacting partners that may drastically modify the BK channel's gating property (Yan and Aldrich, 2010). A 35-kDa leucine-rich repeat-containing protein, LRRC26, was specifically identified in the BKα pull-down components. Knockdown of this protein in LNCaP cells resulted in a complete loss of the BK channel's property of being activated at low voltage in the absence of calcium. Meanwhile, overexpression of LRRC26 in another prostate cancer cell line, PC3, which lacks endogenous LRRC26 expression, converted the endogenous typical BKα channels to the low-voltage–activated LNCaP-type BK channels. It was additionally shown in a heterologous expression system (HEK-293 cells) that LRRC26 was specifically associated with BKα as detected by reciprocal co-immunoprecipitation and shifted the conductance-voltage (G-V) relationship of BK channels to the hyperpolarization direction by ∼140 mV, as was seen in the LNCaP cells. This suggests that direct channel complex formation likely occurs without mediation by other proteins.

LRRC26 is structurally and functionally distinct from the 4β subunits and thus was considered a new type of BK channel auxiliary subunit. Later on, 3 other structurally related leucine-rich repeat-containing proteins, LRRC52, LRRC55, and LRRC38, were reported to be also able to modulate BK channels when co-expressed heterologously with BKα in HEK-293 cells (Yan and Aldrich, 2012) (**Figure 2**). These proteins have also been shown to produce marked shifts in the voltage dependence of BK channel activation in the hyperpolarization direction, although the shifts are smaller than those produced by LRRC26. LRRC52 causes a shift of approximately 100 mV, LRRC55 causes a shift of 50 mV, and LRRC38 causes a shift of 20 mV in the absence of $[Ca^{2+}]_i$ (**Figure 2B**). The ion channel auxiliary subunits, such as the BK or Kv channel β subunits, may exist in multiple paralogous forms

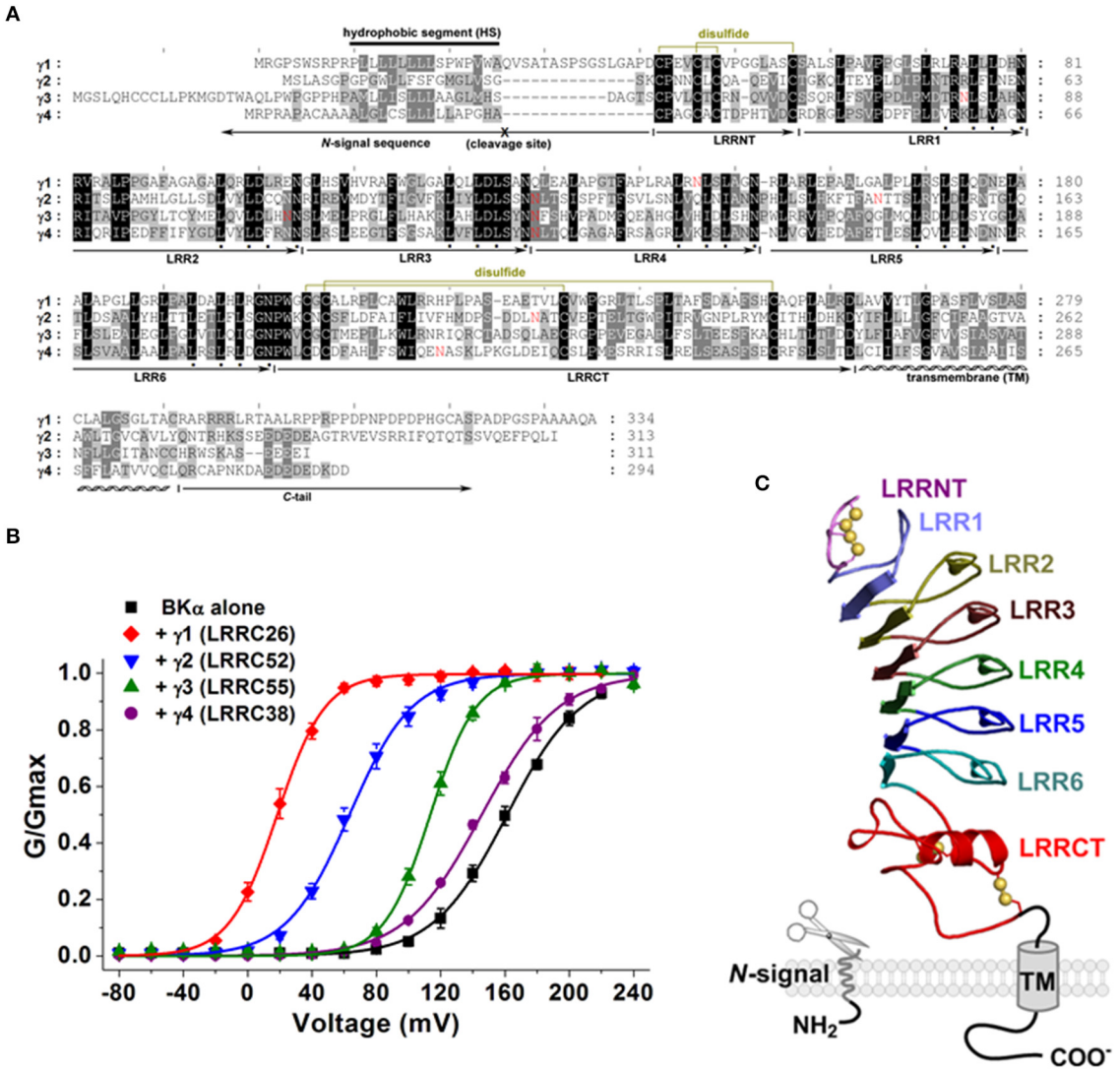


FIGURE 2 | Structure and function of BK channel γ subunits. (A) Protein sequence alignment of BK channel γ subunits in humans. The hydrophobic segments and potential cleavage sites in the *N*-terminal signal peptide sequences are indicated. Potential *N*-glycosylation sites of Asn residues are shown in red. Cysteine pairs (total of 4) for potential disulfide formation are also shown. Key residues of the consensus sequence (LxxLxLxxN) in each leucine-rich repeat unit are marked by a filled square at the bottom. **(B)** Modulatory effects of γ subunits on the voltage dependence of BK channel activation in the absence of intracellular calcium, upon heterologous expression of the γ subunit in HEK-293 cells. **(C)** Predicted leucine-rich repeat domain structure and membrane topology of the γ subunit.

with related modulatory functions. These three LRRC proteins, together with LRRC26, have been thus designated as the γ family of the BK channel auxiliary subunits (Yan and Aldrich, 2012). LRRC26 was named γ1 while LRRC52, LRRC55, and LRRC38 were tentatively named γ2, γ3, and γ4, respectively, according to their capabilities to modify the voltage dependence of BK channel activation. Similar to BK β subunits, these proteins also displayed different tissue-specific expression at the mRNA level (Yan and Aldrich, 2012).

STRUCTURAL CHARACTERISTICS

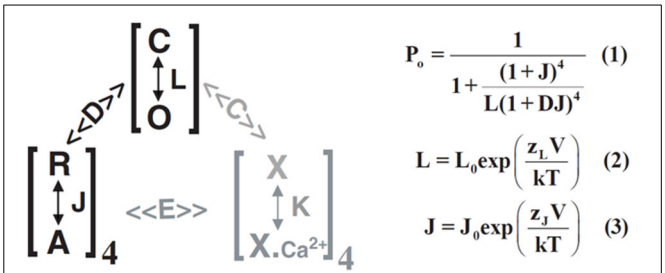
The 4γ subunits have similar molecular weights, around 35 kDa. They are type I single-span membrane proteins containing a classic *N*-terminal cleavable signal peptide for extracellular localization of the *N*-terminal LRR domain in the mature proteins. The signal peptide region was found to be absent in the mature protein. Mutations in this region caused the signal peptide to be retained in the expressed protein and led to a loss of modulatory function in the γ1 subunit, suggesting that proper maturation guided by the signal peptide region is critical for the function of γ subunits (Yan and Aldrich, 2012). The mature proteins of the 4γ subunits all contain a single transmembrane domain, an *N*-terminal extracellular LRRD, and a short *C*-terminal tail. The 4γ subunits share an overall sequence similarity of 35–40% (**Figure 1A**) which are comparable with that among the 4β subunits.

The atomic structure of γ subunits is not yet known. However, a comparison of the amino acid sequences in the LRR domains of γ subunits with those in other LRR-containing proteins provided a good prediction of the structure of γ subunits. The LRR domains of γ subunits all contain 6 LRR units and 2 cysteine-rich regions, a small one called LRRNT, capped on the *N*-terminal side, and a large one called LRRCT, capped on the *C*-terminal side (**Figure 2A**). As in many other LRR-containing proteins, each LRR unit in the γ subunits consists of 24 residues and has a classic consensus sequence of LxxLxLxxN (where x can be any amino acid). Because no structure has been described for an LRR domain containing both LRRNT and LRRCT elements that are similar to those in the γ subunits, structures of lymphocyte receptor B (Kim et al., 2007a) for the LRRNT and LRR regions and structures of mouse TLR4 (Kim et al., 2007b) for the LRRCT region have been referred to in structural modeling (Yan and Aldrich, 2010, 2012). The resultant structural model for the γ subunit LRR domain can be depicted as in **Figure 2C**. According to this structural model, the LRR domain is a banana-shaped structure with a curved parallel β-sheet lining the inner circumference and small helices or turns flanking the convex circumference, formed by 6 LRR units stacked together in the middle. Each LRR unit forms a β-strand lining the concave face and a short α-helix connected by loops flanking the outer circumference. The hydrophobic core of the LRR domain is tightly packed by the parallel inward-pointing leucine residues, shielded by the LRRCT and LRRNT caps on the *N*- and *C*-terminal ends. Both LRRNT and LRRCT contain 2 pairs of fully conserved cysteine residues that in total potentially form 4 disulfide linkages in the favorable oxidizing extracellular environment. Consistent with their predicted extracellular location, the LRR domains of the γ subunits all contain single or multiple consensus N-glycosylation sites: Asn-Xaa-Ser/Thr, where Xaa is not a proline. For the γ1 subunit, N147Q mutation and enzymatic removal of the N-linked glycan by PNGase F resulted in disappearance of an upper glycosylated-mass band in SDS-PAGE (Yan and Aldrich, 2012).

The protein sequences in the LRR domains of γ subunits are closely related but become divergent in the transmembrane and intracellular *C*-terminal tail regions (**Figure 1A**). The single-transmembrane segments of the γ subunits are well predicted from their hydrophobicity and the presence of charged residues on both sides, particularly multiple positively charged residues on the intracellular side following the general "positive-inside rule" for membrane insertion and orientation of membrane proteins. For the *C*-terminal tail regions, in addition to the cluster of positively charged residues adjacent to the transmembrane domain, it is interesting to note that the rest of the amino acid sequence is polyproline (11 proline residues out of 36 residues) for γ1 and polyacidic for γ2, γ3, and γ4.

MODULATORY MECHANISMS

To date, the structures of the whole γ subunit, as well as the whole BKα channel, have not yet been described. The detailed mechanisms underlying how the γ subunits bind to the BKα tetramer and regulate channel function remain largely unexplored and unknown. The gating shift produced by γ1 subunit is equivalent to the effect of ~10 μM $[Ca^{2+}]_i$ on BK channels formed by BKα alone. However, Ca^{2+} and Mg^{2+} sensitivities were shown to be largely unaffected by the γ1 subunit (Yan and Aldrich, 2010). Therefore, the mechanistic actions of the γ1 subunit were investigated and analyzed within the framework of the well-established BK channel allosteric model of voltage-dependent gating in the absence of Ca^{2+}. According to this HA model (Horrigan and Aldrich, 2002), the activation or open probability (P_o) of BK channels by voltage can be simply calculated or described by 5 gating parameters: L_0 and J_0 together with Z_L and Z_J were referred to as equilibrium constants and associated gating charges for the channel pore's closed ↔ open and voltage sensors' resting ↔ activated transitions and *D* was considered the allosteric coupling factors between the pore and the voltage sensors (Horrigan and Aldrich, 2002) (**Figure 3**). Using simulations, and by measuring the kinetics and open probabilities of the channels at very negative voltages to achieve good estimates of the Z_L and L_0 parameters, it was found that the γ1 subunit's modulatory effect can be best explained by a ~20-fold increase in the allosteric coupling *D* factor, whereas the pore's gating parameters L_0 and Z_L are largely unaffected by the γ1 subunit (Yan and Aldrich, 2010). According to this study, the γ1 subunit may mainly affect the coupling between voltage sensors and the pore. However, the possibility cannot be ruled out that the γ1 subunit also slightly affects Z_J, J_0 or L_0, owing to limitations in the accuracy of experimental data obtained at very negative voltages and the assumption used in simulations that only one gating parameter is affected. The γ1 subunit may also act on some other aspect of BK channel gating, such as the two distinct calcium binding events on RCK1 and RCK2 domains (Xia et al., 2002) and the interactions among the two calcium binding sites and the voltage sensor (Qian et al., 2006; Sweet and Cox, 2008; Savalli et al., 2012), which were not addressed by HA model (Horrigan and Aldrich, 2002) and also excluded for consideration in the modeling analyses of the γ1 modulatory effect (Yan and Aldrich, 2010). Regardless of these limitations, γ subunits may serve as good tools to study BK gating mechanisms because little is currently known about the molecular basis underlying allosteric coupling between voltage sensors and the pore in this voltage- and ligand-gated channel.



$$P_o = \frac{1}{1 + \frac{(1+J)^4}{L(1+DJ)^4}} \quad (1)$$

$$L = L_0 \exp\left(\frac{z_L V}{kT}\right) \quad (2)$$

$$J = J_0 \exp\left(\frac{z_J V}{kT}\right) \quad (3)$$

FIGURE 3 | Schematic and equations of the HA allosteric gating model for BK channel activation by voltage. The two processes of voltage-sensor activation (equilibrium constant J) and channel opening (equilibrium constant L) are linked by the allosteric coupling factors D. The calcium binding (equilibrium constant K) and the related allosteric coupling factors C, E are shown in gray. P_o in the equation (1) means channel open probability.

The action of the γ1 subunit is remarkable in its modulatory magnitude and mechanistic simplicity. A recent study indicated that the regulatory mechanism of the γ1 subunit may be fundamentally different from that of the β subunit (Gonzalez-Perez et al., 2014). In contrast to β subunits, which have the ability to regulate the voltage dependence of BK channel activation in the titration-dependent mode, the γ1 subunit exhibited an "all-or-none" regulatory pattern (Gonzalez-Perez et al., 2014). In a classic model, it is understood that auxiliary subunits bind to the BKα subunit one by one with 4-fold symmetry so that the regulatory effect is incremental (Wang et al., 2002). However, the γ1 subunit caused the voltage dependence of channel activation to be either shifted to a full extent or unchanged independent of the molar ratio of the injected BKα:γ1 RNA to *Xenopus oocytes*, although the ratio of these two populations of channels varied (Gonzalez-Perez et al., 2014). It is unknown whether one γ1 subunit per channel complex is sufficient to fully modulate BK channels. Alternatively, the γ1 subunit may preferably exist in a tetrameric form when forming a complex with BKα. Additional studies will be required to determine the detailed mechanisms, particularly the stoichiometry and the interaction sites between the BKα and γ subunits in the tetrameric channel complex.

Not surprisingly, the γ1 subunit can inhibit the effect of some BK activators (Almassy and Begenisich, 2012). The endogenous γ1 subunit was present in native salivary gland parotid acinar cells; it blocked the activating effect of mallotoxin (MTX) but not NS-1619 (Almassy and Begenisich, 2012). Similar blocking effect of the γ1 subunit on MTX action was also observed in HEK-293 cells when the γ1 subunit was heterologously co-expressed with BKα. It was proposed that MTX may displace the γ1 subunit instead of lacking accessibility to the binding site (Almassy and Begenisich, 2012). Further biophysical studies and biochemical binding assays will be needed to clarify the detailed mechanisms. MTX and γ1 are likely exclusively and sterically related in their binding to the BK channels. NS1619 was recently shown to bind to the S6/RCK linker region (Gessner et al., 2012), but little is currently known about the MTX binding site. Identification of the MTX binding site may complement our understanding of the actions of γ subunits on BK channels.

PHYSIOLOGIC RELEVANCE

The tissue-specific distribution patterns of the 4γ subunits at the mRNA level had been investigated using TaqMan quantitative PCR in various human tissues (Yan and Aldrich, 2012). The γ1 subunit was highly expressed in the salivary glands, prostate, and trachea, whereas γ2 (LRRC52) was found predominantly in the testes and γ3 (LRRC55) was found primarily in the nervous system. The γ4 (LRRC38) subunit was mainly observed in skeletal muscle, adrenal glands, and the thymus. These results suggest that like β subunits, γ subunits have different tissue-specific distributions to fit the diverse functional requirements of various tissues and cell types (Yan and Aldrich, 2012). The γ1 subunit's endogenous functional regulation of BK channels has been confirmed in prostate and salivary gland cells (Yan and Aldrich, 2010; Almassy and Begenisich, 2012). The physiological roles of the γ1 subunit in prostate and salivary glands remain to be determined. Conceivably, constitutive activation of BK channels might be required for K^+ flow-mediated fluid secretion in these non-excitable tissues. A very recent study implies that the γ1 subunit in airway epithelial cells may participate in the BK channel-mediated airway hydration for effective mucociliary clearance (Manzanares et al., 2014). The K^+ flow through the apically expressed BK channels in airway epithelial cells provides an electrochemical driving gradient for Cl^- secretion and thus plays a role in airway hydration. It was found that both the mRNA level of the γ1 subunit and the sensitivity of BK channels to mallotoxin were decreased after IFN-γ treatment, implying that the γ1 subunit might be involved in the IFN-γ-mediated reduction in BK channel activity and the resulting mucociliary dysfunction (Manzanares et al., 2014). The γ1 subunit under a different name (CAPC) was reported to be able to suppress tumor growth and metastasis, which may likely involve ion channel–independent function (Liu et al., 2012). It will be necessary to determine how the association of the γ1 subunit with BK channels may affect tumor growth because of the enhanced K^+ channel activity that generally promotes cancer cell proliferation (Pardo and Stuhmer, 2014).

Because of the drastic activating effect caused by the γ1 subunit, expression of this protein even at low levels might exert significant effect on BK channel currents. For example, a low mRNA expression level of the γ1 subunit has been detected in aorta cells (Yan and Aldrich, 2012). A very recent study reported that knockdown of γ1 subunit expression in rat cerebral artery myocytes led to a reduction in the apparent voltage /Ca^{2+} sensitivity and current frequency and amplitude of BK channels, as well as a decrease in the extents of BK channel-specific inhibitor-induced vasoconstriction and activator-induced vasodilation (Evanson et al., 2014). This study suggests that the γ1 subunit may play broad physiological roles that are not limited to non-excitable cells. In excitable cells, the voltage and Ca^{2+} sensitivities of the BK channels are more finely tuned to be properly responsive to different levels of voltage and Ca^{2+} in different cell types, and therefore low expression of this potent BK channel modulator might exert a significant physiological effect. It is worth noting that the γ1 subunit is also expressed in fetal brain tissue (Yan and Aldrich, 2012), and the γ1 subunit might participate in maintaining proper neuronal excitability in the fetal nervous system during early development.

PERSPECTIVES

Our understanding of BK channel γ subunits is still in its infant stage. In particular, very little is known about the physiologic functions and the structural basis underlying the regulatory mechanisms of γ subunits. The discovery of γ subunits adds a new dimension to BK channel regulation and provides a molecular basis for a better understanding of the physiological functions of BK channels in different tissues or cell types. The few published studies examining the modulatory mechanisms and physiological functions of BK channel γ subunits have mainly focused on γ1, the most potent γ subunit. The regulation of BK channels by γ2-4 subunits has so far been demonstrated only in the heterologous expression system. It will be important to determine whether γ2-4 subunits also play any functional or physiological role in BK channel modulation *in vivo*.

The γ2 subunit is also potent in shifting the voltage dependence of BK channel activation. The presence of the γ2 subunit

specifically in the testes among the examined mouse and human tissues suggests that it plays a special role in spermatogenesis or male fertility (Yang et al., 2011; Yan and Aldrich, 2012). A detailed study suggested that the mouse γ2 subunit functions as an accessory subunit of the sperm-specific mouse Slo3 channels (Yang et al., 2011). Co-expression of Slo3 and γ2 in *Xenopus* oocytes generated pH and voltage-dependent currents that are more similar to native KSper than those of the Slo3 channel alone. Moreover, Slo3 deletion in mouse testes and sperm either significantly decreased or eliminated the expression of γ2. In rats, BK channel-like currents and immunostaining of BKα was found to be high in premeiotic germ cells, spermatozoa and primary spermatocytes, but very low in postmeiotic germ cells (Gong et al., 2002). It will be intriguing to determine whether the γ2 subunit also modulates BK channels and whether Slo3 and BK channels can form functional heterotetrameric channels in any stage of germ cells. BK channels and Slo3 belong to the Slo channel family, which also includes 2 more distantly related Na^+-activated channels, Slo2.1 (slick) and Slo2.2 (slack). There is currently no report on the effect of γ subunits on the Slo2.1 or Slo2.2 channels. It remains an open question whether the γ subunits may broadly function as auxiliary proteins of the Slo channel family.

Effective BK channel openers have been sought or explored to treat a variety of diseases such as stroke, epilepsy, psychoses, bladder overactivity, erectile dysfunction, asthma, arterial hypertension, ischemic heart disease, and gastric hypermotility (Nardi and Olesen, 2008). Although the widely used BK channel opener NS1619 can give ∼-40-mV shift in $V_{1/2}$ at a high concentration (30 μM) (Gessner et al., 2012), its specificity was recently questioned because of its direct inhibiting effect on the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Wrzosek, 2014). The γ1 subunit, which is so far the most potent BK channel activator, and the other γ subunits of different potencies provide molecular tools to manipulate BK channel activity *in vivo* through either transgenic or viral delivery of gene expression. This may offer the opportunity to evaluate the therapeutic potential of BK channel activators (openers) of different channel-activating potencies in the treatment of various diseases. Currently, no BK channel–targeted drug has been approved for clinical use in spite of extensive academic and pharmaceutical efforts over the past two decades. Deciphering the biochemical mechanisms underlying BK channel activation by γ subunits will be very useful for the development of new BK channel–targeted drugs.

ACKNOWLEDGMENT

This work is supported by NIH grants NS075118 (Jiusheng Yan) and NS078152 (Jiusheng Yan).

REFERENCES

Almassy, J., and Begenisich, T. (2012). The LRRC26 protein selectively alters the efficacy of BK channel activators. *Mol. Pharmacol.* 81, 21–30. doi: 10.1124/mol.111.075234

Behrens, R., Nolting, A., Reimann, F., Schwarz, M., Waldschutz, R., and Pongs, O. (2000). hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family. *FEBS Lett.* 474, 99–106. doi: 10.1016/S0014-5793(00)01584-2

Berkefeld, H., Sailer, C. A., Bildl, W., Rohde, V., Thumfart, J. O., Eble, S., et al. (2006). BK_{Ca}-Cav channel complexes mediate rapid and localized Ca^{2+}-activated K^+ signaling. *Science* 314, 615–620. doi: 10.1126/science.1132915

Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y., and Aldrich, R. W. (2000). Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. *J. Biol. Chem.* 275, 6453–6461. doi: 10.1074/jbc.275.9.6453

Contreras, G. F., Neely, A., Alvarez, O., Gonzalez, C., and Latorre, R. (2012). Modulation of BK channel voltage gating by different auxiliary beta subunits. *Proc. Natl. Acad. Sci. U.S.A.* 109, 18991–18996. doi: 10.1073/pnas.1216953109

Evanson, K. W., Bannister, J. P., Leo, M. D., and Jaggar, J. H. (2014). LRRC26 is a functional BK channel auxiliary gamma Subunit in arterial smooth muscle cells. *Circ. Res.* 115, 423–431. doi: 10.1161/CIRCRESAHA.115.303407

Fakler, B., and Adelman, J. P. (2008). Control of K(Ca) channels by calcium nano/microdomains. *Neuron* 59, 873–881. doi: 10.1016/j.neuron.2008.09.001

Gessner, G., Cui, Y. M., Otani, Y., Ohwada, T., Soom, M., Hoshi, T., et al. (2012). Molecular mechanism of pharmacological activation of BK channels. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3552–3557. doi: 10.1073/pnas.1114321109

Gessner, G., Schonherr, K., Soom, M., Hansel, A., Asim, M., Baniahmad, A., et al. (2005). BK_{Ca} channels activating at resting potential without calcium in LNCaP prostate cancer cells. *J. Membr. Biol.* 208, 229–240. doi: 10.1007/s00232-005-0830-z

Golowasch, J., Kirkwood, A., and Miller, C. (1986). Allosteric effects of Mg^{2+} on the gating of Ca2+-activated K+ channels from mammalian skeletal muscle. *J. Exp. Biol.* 124, 5–13.

Gong, X. D., Li, J. C., Leung, G. P., Cheung, K. H., and Wong, P. Y. (2002). A BK_{Ca} to K_v switch during spermatogenesis in the rat seminiferous tubules. *Biol. Reprod.* 67, 46–54. doi: 10.1095/biolreprod67.1.46

Gonzalez-Perez, V., Xia, X. M., and Lingle, C. J. (2014). Functional regulation of BK potassium channels by gamma1 auxiliary subunits. *Proc. Natl. Acad. Sci. U.S.A.* 111, 4868–4873. doi: 10.1073/pnas.1322123111

Gu, N., Vervaeke, K., and Storm, J. F. (2007). BK potassium channels facilitate high-frequency firing and cause early spike frequency adaptation in rat CA1 hippocampal pyramidal cells. *J. Physiol.* 580, 859–882. doi: 10.1113/jphysiol.2006.126367

Horrigan, F. T., and Aldrich, R. W. (2002). Coupling between voltage sensor activation, Ca^{2+} binding and channel opening in large conductance (BK) potassium channels. *J. Gen. Physiol.* 120, 267–305. doi: 10.1085/jgp.20028605

Hu, H., Shao, L. R., Chavoshy, S., Gu, N., Trieb, M., Behrens, R., et al. (2001). Presynaptic Ca^{2+}-activated K^+ channels in glutamatergic hippocampal terminals and their role in spike repolarization and regulation of transmitter release. *J. Neurosci.* 21, 9585–9597.

Kim, H. M., Oh, S. C., Lim, K. J., Kasamatsu, J., Heo, J. Y., Park, B. S., et al. (2007a). Structural diversity of the hagfish variable lymphocyte receptors. *J. Biol. Chem.* 282, 6726–6732. doi: 10.1074/jbc.M608471200

Kim, H. M., Park, B. S., Kim, J. I., Kim, S. E., Lee, J., Oh, S. C., et al. (2007b). Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. *Cell* 130, 906–917. doi: 10.1016/j.cell.2007.08.002

Knaus, H. G., Eberhart, A., Kaczorowski, G. J., and Garcia, M. L. (1994a). Covalent attachment of charybdotoxin to the beta-subunit of the high conductance Ca^{2+}-activated K^+ channel. Identification of the site of incorporation and implications for channel topology. *J. Biol. Chem.* 269, 23336–23341.

Knaus, H. G., Folander, K., Garcia-Calvo, M., Garcia, M. L., Kaczorowski, G. J., Smith, M., et al. (1994b). Primary sequence and immunological characterization of beta-subunit of high conductance Ca^{2+}-activated K+ channel from smooth muscle. *J. Biol. Chem.* 269, 17274–17278.

Knaus, H. G., Garcia-Calvo, M., Kaczorowski, G. J., and Garcia, M. L. (1994c). Subunit composition of the high conductance calcium-activated potassium channel from smooth muscle, a representative of the mSlo and slowpoke family of potassium channels. *J. Biol. Chem.* 269, 3921–3924.

Latorre, R., and Miller, C. (1983). Conduction and selectivity in potassium channels. *J. Membr. Biol.* 71, 11–30. doi: 10.1007/BF01870671

Latorre, R., Oberhauser, A., Labarca, P., and Alvarez, O. (1989). Varieties of calcium-activated potassium channels. *Annu. Rev. Physiol.* 51, 385–399. doi: 10.1146/annurev.ph.51.030189.002125

Liu, X. F., Xiang, L., Zhang, Y., Becker, K. G., Bera, T. K., and Pastan, I. (2012). CAPC negatively regulates NF-kappaB activation and suppresses tumor growth and metastasis. *Oncogene* 31, 1673–1682. doi: 10.1038/onc.2011.355

Manzanares, D., Srinivasan, M., Salathe, S. T., Ivonnet, P., Baumlin, N., Dennis, J. S., et al. (2014). IFN-gamma-mediated reduction of large-conductance, Ca^{2+}-activated, voltage-dependent K^+ (BK) channel activity in airway epithelial cells

leads to mucociliary dysfunction. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 306, L453–462. doi: 10.1152/ajplung.00247.2013

Marty, A. (1981). Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature* 291, 497–500. doi: 10.1038/291497a0

Meera, P., Wallner, M., Song, M., and Toro, L. (1997). Large conductance voltage- and calcium-dependent K^+ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0-S6), an extracellular N terminus, and an intracellular (S9-S10) C terminus. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14066–14071. doi: 10.1073/pnas.94.25.14066

Meera, P., Wallner, M., and Toro, L. (2000). A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca^{2+}-activated K^+ channel resistant to charybdotoxin and iberiotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5562–5567. doi: 10.1073/pnas.100118597

Nardi, A., and Olesen, S. P. (2008). BK channel modulators: a comprehensive overview. *Curr. Med. Chem.* 15, 1126–1146. doi: 10.2174/092986708784221412

Nelson, M. T., Cheng, H., Rubart, M., Santana, L. F., Bonev, A. D., Knot, H. J., et al. (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science* 270, 633–637. doi: 10.1126/science.270.5236.633

Pardo, L. A., and Stuhmer, W. (2014). The roles of K^+ channels in cancer. *Nat. Rev. Cancer* 14, 39–48. doi: 10.1038/nrc3635

Qian, X., Niu, X., and Magleby, K. L. (2006). Intra- and intersubunit cooperativity in activation of BK channels by Ca^{2+}. *J. Gen. Physiol.* 128, 389–404. doi: 10.1085/jgp.200609486

Salkoff, L., Butler, A., Ferreira, G., Santi, C., and Wei, A. (2006). High-conductance potassium channels of the SLO family. *Nat. Rev. Neurosci.* 7, 921–931. doi: 10.1038/nrn1992

Savalli, N., Kondratiev, A., De Quintana, S. B., Toro, L., and Olcese, R. (2007). Modes of operation of the BK_{Ca} channel beta2 subunit. *J. Gen. Physiol.* 130, 117–131. doi: 10.1085/jgp.200709803

Savalli, N., Pantazis, A., Yusifov, T., Sigg, D., and Olcese, R. (2012). The contribution of RCK domains to human BK channel allosteric activation. *J. Biol. Chem.* 287, 21741–21750. doi: 10.1074/jbc.M112.346171

Sweet, T. B., and Cox, D. H. (2008). Measurements of the BK_{Ca} channel's high-affinity Ca^{2+} binding constants: effects of membrane voltage. *J. Gen. Physiol.* 132, 491–505. doi: 10.1085/jgp.200810094

Uebele, V. N., Lagrutta, A., Wade, T., Figueroa, D. J., Liu, Y., McKenna, E., et al. (2000). Cloning and functional expression of two families of beta-subunits of the large conductance calcium-activated K^+ channel. *J. Biol. Chem.* 275, 23211–23218. doi: 10.1074/jbc.M910187199

Wallner, M., Meera, P., and Toro, L. (1996). Determinant for beta-subunit regulation in high-conductance voltage-activated and Ca^{2+}-sensitive K^+ channels: an additional transmembrane region at the N terminus. *Proc. Natl. Acad. Sci. U.S.A.* 93, 14922–14927. doi: 10.1073/pnas.93.25.14922

Wallner, M., Meera, P., and Toro, L. (1999). Molecular basis of fast inactivation in voltage and Ca^{2+}-activated K^+ channels: a transmembrane beta-subunit homolog. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4137–4142. doi: 10.1073/pnas.96.7.4137

Wang, Y. W., Ding, J. P., Xia, X. M., and Lingle, C. J. (2002). Consequences of the stoichiometry of Slo1 alpha and auxiliary beta subunits on functional properties of large-conductance Ca^{2+}-activated K^+ channels. *J. Neurosci.* 22, 1550–1561.

Wrzosek, A. (2014). The potassium channel opener NS1619 modulates calcium homeostasis in muscle cells by inhibiting SERCA. *Cell Calcium* 56, 14–24. doi: 10.1016/j.ceca.2014.03.005

Wu, Y., Yang, Y., Ye, S., and Jiang, Y. (2010). Structure of the gating ring from the human large-conductance Ca^{2+}-gated K^+ channel. *Nature* 466, 393–397. doi: 10.1038/nature09252

Xia, X. M., Ding, J. P., and Lingle, C. J. (1999). Molecular basis for the inactivation of Ca^{2+}- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. *J. Neurosci.* 19, 5255–5264.

Xia, X. M., Ding, J. P., Zeng, X. H., Duan, K. L., and Lingle, C. J. (2000). Rectification and rapid activation at low Ca2+ of Ca2+-activated, voltage-dependent BK currents: consequences of rapid inactivation by a novel beta subunit. *J. Neurosci.* 20, 4890–4903.

Xia, X. M., Zeng, X., and Lingle, C. J. (2002). Multiple regulatory sites in large-conductance calcium-activated potassium channels. *Nature* 418, 880–884. doi: 10.1038/nature00956

Yan, J., and Aldrich, R. W. (2010). LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. *Nature* 466, 513–516. doi: 10.1038/nature09162

Yan, J., and Aldrich, R. W. (2012). BK potassium channel modulation by leucine-rich repeat-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7917–7922. doi: 10.1073/pnas.1205435109

Yang, C., Zeng, X. H., Zhou, Y., Xia, X. M., and Lingle, C. J. (2011). LRRC52 (leucine-rich-repeat-containing protein 52), a testis-specific auxiliary subunit of the alkalization-activated Slo3 channel. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19419–19424. doi: 10.1073/pnas.1111104108

Yuan, P., Leonetti, M. D., Hsiung, Y., and Mackinnon, R. (2012). Open structure of the Ca^{2+} gating ring in the high-conductance Ca^{2+}-activated K^+ channel. *Nature* 481, 94–97. doi: 10.1038/nature10670

Zeng, X. H., Xia, X. M., and Lingle, C. J. (2003). Redox-sensitive extracellular gates formed by auxiliary beta subunits of calcium-activated potassium channels. *Nat. Struct. Biol.* 10, 448–454. doi: 10.1038/nsb932

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 July 2014; accepted: 27 September 2014; published online: 15 October 2014.

*Citation: Zhang J and Yan J (2014) Regulation of BK channels by auxiliary γ subunits. Front. Physiol. **5**:401. doi: 10.3389/fphys.2014.00401*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

REVIEW ARTICLE
published: 10 October 2014
doi: 10.3389/fphys.2014.00383

Pharmacological consequences of the coexpression of BK channel α and auxiliary β subunits

Yolima P. Torres[1]*, Sara T. Granados[1,2] and Ramón Latorre[2]*

[1] Departamento de Nutrición y Bioquímica, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá, Colombia
[2] Facultad de Ciencias, Centro Interdisciplinario de Neurociencia de Valparaíso, Universidad de Valparaíso, Valparaíso, Chile

Edited by:
Alex M. Dopico, The University of Tennessee Health Science Center, USA

Reviewed by:
Jianmin Cui, Washington University, USA
Jonathan Lippiat, University of Leeds, UK

***Correspondence:**
Yolima P. Torres, Departamento de Nutrición y Bioquímica, Facultad de Ciencias, Pontificia Universidad Javeriana, Carrera 7 No. 40–62, Bogotá, Colombia
e-mail: yolima.torres@javeriana.edu.co;
Ramón Latorre, Facultad de Ciencias, Centro Interdisciplinario de Neurociencia de Valparaíso, Universidad de Valparaíso, Pasaje Harrington 287 Playa Ancha, Valparaíso 2366103, Chile
e-mail: ramón.latorre@uv.cl

Coded by a single gene (*Slo1*, KCM) and activated by depolarizing potentials and by a rise in intracellular Ca^{2+} concentration, the large conductance voltage- and Ca^{2+}-activated K^+ channel (BK) is unique among the superfamily of K^+ channels. BK channels are tetramers characterized by a pore-forming α subunit containing seven transmembrane segments (instead of the six found in voltage-dependent K^+ channels) and a large C terminus composed of two regulators of K^+ conductance domains (RCK domains), where the Ca^{2+}-binding sites reside. BK channels can be associated with accessory β subunits and, although different BK modulatory mechanisms have been described, greater interest has recently been placed on the role that the β subunits may play in the modulation of BK channel gating due to its physiological importance. Four β subunits have currently been identified (i.e., β1, β2, β3, and β4) and despite the fact that they all share the same topology, it has been shown that every β subunit has a specific tissue distribution and that they modify channel kinetics as well as their pharmacological properties and the apparent Ca^{2+} sensitivity of the α subunit in different ways. Additionally, different studies have shown that natural, endogenous, and synthetic compounds can modulate BK channels through β subunits. Considering the importance of these channels in different pathological conditions, such as hypertension and neurological disorders, this review focuses on the mechanisms by which these compounds modulate the biophysical properties of BK channels through the regulation of β subunits, as well as their potential therapeutic uses for diseases such as those mentioned above.

Keywords: BK channel, *Slo1*, KCNMB, BK β subunits, BK pharmacology, auxiliary subunits

INTRODUCTION

Consisting of 5 families and more than 70 different encoding genes in mammals, the diversity of K^+ channels is amazingly large (for a comprehensive review on K^+ channels, see González et al., 2012). In particular, the Ca^{2+}- and voltage-activated K^+ (BK) channel is a relative of the 6-transmembrane domain voltage-dependent K^+ (Kv) channel family, which is also part of the S4 superfamily encompassing voltage-dependent Na^+ and Ca^{2+} channels. There are, however, several differences between Kv and BK channels that make BK channels unique. First, BK channels are encoded by a single gene (*Slo1*). Second, they contain seven transmembrane domains and, hence, the N-terminus is in contact with the cell external milieu (Meera et al., 1997) (**Figure 1**). Third, the BK channel can be independently activated by Ca^{2+} or voltage, and it can open in the absence of Ca^{2+}. Moreover, it is clear at present that voltage and Ca^{2+} sensors are allosterically coupled to channel opening (as reviewed in Latorre et al., 2010; Horrigan, 2012). In other words, voltage or Ca^{2+} alone can open the channel, but the free energy required to open the channel greatly decreases when both sensors are activated. Finally, unlike Kv channels, where most voltage-sensing charges are contained in the S4 transmembrane segment, only about 50% of the gating charges for BK channels are in S4 and the rest of the voltage-sensing particles reside in S3 and S2 (Ma et al., 2006). Accordingly, the BK voltage sensor can be defined as a decentralized voltage sensor.

The BK channel in mammals is ubiquitously distributed in different tissues and, because it is activated by voltage and Ca^{2+}, it is the perfect molecular machine to reduce or stop excitatory stimuli. For example, the BK channel modulates neurotransmitter release by co-localizing with voltage-dependent Ca^{2+} channels (Robitaille and Charlton, 1992). In vascular smooth muscle cells, BK channels regulate contractile tone. In this case, increments in local Ca^{2+} (i.e., Ca^{2+} sparks) produce BK channel-mediated spontaneous transient outward currents (STOCs), thus hyperpolarizing the membrane and producing muscle relaxation (Jaggar et al., 2000; Ledoux et al., 2006). On the other hand, alterations in BK channels are known to be important in the pathophysiology of hypertension, asthma, diabetes, and epilepsy (as reviewed in Contreras et al., 2013). Because of the profound involvement of BK channels in the health problems described above, their activators, and blockers have potential therapeutic implications. In several cases, the action of these compounds is mediated by their binding to the β subunit or modulated by the presence of these subunits. Hence, the main goal of the present review is to provide an overview of our current knowledge of how such mediation and modulation are accomplished.

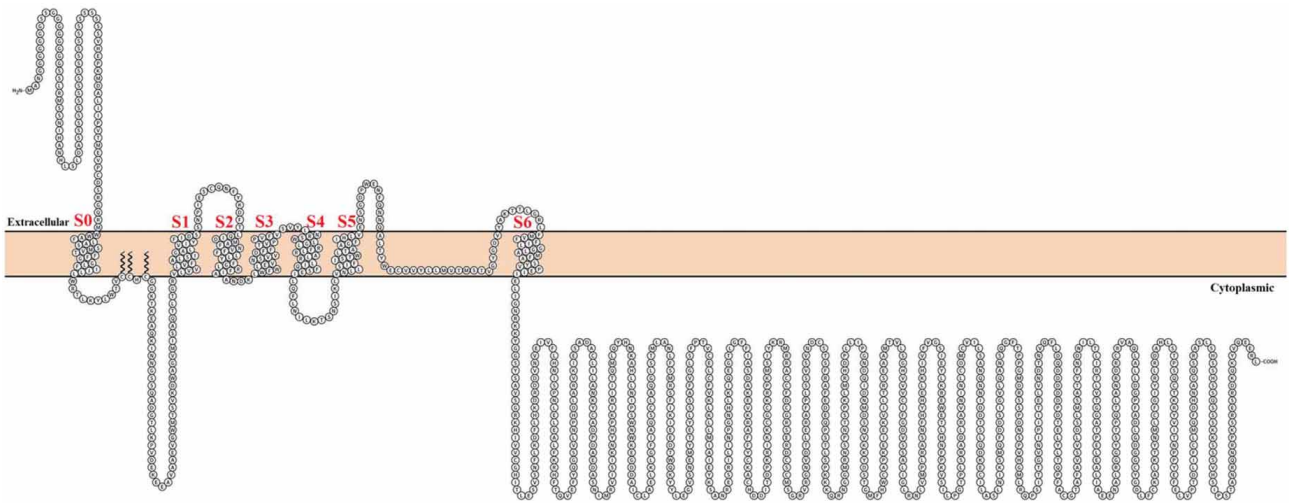


FIGURE 1 | Topological representation of the human Calcium-activated potassium channel α subunit. Using Protter visualizator (Omasits et al., 2014), UniProt protein accession: Q12791.

BK CHANNEL β SUBUNITS

In most tissues, the BK channel is associated with accessory β subunits. To date, four β subunits (β1–β4, encoded by the genes KCNMB1–4) have been identified (**Figure 2**) and their expression depends on cell type (as reviewed in Contreras et al., 2013) (**Table 1**). All β subunits share the same predicted structural characteristics (170–200 residues in length) and they are composed of two transmembrane segments (i.e., TM1 and TM2), intracellular N and C termini and a large extracellular loop (as reviewed in Orio et al., 2002; Torres et al., 2007; Hoshi et al., 2013b). The first β subunit to be identified was β1 in tracheal smooth muscle cells (**Figure 3A**) (Garcia-Calvo et al., 1994; Knaus et al., 1994). In heterologous systems such as *Xenopus oocytes* and cell cultures, the co-expression of β1 with the α subunit leads to an increase in the apparent Ca^{2+} sensitivity of the channel, which slows down activation and deactivation kinetics and causes a lefward shift in G-V relations by −70 to −80 mV at 5–10 μM intracellular Ca^{2+} concentrations (McManus et al., 1995; Wallner et al., 1995; Meera et al., 1996; Tanaka et al., 1997; Brenner et al., 2000a; Cox and Aldrich, 2000; Nimigean and Maglebly, 2000; Bao and Cox, 2005; Orio and Latorre, 2005).

Functional coupling between α and β1 subunits is determined by the S0 transmembrane segment of the BK channel. Cysteine crosslinking experiments have indicated that β1 lies between and can interact with the voltage sensors of two adjacent α subunits, and that TM2 lies in the proximity of S0 (Wallner et al., 1996; Liu et al., 2010). Similar to the β1 subunit, β2 also increases apparent BK channel Ca^{2+} sensitivity and alters its gating kinetics, but the existence of 31 more amino acid residues in the N terminal has shown to promote an inactivation process (**Figure 3B**) (Wallner et al., 1999; Brenner et al., 2000a; Xia et al., 2003; Orio and Latorre, 2005). Although it behaves as an open channel block (Wallner et al., 1999), this N-type inactivation does not promote charge inmobilization, as in the N-type inactivation observed in Shaker K^+ channels (Savalli et al., 2007). It is important to mention that although the β1 subunit can exist in a 1:1 stoichiometry with α subunits, there is a report suggesting that the variability seen in BK inactivation behavior in rat chromaffin cells can originate from a less than a 1:1 assembly of β2 and α subunits (Knaus et al., 1994; Ding et al., 1998; Wang et al., 2002).

The β3 subunit is less similar to the β1 and β2 subunits (**Figure 3C**) and confers distinct modulating characteristics to the α subunit (Riazi et al., 1999; Xia et al., 2000). There are four different β3 subunits (β3a–d), which all originate from alternative splicing of the same gene (i.e., KCNMB3) (Brenner et al., 2000b; Uebele et al., 2000). It was reported that TM1 is closest to αS1 and S2 and TM2 is closest to αS0 (Wu et al., 2013). The expression of β3a, b, and c causes a partial inactivation of potassium currents. Althoug β3a and c induce a similar inactivation process as the β2 subunit, β3b-dependent inactivation is faster. The β3d subunit does not causes inactivation of potassium currents (Brenner et al., 2000b; Uebele et al., 2000).

Similar to β1, TM1 from β4 subunit is nearest to αS1 and S2 meanwhile β4 TM2 is closest to S0 (Wu et al., 2009). This subunit its expressed in the brain and is considered to be a downregulator of BK channels by dramatically decelerating BK channel activation gating kinetics (**Figure 3D**) (Weiger et al., 2000). However, although β4 shifts conductance-voltage curves to the right along the voltage axis at Ca^{2+} concentrations lower that 1 μM, it increases apparent Ca^{2+} sensitivity at Ca^{2+} concentrations larger than 1 μM (Brenner et al., 2000b; Ha et al., 2004; Wang et al., 2006). Additionally, the β4 subunit reduces the voltage dependence of the conductance–voltage relationship as well as the slope of the gating charge-voltage curve (Wang et al., 2006; Contreras et al., 2012).

THE ROLE OF β SUBUNITS IN BK CHANNEL PHARMACOLOGY

BK channels can be modulated by diverse molecules that may induce either an increase or decrease in channel activity. The

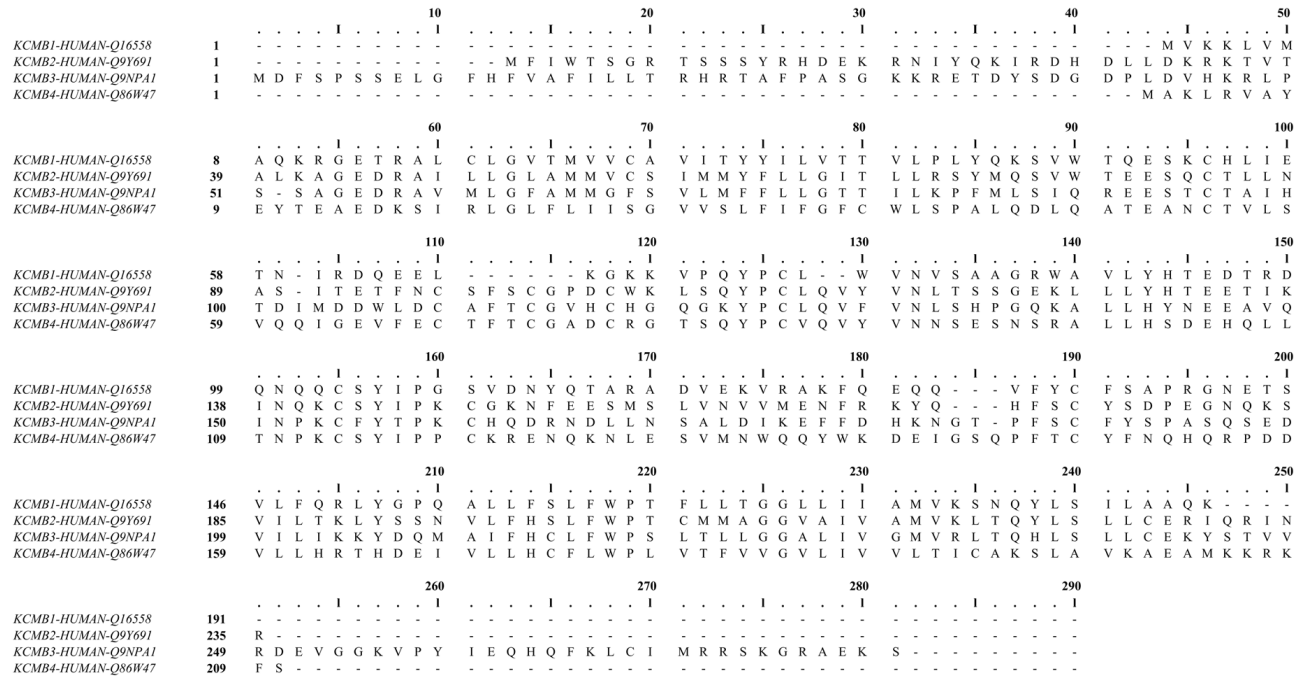


FIGURE 2 | Human calcium-activated potassium channel β subunit alignment. Using MUSCLE software (Edgar, 2004). UniProt protein accession: KCMB1, Q16558; KCMB2, Q9Y691; KCMB3, Q9NPA1; KCMB4, Q86W47.

Table 1 | Tissue expression of the BK channel accessory β subunits.

Subunit	Chromosome location (human)	Gene symbol (human)	UniProt protein accession number (human)	Tissue expression
β1	5q34	KCNMB1	Q16558	Smooth muscle (Knaus et al., 1994), kidney, urinary bladder (Wu and Marx, 2010), brain (Tseng-Crank et al., 1996), myometrium (Wallner et al., 1996)
β2	3q26.32	KCNMB2	Q9Y691	Pancreas (Ohya et al., 2010), kidney, (Uebele et al., 2000), chromafin cells (Xia et al., 1999), brain (Wallner et al., 1999)
β3a	3q26.3-q27	KCNMB3	Q9NPA1-2	Spleen, placenta, pancreas, heart, kidney (Behrens et al., 2000; Brenner et al., 2000b; Uebele et al., 2000; Xia et al., 2000)
β3b	3q26.3-q27	KCNMB3	Q9NPA1-4	Lung, liver, testes, spleen, placenta, pancreas, heart, kidney, brain (Behrens et al., 2000; Brenner et al., 2000b; Xia et al., 2000)
β3c	3q26.3-q27	KCNMB3	Q9NPA1-3	Ovary, brain, lung, liver, spleen, placenta, pancreas, prostate, kidney (Behrens et al., 2000; Brenner et al., 2000b; Xia et al., 2000)
β3d	3q26.3-q27	KCNMB3	Q9NPA1-1	Lung, brain, testes, spleen, placenta, pancreas, kidney (Behrens et al., 2000; Brenner et al., 2000b; Xia et al., 2000)
β4	12q	KCNMB4	Q86W47	Brain, neuronal tissue, Behrens et al., 2000; Brenner et al., 2000b kidney, bladder smooth muscle (Chen and Petkov, 2009)

effects of these molecules can be exerted through direct interactions with the α subunit and, in some cases, by the expression of β subunits, which would modify such effects. Nevertheless, some of these molecules need β subunits to modulate BK channel activity. The effects of various molecules that have been extensively studied as BK channel modulators and whose activity has been proposed to be related to β subunit expression will be described here (**Table 2**). Among the latter, toxins (such as charybdotoxin, iberiotoxin), ethanol, steroids, and fatty acids (like araquidonic acid and metabolites) are also mentioned. Molecules like tungstate, $DiBAC_4$(bis (1,3-dibutylbarbituric acid) trimethine oxonol), nitric oxide (NO) and tetrandrine, whose effects are determined by β subunits and have not been studied in detail will be also described.

TOXIN INTERACTION

BK channels have been described to be sensitive to scorpion-peptide toxins, such as charybdotoxin (ChTX), iberiotoxin

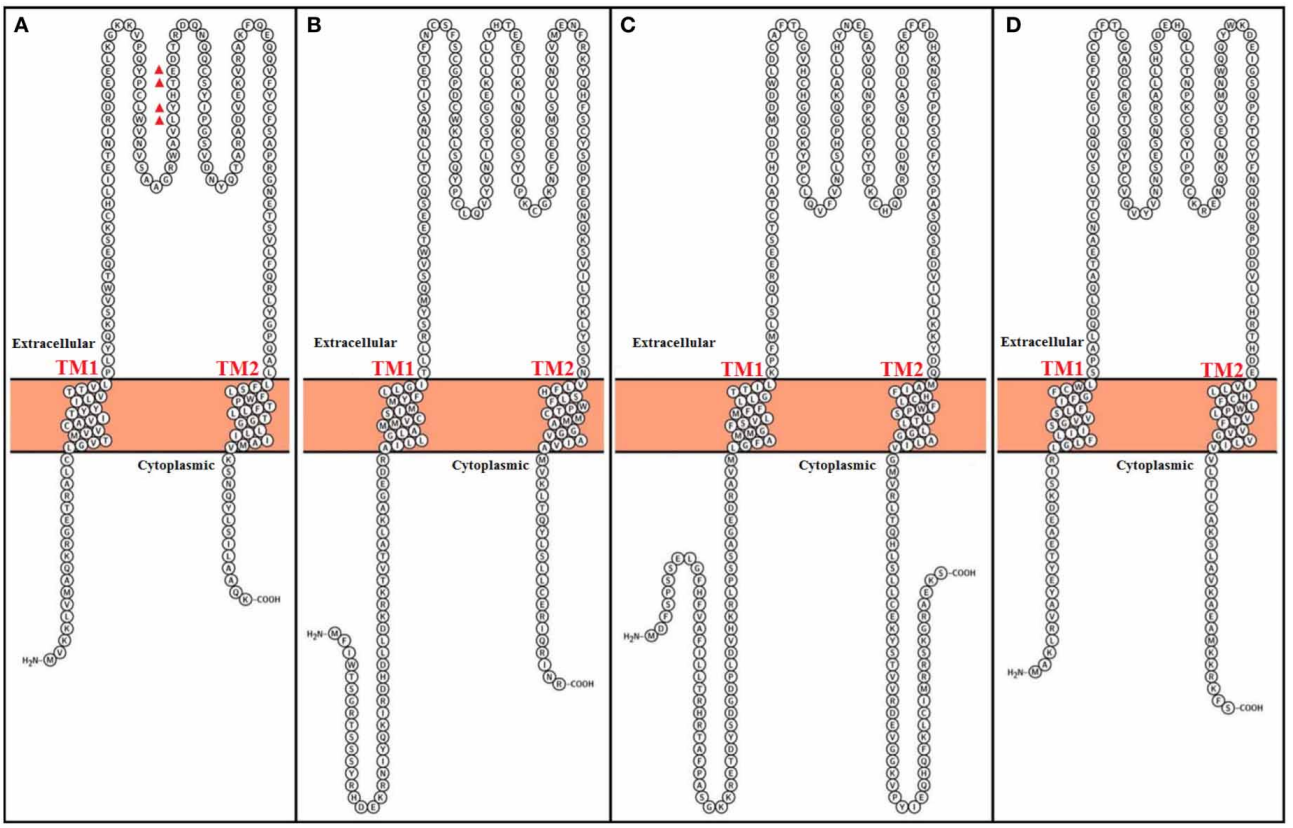


FIGURE 3 | Topological representation of human Calcium-activated potassium channel β subunits. Using Protter visualizator (Omasits et al., 2014). UniProt protein accession: **(A)** KCMB1: Q16558, **(B)** KCMB2: Q9Y691, **(C)** KCMB3: Q9NPA1, **(D)** KCMB4: Q86W47.

(IbTX), slotoxin (SloTX), and martentoxin (**Figure 4**). Toxins can affect channel properties with different affinities and specificities, and their binding has been shown to be modulated by the coexpression of β subunits. For example, while IbTX—which is a 37 amino acid peptide present in the venom of the African scorpion *Buthus tamulus* (Galvez et al., 1990)—is a specific BK channel blocker (Kaczorowski and Garcia, 1999), ChTX—which is a peptide with the same number of amino acid residues isolated from the scorpion *Liurus quinquestriatus*—blocks other potassium channels (Galvez et al., 1990; Kaczorowski and Garcia, 1999). Interestingly, both toxins reversibly block BK channels by externally binding to the mouth of the channel through a bimolecular reaction (Miller et al., 1985; Anderson et al., 1988; Candia et al., 1992; Giangiacomo et al., 1992). IbTX and ChTX blockade is characterized by long-lived (blocked) non-conducting states that separate bursts of activity. These long-lived blocked states define mean blocked times of 10 and 200 s for ChTX and IbTX, respectively. Under similar experimental conditions, the dissociation constant (Kd ∼1.5 nM) for IbTX is nearly 10-fold smaller than for ChTX (Candia et al., 1992; Giangiacomo et al., 1992; Gribkoff et al., 1996; Mullmann et al., 1999).

It has been shown that ChTX and IbTX toxins have different net charges that may affect selectivity by means of a mechanism involving electrostatic interactions with BK channels. Since the channel external vestibule has a fixed negative charge density region, a local electrostatic potential is set up near the toxin binding site, raising the local concentration of the positively-charged toxin (Anderson et al., 1988). Although ChTX and IbTX are equal in size and display a highly homologous sequence (68% homologous), ChTX has a net charge of +5 due to the presence of several basic residues, while the net charge for IbTX is +2, since it has 4 more acidic residues and 1 basic amino acid residue less than the ChTX (**Figure 5**). The latter would explain the different behaviors observed in their toxin-channel electrostatic interactions where the binding of ChTX to BK channel is more sensitive to ion strength variations that IbTX binding (Galvez et al., 1990; Candia et al., 1992; Giangiacomo et al., 1992). Additionally, it has been suggested that an electrostatic mechanism underlies the dissociation of ChTX from the channel (Anderson et al., 1988; MacKinnon and Miller, 1988). The apparent voltage dependence of the channel block is due to a destabilization of the channel-toxin complex, which is mediated by K^+ ions entering the selectivity filter from the internal side when the membrane is depolarized, hence inducing a repulsion force that unbounds the toxin from the channel (MacKinnon and Miller, 1988).

These two toxins plug the BK channel by means of direct interaction between toxin lysine 27 and the channel selectivity filter (Park and Miller, 1992; Mullmann et al., 1999). The above studies

Table 2 | Molecules that regulate BK channel activity through β subunits.

	Ligand	Subunit associated to a modulatory effect on the BK channel activity
Toxins	Charybdotoxin	β1, β2, β3, and β4
	Iberiotoxin	β1 and β4
	Slotoxin	β1 and β4
	Martentoxin	β4
	Vt 3.1	β4
Fatty acids	Arachidonic acid	β1, β2, and β3
	Docosahexaenoic	β1 and β4
	Eicosapentaenoic	β1
	Acil-CoA	β2
Alcohol	Ethanol	β1 and β4
Steroids	17β-Estradiol	β1
	Tamoxifen	β1
	Dehydroepiandrosterone	β2 and β4
	Lithocolic acid	β1
	Dehydrosoyasaponin-I	β1
Others	Tungstate	β1
	$DiBAC_4$	β1, β2, and β4

led to the hypothesis that lysine 27 seems to be in the proximity of one of the external K^+ binding sites. This hypothesis has recently been fully confirmed by Banerjee et al. (2013), who were able to solve the crystal structure of the chimeric channel Kv1.2/Kv2.1 in complex with ChTX. The structure of the Kv-ChTX complex shows that the toxin binds to the extracellular aspect of the selectivity filter and the most extracellular K^+ binding site is devoid of K^+ ions.

Coexpression of BK channel α and β subunits causes a dramatic effect on ChTX-BK interaction properties when compared to the α subunit alone. It has been suggested that extracellular loop from β subunit is involved in the formation of the toxin receptor (Hanner et al., 1997). The affinity of ChTX to α +β1 channels is 50-fold larger than that of channels formed by the α subunit alone. This increase in toxin binding potency is due to a seven-fold decrease in the toxin dissociation rate constant and an increase of about five-fold in the association rate constant (Hanner et al., 1997). To identify the β1 subunit amino acid residues involved in ChTX binding affinity changes, an alanine scanning mutagenesis along the β1 external loop was performed. The results indicated that the L90, Y91, W93, and E94 residues are critical for ChTX high-affinity binding to the α + β1 BK channel. Mutations to alanine of these residues do not change the physical interactions between β1 and α subunits. However, they affect ChTX association and dissociation kinetics, suggesting that the mutated residues may be significantly involved in the large increases observed in ChTX binding affinity in the presence of the β1 subunit (Hanner et al., 1998).

It is worthy of noting that studies have shown that BK channels expressing auxiliary subunits other than β1 change their affinity

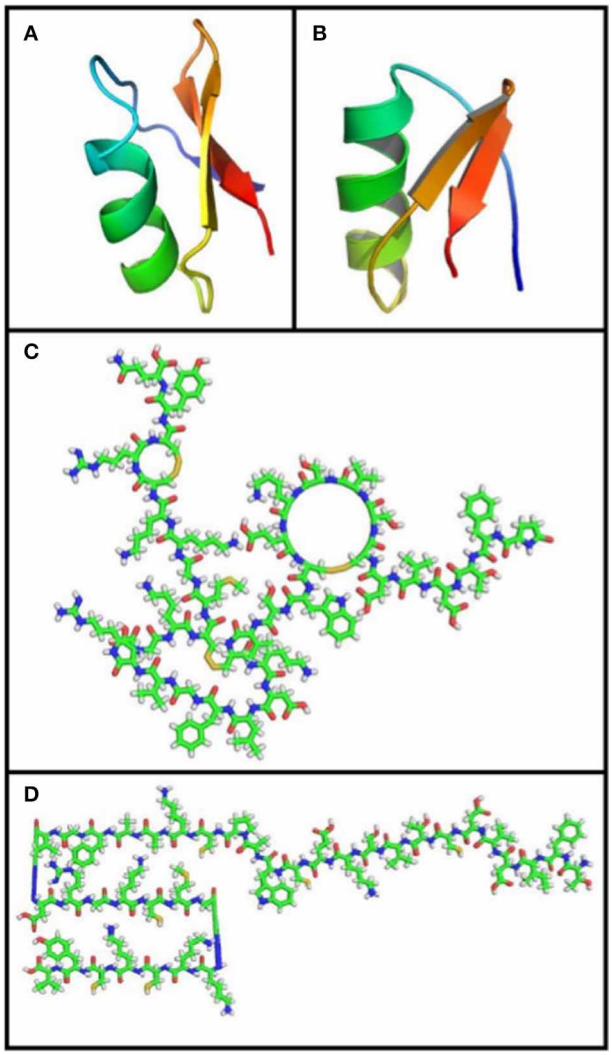


FIGURE 4 | Toxin structure. (A) Charybdotoxin: PDB ID 2CRD; (Bontems et al., 1992). **(B)** Martentoxin: PDB ID 1M2S (Wang et al., 2005). **(C)** Iberiotoxin: PubChem ID 16132435. **(D)** Slotoxin: PubChem ID 16133816. Using PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LL. Green: carbon atoms, white: hydrogen atoms, red: oxygen atoms, blue: nitrogen atoms, yellow: sulfur atoms.

to toxins in an opposite way, that is, by inducing a decrease in the toxin association rate. For instance, Reinhart et al. (1989) describe channels with two different sensitivities to ChTX in brain neurons, namely that while one type of BK channel was blocked with concentrations similar to those required to block the BK channel from skeletal muscle membranes, as describe by Anderson et al. (1988), the others were rather insensitive to the toxin. Further studies on rat supraoptic magnocellular neurons reported by Dopico et al. (1999b) showed similar results, where some BK channels were blocked at ChTX or IbTX concentrations of 10 nM, while the others were insensitive to ChTX even at concentrations as large as 360 nM. To explain such differences in sensitivity, Meera et al. (2000) reported that coexpression of α with the β4 subunit (which is the β subunit present in the

		10										20										30									
		.	.	.	.	I	.	.	.	.	I	.	.	.	.	I	.	.	.	.	I	.	.	.	.	I	.	.	.	.	I
Charybdotoxin-P13487	1	M	K	I	L	S	V	L	L	L	A	L	I	I	C	S	I	V	G	W	S	E	A	-	Q	F	T	N	V	S	C
Iberiotoxin-P24663	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	F	T	D	V	D	C
Slotoxin-P0C182	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	F	I	D	V	D	C
Martentoxin-Q9NBG9	1	M	K	I	F	S	I	L	L	V	A	L	I	I	C	S	I	S	I	C	T	E	A	F	G	L	I	D	V	K	C

		40										50										60									
		.	.	.	.	I	.	.	.	.	I	.	.	.	.	I	.	.	.	.	I	.	.	.	.	I	.	.	.	.	I
Charybdotoxin-P13487	31	T	T	S	K	E	C	W	S	V	C	Q	R	L	H	N	T	S	R	G	K	C	M	N	K	K	C	R	C	Y	S
Iberiotoxin-P24663	8	S	V	S	K	E	C	W	S	V	C	K	D	L	F	G	V	D	R	G	K	C	M	G	K	K	C	R	C	Y	Q
Slotoxin-P0C182	8	T	V	S	K	E	C	W	A	P	C	K	A	A	F	G	V	D	R	G	K	C	M	G	K	K	C	K	C	Y	V
Martentoxin-Q9NBG9	31	F	A	S	S	E	C	W	T	A	C	K	K	V	T	G	S	G	Q	G	K	C	Q	N	N	Q	C	R	C	Y	-

FIGURE 5 | Sequence alignment of the ChTX family of K^+ toxins. Using MUSCLE software (Edgar, 2004). UniProt protein accession: Charybdotoxin, P13487; Iberiotoxin, P24663; Slotoxin, P0C182; Martentoxin, Q9NBG9.

brain) leads to a BK channel phenotype that displays very low ChTX and IbTX sensitivity. Meera et al. (2000) proposed that the molecular basis for toxin insensitivity in BK channels found in neurons is that they are formed by the α + β4 subunit combination (Behrens et al., 2000; Meera et al., 2000). β4 subunit expression induces a rise in the apparent half-maximal inhibitory concentration (IC_{50}) of the BK channel and a lessening of the toxin association rate by 250–1000-fold. To explain the mechanism that may determine the differences in effects induced by the β1 and β4 subunits on ChTX binding to BK channels, the smooth muscle β1 subunit extracellular loop was exchanged with the neuronal β4 subunit loop. The results showed that the β subunit extracellular loop determined the affinity of this toxin to the BK channel. The β4 subunit external loop contains more basic residues (e.g., K120, R121, K125) than the β1 subunits, and their neutralization, or charge reversals (i.e., R121D) greatly increase toxin association and dissociation rates. These results suggest that positively charged residues impose an electrostatic shield on ChTX action (Gan et al., 2008). When comparing β1 and β4 subunits, it has been shown that β4 is missing a conserved Y100 residue, and restoring this residue into the β4 subunit enables the recovery of the α + β4 BK channel's higher sensitivity to ChTX. In addition, tyrosine 294 at the C-terminal end of the pore loop in *mslo*, which interacts with Y36 in ChTX, as well as the missing β4 Y100 residue are possible sites for ChTX channel interaction (Gao and Garcia, 2003; Gan et al., 2008).

These results have also been confirmed by studies evaluating IbTX affinity to BK channels, where the presence of β4 leads to reduced toxin binding to the channels. However, unlike ChTX, the presence of β1 decreases IbTX association rates by about 40-fold and produces a large decrease (∼100-fold) in dissociation rates. The end result of this is that although α + β1 BK channels show less sensitivity to IbTX, binding is essentially irreversible (Meera et al., 2000). These results strongly suggest that differences in toxin residues determine combinatorial effects with the extracellular loop of the β subunit. However, it is important to consider that some reports have found that IbTX behaves similar to ChTX in the presence of β1. This discrepancy can be due to differences in the ionic strengths of the solutions used to perform the experiments (Hanner et al., 1997; Meera et al., 2000).

Similar to the effects observed in channels expressing α + β4 subunits, the expression of β2 was found to trigger a steeper decrease in response rates to ChTX compared to the effect on the α subunit alone [EC_{50} = 58 nM and 1 nM, respectively] (Wallner et al., 1999). This has been further corroborated in chromaffin cells, where the β2 subunit is endogenously expressed (Ding et al., 1998). Additionally, studies evaluating the coexpression of α + β3 channels also report a decrease in the degrees of blockade by ChTX (IC_{50} 80 nM) (Xia et al., 1999, 2000; Zeng et al., 2008), together with a change in the magnitude and kinetics of the blocking reaction (Ding et al., 1998). Different to what has been found when comparing amino acid sequences of β1 and β4, residues proposed to mediate ChTX interaction with β1 are present in the β3 subunit, which suggests that other differences between both subunits are responsible for dissimilarities in ChTX sensitivity (Xia et al., 1999).

Although the previous results strongly suggest that amino acid sequences in the β subunit and toxin loop play an important role in toxin affinity to the BK channel, IbTX, and ChTX surface charge interfaces have been found to be nearly identical. This means that peptide net charge does not contribute to their potassium channel specificities, suggesting that the molecular mechanism responsible for such effects would be related to glycosylations in the β subunit extracellular loop and its proximity to the channel vestibule (Gao and Garcia, 2003).

Another scorpion toxin from the *Centruroides noxius* species that specifically inhibits mammalian BK channels is Slotoxin (SloTX), which has 76% of identity to IbTX and 54% to ChTX (Garcia-Valdes et al., 2001). Like IbTX and ChTX, SloTX interacts through a bimolecular reaction by blocking BK α subunit pore domain. In addition, the toxin can differentiate between the channels expressing α, α + β1, and α + β4 subunits. If the β1 subunit is coexpressed with the α subunit, on-rates of channel blockade are reduced by two orders of magnitude compared to when the BK channel α subunit is alone. Furthermore, off-rates acutely decrease, making SloTX blockade irreversible. Toxin association rates in oocytes coexpressing α + β4 subunits are much slower than when β1 is present. Therefore, α + β4 channels are extremely resistant to SloTX blockade, but once blocked, toxin interaction seems irreversible (Garcia-Valdes et al., 2001).

Martentoxin, which is a peptide isolated from *Buthus martensi Karsch* scorpion venom, can bind to K^+ channels by means of a similar mechanism as other well-known scorpion toxins (Ji et al., 2003; reviewed in Tao et al., 2012). Martentoxin preferentially selects BK over Kv channels with a 1000-fold difference (Shi et al., 2008). After blocking BK channel currents with martentoxin, full recovery is achieved much faster than with ChTX (Ji et al., 2003). Martentoxin is a unique neurotoxin whose activity depends on Ca^{2+} concentrations, and which selectively blocks α + β4 subunit BK channels with a higher preference than channels expressing the α subunit alone (Shi et al., 2008; Tao et al., 2014). At low Ca^{2+} cytoplasmic concentrations, neuronal α + β4 BK channel currents are inhibited by martentoxin, but at high Ca^{2+} concentrations the toxin acts as an agonist. Martentoxin does not shift BK channel (α + β1) conductance-voltage dependence, thus indicating that this toxin does not interact with the voltage sensor, unlike β1 (Tao et al., 2011). After applying 400 nM of martentoxin, the current induced by αBK channels by a +80 mV pulse was not significantly affected, despite the fact that 400 nM of IbTX abolishes it completely (Shi et al., 2008). This result suggests that BK channels expressing only α subunits are insensitive to martentoxin, which could make it a potential selector for BK channel subtypes (Tao et al., 2011).

The toxin Vt3.1 is a disulfide-cross-linked dimer conopeptide isolated from *Conus vitulinus* that acts on BK channels activity by a different mechanism of that reported for toxins like ChTX and IbTX. Toxin Vt3.1 inhibits α BK channels. However, its effect is substantially enhanced when channels are formed by α + β4. This property makes the Vt3.1 toxin an excellent tool for the study of neuronal BK channels where the β4 subunit is highly expressed (Li et al., 2014). Li et al. (2014) have suggested that this toxin affects the gating mechanism of the channel and induces a shift in G-V relation to more positive voltages.

FATTY ACIDS

The well-known role of BK channels in the regulation of vascular tone and their high expression in neurons have increased current interest in evaluating the effects of cardiovascular and nervous system regulators (such as fatty acids) on the biophysical properties of BK channels. For instance, BK channel activation by epoxyeicosatrienoic acids (EET), which are arachidonic acid metabolites, has been observed to result in concentration-dependent coronary artery relaxation (Eckman et al., 1998).

Recent studies have found that unsaturated *cis* fatty acids, such as palmitoleic (PAM), oleic (OA), linoleic (LA), linolenic (ALA), arachidonic (AA), and eicosapentaenoic acids (EPA) (**Figures 6A–F**), significantly heighten BK channel activity by increasing their open probability (P_0) without affecting channel voltage sensitivity or unitary conductance (Ahn et al., 1994; Denson et al., 2000; Clarke et al., 2002; Zheng et al., 2005). However, saturated and *trans*-unsaturated fatty acids have no effect on channel activity. In addition, it has been demonstrated that AA metabolites, such as 11,12-EET, and 14,15-EET (**Figures 6G,H**), increase BK channel P_0 in cells from both pig coronary arteries and rat pituitary GH3 with no effect on their unitary conductance and Ca^{2+} sensitivity, hence providing evidence for the direct influence of EET on the channel protein (Baron et al., 1997; Wu et al., 2000a). This was further corroborated by using inhibitors such as ChTX and TEA, which diminish or abolish the effects induced by EET on P_0 in bovine coronary arterial smooth muscle cells (Campbell et al., 1996). On the other hand, results observed by Fukao et al. (2001) suggest that 11, 12-EET directly acts on the α subunit independently from the β-subunit. The role of the α subunit complex as a high affinity receptor for fatty acids has been confirmed with docosahexaenoic acid (DHA), which activates BK channels from excised patches in a reversible way (Hoshi et al., 2013a). Recent studies using human BK mutants (Y318S) showed a significant decrease in the amplifying effect of DHA (**Figure 6I**) and EPA currents, suggesting that this residue located in the S6 segment is either part of the fatty acid binding site or constitutes an important piece of the coupling system that transforms chemical binding energy into pore-opening energy (Hoshi et al., 2013a). However, no changes were observed when AA or ALA was used (Hoshi et al., 2013c), indicating that additional α subunit residues or auxiliary subunits may be required for the enhancing effects of these fatty acids on BK channels.

In order to increase BK channel activity, studies thus far have shown that fatty acids must have the following molecular characteristics: more than 8 carbon atoms, one or more *cis* unsaturations and a negative charge (Ahn et al., 1994; Denson et al., 2000; Zheng et al., 2005). Neutral and short-chain lipids have no effect on P_0, while positively charged lipids (like sphingosine) produced a decrease in NP_0. Increases in P_0 result from decreases in mean closed time (τ_c) without modifying mean open time (τ_o) (Clarke et al., 2002, 2003).

Similar to the differential effects seen from toxins in the presence of auxiliary subunits, the effects of fatty acids on BK channels are modified by β subunit expression where fatty acids interacts directly with β subunits (Martín et al., 2013). When examining AA effects on BK channels formed by α + β2 or α + β3 subunits, a potentiation in the current and a slowdown in inactivation (α + β2 channels) were displayed. It is thought that this effect could be mediated by the direct interaction of AA with the channel rather than through its metabolites (Sun et al., 2007). When BK channels are modulated by AA, β subunits have important implications for the adequate function of the nervous system, considering that the expression of β2 and β3 are specific to certain types of neurons. In the absence of β2 or β3 or in the presence of β4, AA does not induce a significant response to BK channels. Furthermore, AA causes an increase in P_0 as well as in the number of open channels. Effects on peak current amplitude and α + β2 current inactivation kinetics have been observed to be dose-dependent in the range of 1–50 μM of AA. At higher AA doses, a complete loss of the inactivation component was evident. Data supports the premise that the effects of AA are mediated by changes in trypsin-sensitive inactivation and that there is no obvious fatty acid-mediated activation effect upon removing inactivation. As a whole, the β2-dependent BK channel inactivation gate is a specific molecular target for AA and other unsaturated fatty acids (Sun et al., 2007). In addition, it has been shown that DHA and oleic acid (OA) can also activate α + β2 current and slow down inactivation kinetics, whereas saturated fatty acids (i.e., palmitic, stearic, and caprylic acid) seem to have

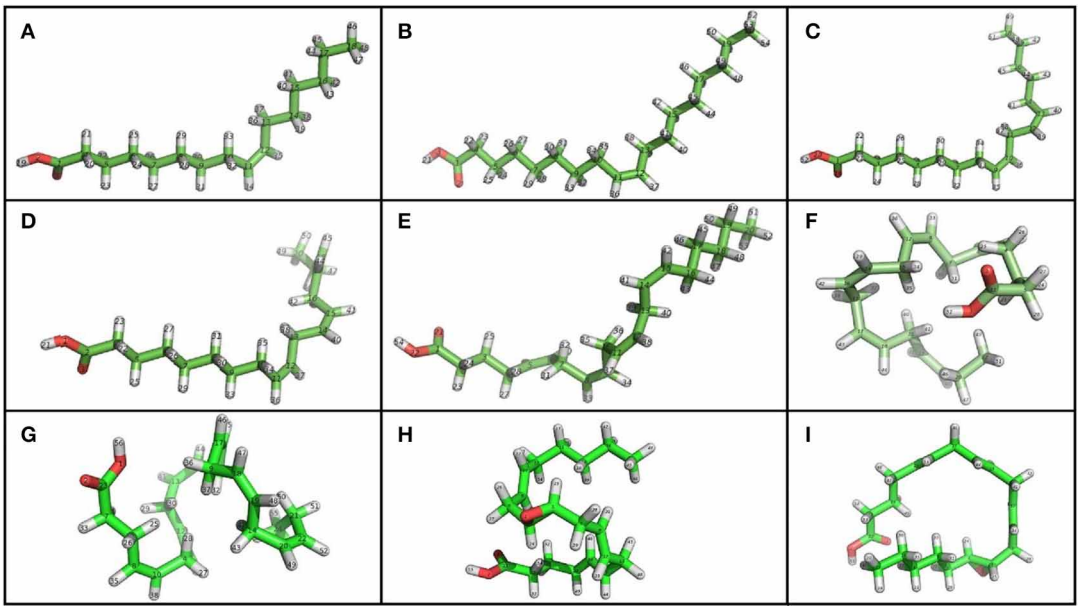


FIGURE 6 | Chemical structure of unsaturated fatty acids that modulate BK channel activity. (A) PAM, Palmitoleic Acid; **(B)** OA, Oleic Acid; **(C)** LA, Linoleic Acid; **(D)** ALA, Linolenic Acid; **(E)** AA, Arachidonic Acid; **(F)** EPA, Eicosapentaenoic Acid; **(G)** DHA, Docosahexaenoic acid; **(H)** 11, 12 EET, 11, 12 Epoxyeicosatrienoic acid; **(I)** 14, 15 EET, 14, 15 Epoxyeicosatrienoic acid. Using PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LL. *Green, carbon atoms; white, hydrogen atoms; red, oxygen atoms.*

no effects. Of all the fatty acids tested, OA is the most potent in enhancing BK currents.

In native human vascular smooth muscle cells, where the β1 subunit is expressed, single channel recordings have shown an important rise in the P_0 of BK channels induced by AA acid (Martín et al., 2013). In addition, AA accelerates voltage-dependent activation kinetics of channel. Studies in HEK 293T cells have concluded that β1 subunit expression is needed for AA to activate the channel, since no effect has been seen when the β1 subunit is not expressed. Therefore, the β1 subunit is required to mediate AA effects on BK channels by a mechanism that involves modifying gating ring operation independently from channel voltage sensitivity changes (Martín et al., 2013). In conclusion, these results strongly suggest that BK channel activation by AA requires the expression of β1, β2, or β3 subunits. Although such mechanisms have not yet been fully established, Martín et al. (2013) have suggested a mechanism for AA action that involves an increase in P_0, while Sun et al. (2007) have proposed that AA prevents inactivation when BK channels are formed by the α + β2 subunit.

The degree of DHA modulation, on the other hand, depends on which β subunit is associated with the α subunit. For instance, when β1 or β4 are expressed, a large increase in peak current can be observed, but in α + β2 or αBK channels the response is rather limited. In addition, DHA differentially influences activation or deactivation kinetics, depending on which β subunit is present (β1, β2, and β4) (Hoshi et al., 2013c). It has been reported that DHA and EPA activate BK channels expressing β1 subunits (Lai et al., 2009; Hoshi et al., 2013c), and DHA has a ∼20-fold current-enhancing effect on channels expressing α + β1. This effect is associated with a leftward shift in half-activation voltage, which also destabilizes the closed conformation of the conduction gate and decreases the activation time constant. The following two important residues determine the modulatory effects exerted by DHA in β1 and β4 subunits: one residue is in the N terminus (R11 in β1 and E12 in β4) and the other in TM1 (C18 in β1 and R19 in β4). The exchange of these residues with the equivalent amino acids in β2 allows this subunit (as is the case of β1 and β4) to behave as an enhancer of the DHA effect (Hoshi et al., 2013c).

In contrast to the enhancing effect found from fatty acids, a metabolic intermediary in the synthesis of fatty acids (i.e., acyl-CoA) has been shown to interact with the β2 subunit in inhibiting BK currents (Sun et al., 2008). In cerebral ischemic damage, selective AA reincorporation and accumulation into brain membranes has been observed (Rabin et al., 1997). In addition, it is acknowledged from studies in animal models that BK channel activation can potentially protect neurons under ischemic conditions (Rabin et al., 1997; Gribkoff et al., 2001). Moreover, the effects of unsaturated fatty acids on BK channel activity may influence neuronal survival/death, as their concentration may undergo rapid changes under certain pathophysiological conditions (Sun et al., 2007). Therefore, BK channel modulation by acyl-CoA or unsaturated fatty acids may control events leading to cell protection or cell death. Understanding the role of lipids under ion channel modulation conditions may lead to new therapeutic uses (Sun et al., 2008).

ETHANOL

Ethanol is a well-known modulator of the BK channel activity, as it has been described for several cells types such as neurons from rat dorsal root ganglia, nucleus accumbens and neurohypophysial

nerve terminals (reviewed in Dopico et al., 1999a; Gruss et al., 2001; Knott et al., 2002; Martin et al., 2004). Changes in neuropeptide release (Knott et al., 2002) and cerebrovascular tone variations (Bukiya et al., 2009) are some of the reported effects of ethanol, all of which are reversible and seen at clinically relevant concentrations. It was initially proposed that such effects were related to the number of functional channels, changes in single channel conductance or modifications in gating properties. Subsequently, however, it was shown that gating properties were modified by the direct binding of ethanol to BK channels and by the activation of intracellular signaling cascades that may regulate the channel (Dopico et al., 1996). More recently two reports have described BK amino acid residues required for activation (Bukiya et al., 2014; Davis et al., 2014). The BK single-point mutant T352I located in RCK1 was found to be utterly insensitive to ethanol, with no changes in conductance, selectivity or gating (Davis et al., 2014). On the other hand, Bukiya et al. (2014) unveiled an alcohol-sensing site, site that is only accessible to ethanol in the presence of Ca^{2+}. This site is located in the Ca^{2+}-sensing tail domain and molecular modeling suggest that residue K361forms an essential hydrogen bond with ethanol only in the presence of Ca^{2+}.

Studies on *mslo* (Dopico et al., 1998; reviewed in Dopico et al., 1999a), *hslo* (Feinberg-Zadek and Treistman, 2007; Feinberg-Zadek et al., 2008) and *bslo* channels (Dopico, 2003) have shown a concentration-dependent increase in NP_0 related to gating channel properties and not to changes in ionic conductance (Dopico et al., 1996, 1998). BK channel response is known to be voltage-independent, but is inextricably affected by intracellular Ca^{2+} concentration changes. For instance, the potentiation of *mslo* activity induced by ethanol decreases when intracellular Ca^{2+} increases, meaning that BK channel activation by this alcohol is more marked when intracellular Ca^{2+} is near basal conditions (Dopico et al., 1996, 1999a). Effects on NP_0 are induced by a combination of increases in τ_0 and decreases in τ_c (Dopico et al., 1998).

It has also been observed that ethanol can increase BK channel activity in a tissue-dependent manner (Dopico et al., 1996, 1999b; Gruss et al., 2001; Knott et al., 2002), which was initially related to BK isoforms and/or differential β subunit expression in various tissues (Dopico, 2003). Of the cloned β subunits, only β1, and β4 were studied and showed different responses. In vascular smooth muscles, where the β1 subunit is predominant (reviewed in Orio et al., 2002), ethanol effects depend on intracellular Ca^{2+} concentrations (Bukiya et al., 2009). For instance, when a BK channel-forming α-subunit isoform cloned from rat cerebral artery myocytes (cbv1) is expressed in *Xenopus laevis*, current potentiation after ethanol exposure (as seen by decreases in $V_{1/2}$) is evident at Ca^{2+} concentrations less than 20 μM. On the other hand, rises in Ca^{2+} concentrations diminish potentiation and concentrations' over 30 μM result in inhibition. The latter response denotes that basal Ca^{2+} concentrations promote BK channel potentiation, whereas high concentrations (>10 μM) induce inhibition. Ethanol does not generate changes in channel unitary current amplitude, which implies that its effect on cbv1 channels is limited to modifying gating. Finally, the effect of ethanol depends on the intracellular Ca^{2+} levels sensed by the α subunit (Bukiya et al., 2009).

In studies, where cbv1 was co-expressed with the β1 subunit, shifts from ethanol induced activation to inhibition were found at Ca^{2+} concentrations less than 3 μM. The latter implies that β1 subunit expression induces BK channel current inhibition by ethanol at intracellular Ca^{2+} levels similar to those reached during cerebral myocyte contraction (4–30 μM). These results indicate that β1 subunit effect on ethanol activity is mediated with an allosteric mechanism where β1 acts like a transducer that couples changes in Ca^{2+} concentration to BK channel activation (Bukiya et al., 2009). Similar results were also found in myocytes, indicating that the β1 subunit would be responsible for ethanol-induced BK current inhibition at membrane potentials and Ca^{2+} concentrations reached during cell contraction (Bukiya et al., 2009).

In experiments performed on soma and dendrites of nucleus accumbens neurons, BK channels exhibit a dual behavior, where somatic channels are sensitive to ethanol, while dendritic channels are not. In other words, when the β4 subunit is expressed, ethanol has a potentiating effect, whereas when β1 is expressed, this effect is not exerted. These results were further confirmed by heterologous expressions of α and β4 subunits in HEK293 cells (Martin et al., 2004; Treistman and Martin, 2009). The increase of the α + β4 channel's NP_0 results from a rise in τ_o and a fall in τ_c (Feinberg-Zadek and Treistman, 2007).

The effect of β1 and β4 in the response of BK channels to ethanol has been related to alcohol tolerance, implying a loss of effectiveness with time, which is a key feature of addiction (Martin et al., 2008). The mechanism by which β subunits determine BK channel potentiation seems to also modulate alcohol sensitivity, which may contribute to explaining the roles of genetic predisposition and tissue-dependent expression of BK channels (Feinberg-Zadek and Treistman, 2007). For example, β1 determines low BK channel sensitivity to ethanol (Feinberg-Zadek et al., 2008), whereas β4 subunit expression in BK channels potentiates the response to ethanol. This suggests that in the absence of the β4 subunit, acute tolerance of BK channels is induced, while the presence of this subunit abolishes it. Accordingly, there is a strong association between alcohol tolerance and predisposition to alcoholism. Therefore, the β4 subunit could determine specific differences related to alcohol abuse and alcoholism, hence making it a potential therapeutic target (Martin et al., 2008). Intermittent chronic alcohol exposure could increase expression of β1 subunits in local brain regions. The latter could be used as a good strategy in alcohol-dependent subjects to selectively alter their motivational drive to excessively consume alcohol (Kreifeldt et al., 2013).

STEROIDS

Steroids usually exert their action through specific receptors within cells. The lipophilic nature of these components benefits their entrance across the cellular membrane to the cytoplasm, allowing them to bind to receptors acting as transcription factors in the nucleus. (Thiede et al., 2012). Nevertheless, there are certain rapid effects that are independent of this nuclear signalization and start in membrane receptors (Simoncini et al., 2004).

17β-estradiol (E2) (**Figure 7A**) has been shown to activate BK channels in a nuclear receptor-independent manner. This activation is caracterized by an increase in potassium currents, accelerated current kinetics and a rise in P_0 to less positive potentials

at 1–20 μM of E2 and at low Ca^{+2} concentrations (∼100 nM) (Valverde et al., 1999; De Wet et al., 2006). The observed increments in BK activity are related to the expression of auxiliary β subunits, that can act like a receptor for E2 (King et al., 2006). Such increments can be β1-dependent at <1 μM of E2 or β1-, β2-, and β4- dependent at 1–30 μM, (Behrens et al., 2000; King et al., 2006), which suggests the existence of an E2 membrane receptor that can distinguish between different β subunits. (King et al., 2006). The action of E2 on BK channels is determined by type of β subunit expression, and it can be observed in native (Holdiman et al., 2002; Coiret et al., 2005; Ohya et al., 2005), and heterologous expression (Valverde et al., 1999; De Wet et al., 2006).

Although E2 can activate the BK channel when the β1 subunit is present, the exact binding site has not yet been identified. E2 activation can be induced when E2 is conjugated to a protein that is impermeable to the membrane, but only when it is applied to the outside of the cell, suggesting that the binding site is located in the extracellular region of the β1 subunit (Valverde et al., 1999). Regulation in proteasomal degradation of the β1 subunit in the presence of E2 also suggests the importance of this subunit for cellular metabolism (Korovkina et al., 2004; Nagar et al., 2005).

After identifying the stimulating activity of E2 on BK channel properties, research has focused on other molecules that can exert the same characteristics. It has been shown that Tamoxifen (**Figure 7B**), which is an agonist of estrogen receptors that is widely used for breast cancer therapy, also activates the BK channel, but is at least 10 times more potent than E2, causing a reversible and fast effect at concentrations as low as 0.1 μM (Dick et al., 2001). The E2-mediated increase in P_0 was also observed in the absence of cytoplasmic signals, as this effect was observed in BK channels incorporated into lipid bilayers, as well as in outside out and inside out patches. Changes in channel activation, however, were not seen when the BK α subunit was alone, as confirmed in experiments with β1—knockout mice cells (Dick and Sanders, 2001). Ethylbromide tamoxifen, which is a molecule that does not permeate the membrane, was observed to elevate potassium current at +40 mV and also raise the open probability when applied to the outside of the membrane, indicating that there may be an extracellular binding site (Dick et al., 2002) just as was proposed for E2 (Valverde et al., 1999).

Dehydroepiandrosterone (DHEA; **Figure 7C**), which is an adrenal androgen, is another steroid derivative that activates BK channels, for which such sensitivity is provided by the β2 subunit. Corticoesterone (**Figure 7D**) at physiological concentrations can also quickly and reversibly activate BK channels when expressed with the β4 subunit. Under conditions of constant calcium concentration, it primarily acts by shifting the voltage activation curve to the left. This finding has suggested that β subunits differentiate between steroids, but that BK channels do not respond when α subunits are present alone (King et al., 2006), which has led to an increased interest in researching the molecules targeting the β1 subunit as a potential modulator of the BK channel. Lithocholic acid (**Figure 7E**), being a cholane at micromolar concentrations (EC_{50} = 45 μM), acts as a BK channel activator only in channels formed by α + β1 (Bukiya et al., 2013).

The binding site of these molecules is another important research focus, due to its pharmacological potential. Structural molecular models and structure–activity relationship (SAR) studies have led to the identification of a binding site for lithocolic acid in the β1 subunit TM2 domain (Bukiya et al., 2011). The search for similarities in such models and structural databases is

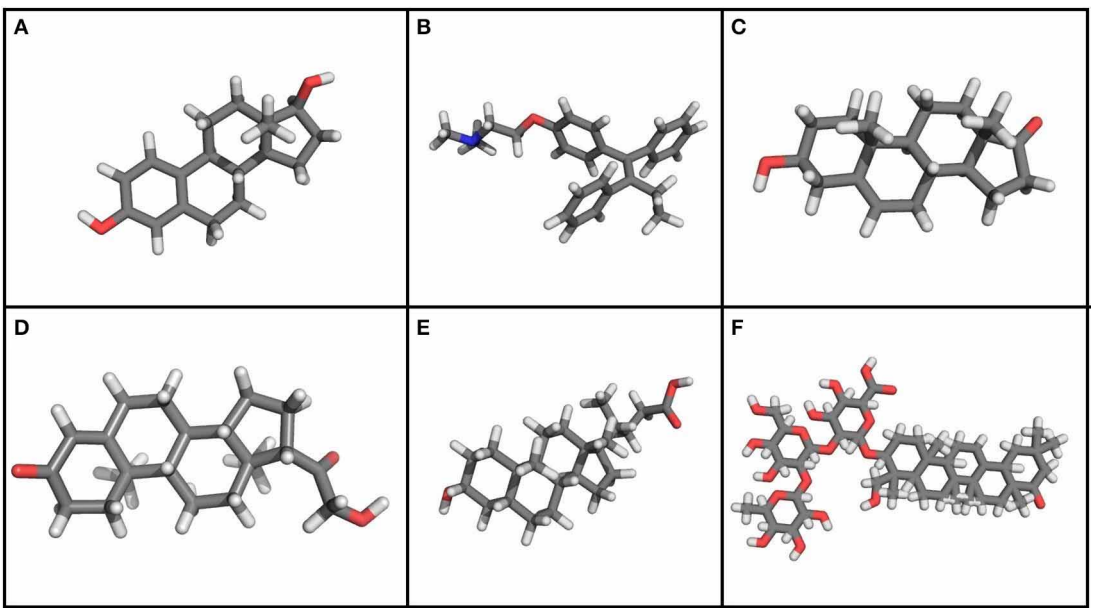


FIGURE 7 | Chemical structure of steroids and other molecules that modulate BK channel activity. (A) 17β-Estradiol. **(B)** Tamoxifen. **(C)** Dehydroepiandrosterone. **(D)** Corticoesterone. **(E)** Lithocholic acid. **(F)** Dehydrosoyasaponin-I. Using PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LL. *Gray, carbon atoms; white, hydrogen atoms; red, oxygen atoms; blue, nitrogen atoms.*

helpful for the identification of other molecules, such as sodium 3-hydroxyolean-12-en-30-oate (HENA). This compound is not a steroid, but it activates BK channels because it contains the structural characteristics considered to be necessary for its activation and, interestingly, it also activates BK channels only when β1 subunits are expressed (Bukiya et al., 2013).

Dehydrosoyasaponin-I (DHS-I) (**Figure 7F**) isolated from *Desmodium adscendens* is a reversible and potent activator of the BK channel. When applied to the intracellular side of the membrane, it causes a shift in both calcium- and voltage-dependent activation curves. It was identified because it inhibits ChTX binding to the channel in bovine tracheal smooth muscle membranes (McManus et al., 1993). A triterpene glycoside was the first and most potent activator to be identified at nanomolecular (100 nM) concentrations, causing an 80 mV lefward shift in $V_{1/2}$ and an increase in open probability by means of a direct mechanism (Giangiacomo et al., 1998). The identification of this effect in smooth muscle membranes has led to report that such activation depends on the expression of the β1 subunit, although it is unclear whether this effect is determined by other subunits as well (Bukiya et al., 2012).

OTHER MOLECULES

Tungstate is a molecule commonly used as and antihypertensive agent (Swei et al., 1999) that modulates BK channels expressing α subunits alone or coexpressed with β subunits (β1 – β4) in a concentration-dependent manner (Fernández-Mariño et al., 2012). When comparing 0.1 and 1 mM of tungstate, it was observed that both concentrations can induce a decrease in $V_{1/2}$, but reductions in current amplitude are seen only at higher concentrations. $V_{1/2}$ declines are calcium-dependent and are only seen in the presence of β1 or β4 subunits, but not in channels containing β2 or β3 subunits (Fernández-Mariño et al., 2012). The tungstate putative binding site is located in the BK channel α subunit, but residues from the β1 extracellular loop are also required for the appropriate activation of the channel. It has been proposed that extracellular loop residues are involved in maintaining the BK channel structure needed for tungstate to bind to the channel (Fernández-Mariño et al., 2014).

Another BK channel activator is $DiBAC_4$ (bis (1,3)-dibutylbarbituric acid) trimethine oxonol), which is a voltage-sensitive fluorescent dye that significantly increases whole-cell BK α + β1 currents in HEK293 cells and in native channels from urinary bladder smooth muscle cells. $DiBAC_4$ shifts the activation voltage to hyperpolarizing potentials without causing changes in single-channel conductance. Morimoto et al. (2007) reported that $DiBAC_4$ activates whole-cell rBKα + β1 and rBKα + β4 currents partially blocking rBKα + β2 currents with no effects on channels where the α subunit is alone (Morimoto et al., 2007). Nonetheless, Scornik et al. (2013) suggested that the $DiBAC_4$ binding site should be in the α subunit, since $DiBAC_4$ selectively enhances BK channel activity in the presence or absence of the β1 subunit (Scornik et al., 2013). It has been shown that $DiBAC_4$ can increase deactivation time constants without changing the voltage dependence of deactivation. Although the latter effects do not necessarily require the presence of β1 subunits, these subunits enhance BK channel response to $DiBAC_4$. It has also been established that leftward shifts in conductance-voltage curves induced by $DiBAC_4$ are significantly larger in channels expressing α + β1 than in those expressing α subunits alone. Moreover, β1 subunits promote a four-fold decrease in the K_d of $DiBAC_4$. Such findings could envision a new scope for the role β1 subunits in cardiovascular pharmacology (Bosch et al., 2014).

Additionally, NO, being an endogenous vasodilatation gas, can rapidly, reversibly, and significantly increase BK open channel probability from both sides of the membrane (Bolotina et al., 1994; Peng et al., 1996). Carbon monoxide (CO), which is another endogenous like NO, can increase native BK channel activity. However, stimulatory effects of CO and NO rely on specific interactions, where CO interacts with α and NO with β subunits (Wu et al., 2002). The alkaloid tetrandine is a blocker of *hslo* current and its action as a BK inhibitor is potentiated in channels coexpressing α and β subunits (Dworetzky et al., 1996). In endothelial human cells, it was found that tetrandrine shifted the G-V to more positive potentials and inhibited the maximal P_0 (Wu et al., 2000b). BK channel expression in human gliomas appears to be correlated with tumor malignancy grade and is involved in the proliferation of human osteoblasts (Henney et al., 2009; Chen and Tseng, 2010).

CONCLUDING REMARKS

BK channels are related to different pathophysiological processes, such as changes in vascular tone regulation, diabetes, kidney, and nervous system diseases. The expression of auxiliary β subunits plays an important role in these processes because of the modulatory effects of these subunits on BK channel activity, inducing changes in their biophysical properties. Furthermore, β subunits act like binding sites for various molecules that regulate BK channel activity, prompting an increase or decrease in the effects induced by drugs. This knowledge would be important in drug design, since it may be possible to find molecules that induce specific modulatory effects on BK channel properties, depending on the tissue types where they are expressed. That is particularly important in smooth muscles and in the nervous system, where β1 and β4 are highly expressed and have been found to significantly regulate the biophysical properties of BK channels.

ACKNOWLEDGMENT

This work is supported by FONDECYT Grants 1110430 (Ramón Latorre), The Centro Interdisciplinario de Neurociencia de Valparaíso (CINV) is a Millennium Institute supported by the Millennium Scientific Initiative of the Chilean Ministry of Economy, Development and Tourism. Pontificia Universidad Javeriana, Grant ID5571 (Yolima P. Torres); COLCIENCIAS doctoral fellowships (Sara T. Granados).

REFERENCES

Ahn, D. S., Kim, Y. B., Lee, Y. H., Kang, B. S., and Kang, D. H. (1994). Fatty acids directly increase the activity of $Ca^{(2+)}$-activated K^+ channels in rabbit coronary smooth muscle cells. *Yonsei Med. J.* 35, 10–24.

Anderson, C. S., MacKinnon, R., Smith, C., and Miller, C. (1988). Charybdotoxin block of single Ca^{2+}-activated K^+ channels. Effects of channel gating, voltage, and ionic strength. *J. Gen. Physiol.* 91, 317–333.

Banerjee, A., Lee, A., Campbell, E., and MacKinnon, R. (2013). Structure of a pore-blocking toxin in complex with a eukaryotic voltage-dependent K^+ channel. *Elife* 2:e00594. doi: 10.7554/eLife.00594

Bao, L., and Cox, D. H. (2005). Gating and ionic currents reveal how the BKCa channel's Ca^{2+} sensitivity is enhanced by its beta1 subunit. *J. Gen. Physiol.* 126, 393–412. doi: 10.1085/jgp.200509346

Baron, A., Frieden, M., and Bény, J. L. (1997). Epoxyeicosatrienoic acids activate a high-conductance, $Ca^{(2+)}$-dependent K^+ channel on pig coronary artery endothelial cells. *J. Physiol.* 504, 537–543.

Behrens, R., Nolting, A., Reimann, F., Schwarz, M., Waldschütz, R., and Pongs, O. (2000). hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel β subunit family. *FEBS Lett.* 474, 99–106. doi: 10.1016/S0014-5793(00)01584-2

Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J., and Cohen, R. A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 368, 850–853.

Bontems, F., Gilquin, B., Roumestand, C., Ménez, A., and Toma, F. (1992). Analysis of side-chain organization on a refined model of charybdotoxin: structural and functional implications. *Biochemistry* 31, 7756–7764. doi: 10.1021/bi00149a003

Bosch Calero, C., Selga, E., Brugada, R., Scornik, F. S., and Pérez, G. J. (2014). The smooth muscle-type β1 subunit potentiates activation by DiBAC4(3) in recombinant BK channels. *Channels (Austin)* 8, 95–102. doi: 10.4161/chan.27212

Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y., and Aldrich, R. W. (2000b). Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. *J. Biol. Chem.* 275, 6453–6461. doi: 10.1074/jbc.275.9.6453

Brenner, R., Peréz, G. J., Bonev, A. D., Eckman, D. M., Kosek, J. C., Wiler, S. W., et al. (2000a). Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature* 407, 870–876. doi: 10.1038/35038011

Bukiya, A. N., Kuntamallappanavar, G., Edwards, J., Singh, A. D., Shivakumar, B., and Dopico, A. M. (2014). An alcohol-sensing site in the calcium-and voltage-gated, large conductance potassium (BK) channel. *Proc. Natl. Acad. Sci. U.S.A.* 111, 9313–9318. doi: 10.1073/pnas.1317363111

Bukiya, A. N., McMillan, J. E., Fedinec, A. L., Patil, S. A., Miller, D. D., Leffler, C. W., et al. (2013). Cerebrovascular dilation via selective targeting of the cholane steroid-recognition site in the BK channel β1-subunit by a novel nonsteroidal agent. *Mol. Pharmacol.* 83, 1030–1044. doi: 10.1124/mol.112.083519

Bukiya, A. N., Patil, S. A., Li, W., Miller, D. D., and Dopico, A. M. (2012). Calcium- and voltage-gated potassium (BK) channel activators in the 5β-cholanic acid-3α-ol analogue series with modifications in the lateral chain. *Chem. Med. Chem.* 7, 1784–1792. doi: 10.1002/cmdc.201200290

Bukiya, A. N., Singh, A. K., Parrill, A. L., and Dopico, A. M. (2011). The steroid interaction site in transmembrane domain 2 of the large conductance, voltage- and calcium-gated potassium (BK) channel accessory 1 subunit. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20207–20212. doi: 10.1073/pnas.1112901108

Bukiya, A. N., Vaithianathan, T., Toro, L., and Dopico, A. M. (2009). Channel beta2-4 subunits fail to substitute for beta1 in sensitizing BK channels to lithocholate. *Biochem. Biophys. Res. Commun.* 390, 995–1000. doi: 10.1016/j.bbrc.2009.10.091

Campbell, W. B., Gebremedhin, D., Pratt, P. F., and Harder, D. R. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Cir. Res.* 78, 415–423.

Candia, S., Garcia, M. L., and Latorre, R. (1992). Mode of action of iberiotoxin, a potent blocker of the large conductance $Ca^{(2+)}$-activated K^+ channel. *Biophys. J.* 63, 583–590.

Chen, M., and Petkov, G. V. (2009). Identification of large conductance calcium activated potassium channel accessory beta4 subunit in rat and mouse bladder smooth muscle. *J. Urol.* 182, 374–381. doi: 10.1016/j.juro.2009.02.109

Chen, Y., and Tseng, S. H. (2010). The Potential of Tetrandrine against Gliomas. *Anticancer Agents Med. Chem.* 10, 534–542. doi: 10.2174/1871520107934 98609

Clarke, A. L., Petrou, S., Walsh, J. V. Jr., and Singer, J. J. (2002). Modulation of BK(Ca) channel activity by fatty acids: structural requirements and mechanism of action. *Am. J. Physiol. Cell. Physiol.* 283, C1441–C1453. doi: 10.1152/ajpcell.00035.2002

Clarke, A. L., Petrou, S., Walsh, J. V. Jr., and Singer, J. J. (2003). Site of action of fatty acids and other charged lipids on BKCa channels from arterial smooth muscle cells. *Am. J. Physiol. Cell. Physiol.* 284, C607–C619. doi: 10.1152/ajpcell.00364.2002

Coiret, G., Matifat, F., Hague, F., and Ouadid-Ahidouch, H. (2005). 17-beta-estradiol activates maxi-K channels through a non-genomic pathway in human breast cancer cells. *FEBS Lett.* 579, 2995–3000. doi: 10.1016/j.febslet.2005.02.085

Contreras, G. F., Castillo, K., Enrique, N., Carrasquel-Ursualez, W., Castillo, J. P., Milesi, V., et al. (2013). A BK (*Slo1*) channel journey from molecule to physiology. *Channels (Austin)* 7, 442–458. doi: 10.4161/chan.26242

Contreras, G. F., Neely, A., Alvarez, O., Gonzalez, C., and Latorre, R. (2012). Modulation of BK channel voltage gating by different auxiliary β subunits. *Proc. Natl. Acad. Sci. U.S.A.* 109, 18991–18996. doi: 10.1073/pnas.1216953109

Cox, D. H., and Aldrich, R. W. (2000). Role of the beta1 subunit in large-conductance $Ca^{(2+)}$-activated $K^{(+)}$ channel gating energetics. Mechanisms of enhanced $Ca^{(2+)}$ sensitivity. *J. Gen. Physiol.* 116, 411–432. doi: 10.1085/jgp.116.3.411

Davis, S. J., Soctt, L. L., Hu, K., and Pierce-Shimomura, J. T. (2014). Conserved single residue in the BK potassium channel required for activation by alcohol and intoxication in *C. elegans*. *J. Neurosci.* 9562–9573. doi: 10.1523/JNEUROSCI.0838-14.2014

Denson, D. D., Wang, X., Worrell, R. T., and Eaton, D. C. (2000). Effects of fatty acids on BK channels in GH(3) cells. *Am. J. Physiol. Cell. Physiol.* 279, C1211–C1219.

De Wet, H., Allen, M., Holmes, C., Stobbart, M., Lippiat, J. D., and Callaghan, R. (2006). Modulation of the BK channel by estrogens: examination at single channel level. *Mol. Membr. Biol.* 23, 420–429. doi: 10.1080/09687860600802803

Dick, G. M., Hunter, A. C., and Sanders, K. M. (2002). Ethylbromide tamoxifen, a membrane-impermeant antiestrogen, activates smooth muscle calcium-activated large-conductance potassium channels from the extracellular side. *Mol. Pharmacol.* 61, 1105–1113. doi: 10.1124/mol.61.5.1105

Dick, G. M., Rossow, C. F., Smirnov, S., Horowitz, B., and Sanders, K. M. (2001). Tamoxifen activates smooth muscle BK channels through the regulatory beta 1 subunit. *J. Biol. Chem.* 276, 34594–34599. doi: 10.1074/jbc.M104689200

Dick, G. M., and Sanders, K. M. (2001). (Xeno) estrogen sensitivity of smooth muscle BK channels conferred by the regulatory beta1 subunit: a study of beta1 knockout mice. *J. Biol. Chem.* 276, 44835–44840. doi: 10.1074/jbc.M106851200

Ding, J. P., Li, Z. W., and Lingle, C. J. (1998). Inactivating BK channels in rat chromaffin cells may arise from heteromultimeric assembly of distinct inactivation-competent and noninactivating subunits. *Biophys. J.* 74, 268–289.

Dopico, A. M. (2003). Ethanol sensitivity of BK(Ca) channels from arterial smooth muscle does not require the presence of the beta1-subunit. *Am. J. Physiol. Cell. Physiol.* 284, C1468–C1480. doi: 10.1152/ajpcell.00421.2002

Dopico, A. M., Anantharam, V., and Treistman, S. N. (1998). Ethanol increases the activity of Ca(++)-dependent K^+ (*mslo*) channels: functional interaction with cytosolic Ca++. *J. Pharmacol. Exp. Ther.* 284, 258–268.

Dopico, A. M., Chu, B., Lemos, J. R., and Treistman, S. N. (1999a). Alcohol modulation of calcium-activated potassium channels. *Neurochem. Int.* 35, 103–106.

Dopico, A. M., Lemos, J. R., and Treistman, S. N. (1996). Ethanol increases the activity of large conductance, $Ca^{(2+)}$-activated K^+ channels in isolated neurohypophysial terminals. *Mol. Pharmacol.* 49, 40–48.

Dopico, A. M., Widmer, H., Wang, G., Lemos, J. R., and Treistman, S. N. (1999b). Rat supraoptic magnocellular neurones show distinct large conductance, Ca^{2+}-activated K^+ channel subtypes in cell bodies versus nerve endings. *J. Physiol.* 519, 101–114.

Dworetzky, S. I., Boissard, C. G., Lum-Ragan, J. T., McKay, M. C., Post-Munson, D. J., Trojnacki, J. T., et al. (1996). Phenotypic alteration of a human BK (*hslo*) channel by hSlob subunit coexpression: changes in blocker sensitivity, activation/ relaxation and inactivation kinetics, and protein kinase A modulation. *J. Neurosci.* 16, 4543–4550.

Eckman, D. M., Hopkins, N., McBride, C., and Keef, K. D. (1998). Endothelium-dependent relaxation and hyperpolarization in guinea-pig coronary artery: role of epoxyeicosatrienoic acid. *Br. J. Pharmacol.* 124, 181–189.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Feinberg-Zadek, P. L., Martin, G., and Treistman, S. N. (2008). BK channel subunit composition modulates molecular tolerance to ethanol. *Alcohol Clin. Exp. Res.* 32, 1207–1216. doi: 10.1111/j.1530-0277.2008.00704.x

Feinberg-Zadek, P. L., and Treistman, S. N. (2007). Beta-subunits are important modulators of the acute response to alcohol in human BK channels. *Alcohol Clin. Exp. Res.* 31, 737–744. doi: 10.1111/j.1530-0277.2007.00371.x

Fernández-Mariño, A. I., Porras-González, C., González-Rodríguez, P., Selent, J., Pastor, M., Ureña, J., et al. (2012). Tungstate activates BK channels in a β

subunit- and Mg2+-dependent manner: relevance for arterial vasodilatation. *Cardiovasc. Res.* 95, 29–38. doi: 10.1093/cvr/cvs139

Fernández-Mariño, A. I., Valverde, M. A., and Fernández-Fernández, J. M. (2014). BK channel activation by tungstate requires the β1 subunit extracellular loop residues essential to modulate voltage sensor function and channel gating. *Pflügers Arch.* 466, 1365–1375. doi: 10.1007/s00424-013-1379-9

Fukao, M., Mason, H. S., Kenyon, J. L., Horowitz, B., and Keef, K. D. (2001). Regulation of BK(Ca) channels expressed in human embryonic kidney 293 cells by epoxyeicosatrienoic acid. *Mol. Pharmacol.* 59, 16–23. doi: 10.1124/mol.59.1.16

Galvez, A., Gimenez-Gallego, G., Reuben, J. P., Roy-Contancin, L., Feigenbaum, P., Kaczorowski, G. J., et al. (1990). Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion *Buthus tamulus*. *J. Biol. Chem.* 265, 11083–11090.

Gan, G., Yi, H., Chen, M., Sun, L., Li, W., Wu, Y., et al. (2008). Structural basis for toxin resistance of beta4-associated calcium-activated potassium (BK) channels. *J. Biol. Chem.* 283, 24177–24184. doi: 10.1074/jbc.M800179200

Gao, Y. D., and Garcia, M. L. (2003). Interaction of agitoxin2, charybdotoxin, and iberiotoxin with potassium channels: selectivity between voltage-gated and Maxi-K channels. *Proteins* 52, 146–154. doi: 10.1002/prot.10341

Garcia-Calvo, M., Knaus, H. G., McManus, O. B., Giangiacomo, K. M., Kaczorowski, G. J., and Garcia, M. L. (1994). Purification and reconstitution of the high-conductance, calcium-activated potassium channel from tracheal smooth muscle. *J. Biol. Chem.* 269, 676–682.

Garcia-Valdes, J., Zamudio, F. Z., Toro, L., and Possani, L. D. (2001). Slotoxin, alphaKTx1.11, a new scorpion peptide blocker of MaxiK channels that differentiates between alpha and alpha+beta (beta1 or beta4) complexes. *FEBS Lett.* 505, 369–373. doi: 10.1016/S0014-5793(01)02791-0

Giangiacomo, K. M., Garcia, M. L., and McManus, O. B. (1992). Mechanism of iberiotoxin block of the large-conductance calcium-activated potassium channel from bovine aortic smooth muscle. *Biochemistry* 31, 6719–6727.

Giangiacomo, K. M., Kamassah, A., Harris, G., and McManus, O. B. (1998). Mechanism of maxi-K channel activation by dehydrosoyasaponin-I. *J. Gen. Physiol.* 112, 485–501.

González, C., Baez-Nieto, D., Valencia, I., Oyarzún, I., Rojas, P., Naranjo, D., et al. (2012). $K^{(+)}$ channels: function-structural overview. *Compr. Physiol.* 2, 2087–2149. doi: 10.1002/cphy.c110047

Gribkoff, V. K., Lum-Ragan, J. T., Boissard, C. G., Post-Munson, D. J., Meanwell, N. A., Starrett, J. E. Jr., et al. (1996). Effects of channel modulators on cloned large-conductance calcium-activated potassium channels. *Mol. Pharmacol.* 50, 206–217.

Gribkoff, V. K., Starrett, J. E. Jr., Dworetzky, S. I., Hewawasam, P., Boissard, C. G., Cook, D. A., et al. (2001). Targeting acute ischemic stroke with a calcium-sensitive opener of maxi-K potassium channels. *Nat. Med.* 7, 471–477. doi: 10.1038/86546

Gruss, M., Henrich, M., König, P., Hempelmann, G., Vogel, W., and Scholz, A. (2001). Ethanol reduces excitability in a subgroup of primary sensory neurons by activation of BK(Ca) channels. *Eur. J. Neurosci.* 14, 1246–1256. doi: 10.1046/j.0953-816x.2001.01754.x

Ha, T. S., Heo, M. S., and Park, C. S. (2004). Functional effects of auxiliary beta4-subunit on rat large-conductance $Ca^{(2+)}$-activated $K^{(+)}$ channel. *Biophys. J.* 86, 2871–2882. doi: 10.1016/S0006-3495(04)74339-8

Hanner, M., Schmalhofer, W. A., Munujos, P., Knaus, H. G., Kaczorowski, G. J., and Garcia, M. L. (1997). The beta subunit of the high-conductance calcium-activated potassium channel contributes to the high-affinity receptor for charybdotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 94, 2853–2858.

Hanner, M., Vianna-Jorge, R., Kamassah, A., Schmalhofer, W. A., Knaus, H. G., Kaczorowski, G. J., et al. (1998). The beta subunit of the high conductance calcium-activated potassium channel. Identification of residues involved in charybdotoxin binding. *J. Biol. Chem.* 273, 16289–16296.

Henney, N. C., Elford, C., Reviriego, P., Campbell, A. K., Wann, K. T., and Evans, B. A. J. (2009). A large-conductance (BK) potassium channel subtype affects both growth and mineralization of human osteoblasts. *Am. J. Physiol. Cell. Physiol.* 297, C1397–C1408. doi: 10.1152/ajpcell.00311.2009

Holdiman, A. J., Fergus, D. J., and England, S. K. (2002). 17beta-Estradiol upregulates distinct maxi-K channel transcripts in mouse uterus. *Mol. Cell. Endocrinol.* 192, 1–6. doi: 10.1016/S0303-7207(02)00136-3

Horrigan, F. T. (2012). Perspectives on: conformational coupling in ion channels: conformational coupling in BK potassium channels. *J. Gen. Physiol.* 140, 625–634. doi: 10.1085/jgp.201210849

Hoshi, T., Tian, Y., Xu, R., Heinemann, S. H., and Hou, S. (2013c). Mechanism of the modulation of BK potassium channel complexes with different auxiliary subunit compositions by the omega-3 fatty acid DHA. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4822–4827. doi: 10.1073/pnas.1222003110

Hoshi, T., Wissuwa, B., Tian, Y., Tajima, N., Xu, R., Bauer, M., et al. (2013a). Omega-3 fatty acids lower blood pressure by directly activating large-conductance Ca²?-dependent K? channels. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4816–4821. doi: 10.1073/pnas.1221997110

Hoshi, T., Xu, R., Hou, S., Heinemann, S. H., and Tian, Y. (2013b). A point mutation in the human *Slo1* channel that impairs its sensitivity to omega-3 docosahexaenoic acid. *J. Gen. Physiol.* 142, 507–522. doi: 10.1085/jgp.201311061

Jaggar, J. J., Porter, V. A., Lederer, W. J., and Nelson, M. T. (2000). Calcium sparks in smooth muscle. *Am. J. Physiol. Cell. Physiol.* 278, C235–C256.

Ji, Y. H., Wang, W. X., Ye, J. G., He, L. L., Li, Y. J., Yan, Y. P., et al. (2003). Martentoxin, a novel K^+-channel-blocking peptide: purification, cDNA and genomic cloning, and electrophysiological and pharmacological characterization. *J. Neurochem.* 84, 325–335. doi: 10.1046/j.1471-4159.2003.01516.x

Kaczorowski, G. J., and Garcia, M. L. (1999). Pharmacology of voltage-gated and calcium-activated potassium channels. *Curr. Opin. Chem. Biol.* 3, 448–458.

King, J. T., Lovell, P. V., Rishniw, M., Kotlikoff, M. I., Zeeman, M. L., and McCobb, D. P. (2006). Beta2 and beta4 subunits of BK channels confer differential sensitivity to acute modulation by steroid hormones. *J. Neurophysiol.* 95, 2878–2888. doi: 10.1152/jn.01352.2005

Knaus, H. G., Garcia-Calvo, M., Kaczorowski, G. J., and Garcia, M. L. (1994). Subunit composition of the high conductance calcium-activated potassium channel from smoth muscle, a representative of the *mslo* and slowpoke family of potassium channels. *J. Biol. Chem.* 269, 3921–3924.

Knott, T. K., Dopico, A. M., Dayanithi, G., Lemos, J., and Treistman, S. N. (2002). Integrated channel plasticity contributes to alcohol tolerance in neurohypophysial terminals. *Mol. Pharmacol.* 62, 135–142. doi: 10.1124/mol.62.1.135

Korovkina, V. P., Brainard, A. M., Ismail, P., and Schmidt, T. J. (2004). Estradiol binding to maxi-K channels induces their down-regulation via proteasomal degradation. *J. Biol. Chem.* 279, 1217–1223. doi: 10.1074/jbc.M309158200

Kreifeldt, M., Le, D., Treistman, S. N., Koob, G. F., and Contet, C. (2013). BK channel β1 and β4 auxiliary subunits exert opposite influences on escalated ethanol drinking in dependent mice. *Front. Integr. Neurosci.* 7:105. doi: 10.3389/fnint.2013.00105

Lai, L. H., Wang, R. X., Jiang, W. P., Yang, X. J., Song, J. P., Li, X. R., et al. (2009). Effects of docosahexaenoic acid on large-conductance Ca^{2+}-activated K^+ channels and voltage-dependent K^+ channels in rat coronary artery smooth muscle cells. *Acta Pharmacol. Sin.* 30, 314–320. doi: 10.1038/aps.2009.7

Latorre, R., Morera, F. J., and Zaelzer, C. (2010). Allosteric interactions and the modular nature of the voltage- and Ca^{2+}-activated (BK) channel. *J. Physiol.* 588, 3141–3148. doi: 10.1113/jphysiol.2010.191999

Ledoux, J., Werner, M. E., Brayden, J. E., and Nelson, M. T. (2006). Calcium-activated potassium channels and the regulation of vascular tone. *Physiology (Bethesda)* 21, 69–78. doi: 10.1152/physiol.00040.2005

Li, M., Chang, S., Yang, L., Shi, J., McFarland, K., Yang, X., et al. (2014). Conopeptide Vt3.1 preferentially inhibits BK potassium channels containing β4 subunits via Electrostatic. *J. Biol. Chem.* 289, 4735–4742. doi: 10.1074/jbc.M113.535859

Liu, G., Niu, X., Wu, R. S., Chudasama, N., Yao, Y., Jin, X., et al. (2010). Location of modulatory beta subunits in BK potassium channels. *J. Gen. Physiol.* 135, 449–459. doi: 10.1085/jgp.201010417

Ma, Z., Lou, X. J., and Horrigan, F. T. (2006). Role of charged residues in the S1–S4 voltage sensor of BK channels. *J. Gen. Physiol.* 127, 309–328. doi: 10.1085/jgp.200509421

MacKinnon, R., and Miller, C. (1988). Mechanism of charybdotoxin block of the high-conductance, Ca^{2+}- activated K^+ channel. *J. Gen. Physiol.* 91, 335–349.

Martin, G. E., Hendrickson, L. M., Penta, K. L., Friesen, R. M., Pietrzykowski, A. Z., Tapper, A. R., et al. (2008). Identification of a BK channel auxiliary protein controlling molecular and behavioral tolerance to alcohol. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17543–17548. doi: 10.1073/pnas.0801068105

Martin, G., Puig, S., Pietrzykowski, A., Zadek, P., Emery, P., and Treistman, S. (2004). Somatic localization of a specific large-conductance calcium-activated

potassium channel subtype controls compartmentalized ethanol sensitivity in the nucleus accumbens. *J. Neurosci.* 24, 6563–6572. doi: 10.1523/JNEUROSCI.0684-04.2004

Martín, P., Moncada, M., Enrique, N., Asuaje, A., Valdez Capuccino, J. M., Gonzalez, C., et al. (2013). Arachidonic acid activation of BKCa (*Slo1*) channels associated to the β (1)-subunit in human vascular smooth muscle cells. *Pflügers Arch.* 105, 17543–17548. doi: 10.1007/s00424-013-1422-x

McManus, O. B., Harris, G. H., Giangiacomo, K. M., Feigenbaum, P., Reuben, J. P., Addy, M. E., et al. (1993). An activator of calcium-dependent potassium channels isolated from a medicinal herb. *Biochemistry* 32, 6128–6133.

McManus, O. B., Helms, L. M., Pallanck, L., Ganetzky, B., Swanson, R., and Leonard, R. J. (1995). Functional role of the beta subunit of high conductance calcium-activated potassium channels. *Neuron* 14, 645–650.

Meera, P., Wallner, M., Jiang, Z., and Toro, L. (1996). A calcium switch for the functional coupling between alpha (*hslo*) and beta subunits (Kv, cabeta) of maxi K channels. *FEBS Lett.* 385, 127–128.

Meera, P., Wallner, M., Song, M., and Toro, L. (1997). Large conductance voltage- and calcium-dependent K^+ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0-S6), an extracellular N terminus, and an intracellular (S9-S10) C terminus. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14066–14071.

Meera, P., Wallner, M., and Toro, L. (2000). A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca^{2+}-activated K^+ channel resistant to charybdotoxin and iberiotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5562–5567. doi: 10.1073/pnas.100118597

Miller, C., Moczydlowski, E., Latorre, R., and Phillips, M. (1985). Charybdotoxin, a protein inhibitor of single Ca^{2+} channels from mammalian skeletal muscle. *Nature* 313, 316–318.

Morimoto, T., Sakamoto, K., Sade, H., Ohya, S., Muraki, K., and Imaizumi, Y. (2007). Voltage-sensitive oxonol dyes are novel large-conductance Ca^{2+}-activated K^+ channel activators selective for beta1 and beta4 but not for beta2 subunits. *Mol. Pharmacol.* 71, 1075–1088. doi: 10.1124/mol.106.031146

Mullmann, T. J., Munujos, P., Garcia, M. L., and Giangiacomo, K. M. (1999). Electrostatic mutations in iberiotoxin as a unique tool for probing the electrostatic structure of the maxi-K channel outer vestibule. *Biochemistry* 38, 2395–2402.

Nagar, D., Liu, X. T., and Rosenfeld, C. R. (2005). Estrogen regulates beta1-subunit expression in $Ca^{(2+)}$-activated $K^{(+)}$ channels in arteries from reproductive tissues. *Am. J. Physiol. Heart Circ. Physiol.* 289, H1417–H1427. doi: 10.1152/ajpheart.01174.2004

Nimigean, C. M., and Maglebly, K. L. (2000). Functional coupling of the beta(1) subunit to the large conductance $Ca^{(2+)}$-activated $K^{(+)}$ channel in the absence of $Ca^{(2+)}$. Increased $Ca^{(2+)}$ sensitivity from a $Ca^{(2+)}$-independent mechanism. *J. Gen. Physiol.* 115, 719–736. doi: 10.1085/jgp.115.6.719

Ohya, S., Fujimori, T., Kimura, T., Yamamura, H., and Imaizumi, Y. (2010). Novel spliced variants of large-conductance $Ca^{(2+)}$-activated $K^{(+)}$-channel β2-subunit in human and rodent pancreas. *J. Pharmacol. Sci.* 114, 198–205. doi: 10.1254/jphs.10159FP

Ohya, S., Kuwata, Y., Sakamoto, K., Muraki, K., and Imaizumi, Y. (2005). Cardioprotective effects of estradiol include the activation of large-conductance Ca^{2+}-activated K^+ channels in cardiac mitochondria. *Am. J. Physiol. Heart. Circ. Physiol.* 289, H1635–H1642. doi: 10.1152/ajpheart.00016.2005

Omasits, U., Ahrens, C. H., Müller, S., and Wollscheid, B. (2014). Protter: interactive protein feature visualization and integration with experimental proteomic data. *Bioinformatics* 30, 884–886. doi: 10.1093/bioinformatics/btt607

Orio, P., and Latorre, R. (2005). Differential effects of beta 1 and beta 2 subunits on BK channel activity. *J. Gen. Physiol.* 125, 395–411. doi: 10.1085/jgp.200409236

Orio, P., Rojas, P., Ferreira, G., and Latorre, R. (2002). New disguises for an old channel: MaxiK channel beta-subunits. *News Physiol. Sci.* 17, 156–161. doi: 10.1152/nips.01387.2002

Park, C. S., and Miller, C. (1992). Interaction of charybdotoxin with permeant ions inside the pore of a K^+ channel. *Neuron* 9, 307–313.

Peng, W., Hoidal, J. R., and Farrukh, I. S. (1996). Regulation of $Ca^{(2+)}$-activated K^+ channels in pulmonary vascular smooth muscle cells: role of nitric oxide. *J. Appl. Physiol.* 81, 1264–1272.

Rabin, O., Deutsch, J., Grange, E., Pettigrew, K. D., Chang, M. C., Rapoport, S. I., et al. (1997). Changes in cerebral acyl-CoA concentrations following ischemia-reperfusion in awake gerbils. *J. Neurochem.* 68, 2111–2118.

Reinhart, P. H., Chung, S., and Levitan, I. B. (1989). A family of calcium-dependent potassium channels from rat brain. *Neuron* 2, 1031–1041.

Riazi, M. A., Brinkman-Mill, P., Johnson, A., Naylor, S. L., Minoshima, S., Shimizu, N., et al. (1999). Identification of a putative regulatory subunit of a calcium-activated potassium channel in the dup(3q) syndrome region and a related sequence on 22q11.2. *Genomics* 65, 90–94.

Robitaille, R., and Charlton, M. P. (1992). Presynaptic Calcium Signals and Transmitter by Calcium-activated Potassium Channels. *J. Neurosci.* 12, 297–305.

Savalli, N., Kondratiev, A., de Quintana, S. B., Toro, L., and Olcese, R. (2007). Modes of operation of the BKCa channel beta2 subunit. *J. Gen. Physiol.* 130, 117–131. doi: 10.1085/jgp.200709803

Scornik, F. S., Bucciero, R. S., Wu, Y., Selga, E., Bosch Calero, C., Brugada, R., et al. (2013). DiBAC4(3) hits a "sweet spot" for the activation of arterial large-conductance Ca^{2+}-activated potassium channels independently of the β1-subunit. *Am. J. Physiol. Heart. Circ. Physiol.* 304, H1471–H1482. doi: 10.1152/ajpheart.00939.2012

Shi, J., He, H. Q., Zhao, R., Duan, Y. H., Chen, J., Chen, Y., et al. (2008). Inhibition of martentoxin on neuronal BK channel subtype (alpha+beta4): implications for a novel interaction model. *Biophys. J.* 94, 3706–3713. doi: 10.1529/biophysj.107.122150

Simoncini, T., Mannella, P., Fornari, L., Caruso, A., Varone, G., and Genazzani, A. R. (2004). Genomic and non-genomic effects of estrogens on endothelial cells. *Steroids* 69, 537–542. doi: 10.1016/j.steroids.2004.05.009

Sun, X., Yao, H., Zhou, D., Gu, X., and Haddad, G. G. (2008). Modulation of *hslo* BK current inactivation by fatty acid esters of CoA. *J. Neurochem.* 104, 1394–1403. doi: 10.1111/j.1471-4159.2007.05083.x

Sun, X., Zhou, D., Zhang, P., Moczydlowski, E. G., and Haddad, G. G. (2007). Beta-subunit-dependent modulation of *hslo* BK current by arachidonic acid. *J. Neurophysiol.* 97, 62–69. doi: 10.1152/jn.00700.2006

Swei, A., Lacy, F., Delano, F. A., Parks, D. A., and Schmid-Schönbein, G. W. (1999). A mechanism of oxygen free radical production in the Dahl hypertensive rat. *Microcirculation* 6, 179–187.

Tanaka, Y., Meera, P., Song, M., Knaus, H. G., and Toro, L. (1997). Molecular constituents of maxi KCa channels in human coronary smooth muscle: predominant alpha + beta subunit complexes. *J. Physiol.* 502, 545–557.

Tao, J., Shi, J., Liu, Z. R., and Ji, Y. H. (2012). Martentoxin: a unique ligand of BK channels. *Sheng Li Xue Bao* 64, 355–364.

Tao, J., Shi, J., Yan, L., Chen, Y., Duan, Y. H., Ye, P., et al. (2011). Enhancement effects of martentoxin on glioma BK channel and BK channel (α+β1) subtypes. *PLoS ONE* 6:e15896. doi: 10.1371/journal.pone.0015896

Tao, J., Zhou, Z. L., Wu, B., Shi, J., Chen, X. M., and Ji, Y. H. (2014). Recombinant expression and functional characterization of martentoxin: a selective inhibitor for BK channel (α + β4). *Toxins* 6, 1419–1433. doi: 10.3390/toxins6041419

Thiede, A., Gellerich, F. N., Schönfeld, P., and Siemen, D. (2012). Complex effects of 17β-estradiol on mitochondrial function. *Biochim. Biophys. Acta* 1817, 1747–1753. doi: 10.1016/j.bbabio.2012.02.034

Torres, Y. P., Morera, F. J., Carvacho, I., and Latorre, R. (2007). A marriage of convenience: beta-subunits and voltage-dependent K^+ channels. *J. Biol. Chem.* 282, 24485–24489. doi: 10.1074/jbc.R700022200

Treistman, S. N., and Martin, G. E. (2009). BK Channels: mediators and models for alcohol tolerance. *Trends Neurosci.* 32, 629–637. doi: 10.1016/j.tins.2009.08.001

Tseng-Crank, J., Godinot, N., Johansen, T. E., Ahring, P. K., Strøbaek, D., Mertz, R., et al. (1996). Cloning, expression, and distribution of a $Ca^{(2+)}$-activated K^+ channel beta-subunit from human brain. *Proc. Natl. Acad. Sci. U.S.A.* 93, 9200–9205.

Uebele, V. N., Lagrutta, A., Wade, T., Figueroa, D. J., Liu, Y., McKenna, E., et al. (2000). Cloning and functional expression of two families of beta-subunits of the large conductance calcium-activated K^+ channel. *J. Biol. Chem.* 275, 23211–23218. doi: 10.1074/jbc.M910187199

Valverde, M. A., Rojas, P., Amigo, J., Cosmelli, D., Orio, P., Bahamonde, M. I., et al. (1999). Acute activation of Maxi-K channels (*hslo*) by estradiol binding to the beta subunit. *Science* 285, 1929–1931.

Wallner, M., Meera, P., Ottolia, M., Kaczorowski, G. J., Latorre, R., Garcia, M. L., et al. (1995). Characterization of and modulation by a beta-subunit of a human maxi KCa channel cloned from myometrium. *Receptors Channels* 3, 185–199.

Wallner, M., Meera, P., and Toro, L. (1996). Determinant for beta-subunit regulation in high-conductance voltage-activated and $Ca^{(2+)}$-sensitive K^+ channels: an additional transmembrane region at the N terminus. *Proc. Natl. Acad. Sci. U.S.A.* 93, 14922–14927.

Wallner, M., Meera, P., and Toro, L. (1999). Molecular basis of fast inactivation in voltage and Ca^{2+}-activated K^+ channels: a transmembrane beta-subunit homolog. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4137–4142.

Wang, B., Rothberg, B. S., and Brenner, R. (2006). Mechanism of beta4 subunit modulation of BK channels. *J. Gen. Physiol.* 127, 449–465. doi: 10.1085/jgp.200509436

Wang, Y., Chen, X., Zhang, N., Wu, G., and Wu, H. (2005). The solution structure of BmTx3B, a member of the scorpion toxin subfamily alpha-KTx 16. *Proteins* 58, 489–497. doi: 10.1002/prot.20322

Wang, Y. W., Ding, J. P., Xia, X. M., and Lingle, C. J. (2002). Consequences of the stoichiometry of *Slo1* alpha and auxiliary beta subunits on functional properties of large-conductance Ca^{2+}-activated K^+ channels. *J. Neurosci.* 22, 1550–1561.

Weiger, T. M., Holmgvist, M. H., Levitan, I. B., Clark, F. T., Sprague, S., and Huang, W. J., et al. (2000). A novel nervous system beta subunit that downregulates human large conductance calcium-dependent potassium channels. *J. Neurosci.* 20, 3563–3570.

Wu, L., Cao, K., Lu, Y., and Wang, R. (2002). Different mechanisms underlying the stimulation of K(Ca) channels by nitric oxide and carbon monoxide. *J. Clin. Invest.* 110, 691–700. doi: 10.1172/JCI15316

Wu, R. S., Chudasama, N., Zakharov, S. I., Doshi, D., Motoike, H., Liu, G., et al. (2009). Location of the β4 transmembrane helices in the BK potassium channel. *J. Neurosci.* 29, 8321–8328. doi: 10.1523/JNEUROSCI.6191-08.2009

Wu, R. S., Liu, G., Zakharov, S. I., Chudasama, N., Motoike, H., Karlin, A., et al. (2013). Positions of β2 and β3 subunits in the large-conductance calcium-and voltage-activated BK potassium channel. *J. Gen. Physiol.* 141, 105–117. doi: 10.1085/jgp.201210891

Wu, R. S., and Marx, S. O. (2010). The BK potassium channel in the vascular smooth muscle and kidney: α- and β-subunits. *Kidney Int.* 78, 963–974. doi: 10.1038/ki.2010.325

Wu, S. N., Li, H. F., and Chiang, H. T. (2000a). Actions of epoxyeicosatrienoic acid on large-conductance $Ca^{(2+)}$-activated $K^{(+)}$ channels in pituitary GH(3) cells. *Biochem. Pharmacol.* 60, 251–262. doi: 10.1016/S0006-2952(00)00317-8

Wu, S. N., Li, H. F., and Lo, Y. C. (2000b). Characterization of Tetrandrine-Induced Inhibition of Large- Conductance Calcium-Activated Potassium Channels in a Human Endothelial Cell Line (HUV-EC-C). *J. Pharmacol. Exp. Ther.* 292, 188–195.

Xia, X. M., Ding, J. P., and Lingle, C. J. (1999). Molecular basis for the inactivation of Ca^{2+}- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. *J. Neurosci.* 19, 5255–5264.

Xia, X. M., Ding, J. P., and Lingle, C. J. (2003). Inactivation of BK channels by the NH2 terminus of the beta2 auxiliary subunit: an essential role of a terminal peptide segment of three hydrophobic residues. *J. Gen. Physiol.* 121, 125–148. doi: 10.1085/jgp.20028667

Xia, X. M., Ding, J. P., Zeng, X. H., Duan, K. L., and Lingle, C. J. (2000). Rectification and rapid activation at low Ca^{2+} of Ca^{2+}-activated, voltage-dependent BK currents: consequences of rapid inactivation by a novel beta subunit. *J. Neurosci.* 20, 4890–4903.

Zeng, X., Xia, X. M., and Lingle, C. J. (2008). Species-specific Differences among KCNMB3 BK beta3 auxiliary subunits: some beta3 N-terminal variants may be primate-specific subunits. *J. Gen. Physiol.* 132, 115–129. doi: 10.1085/jgp.200809969

Zheng, H. F., Li, X. L., Jin, Z. Y., Sun, J. B., Li, Z. L., and Xu, W. X. (2005). Effects of unsaturated fatty acids on calcium-activated potassium current in gastric myocytes of guinea pigs. *World J. Gastroenterol.* 11, 672–675.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 July 2014; accepted: 16 September 2014; published online: 10 October 2014.

*Citation: Torres YP, Granados ST and Latorre R (2014) Pharmacological consequences of the coexpression of BK channel α and auxiliary β subunits. Front. Physiol. **5**:383. doi: 10.3389/fphys.2014.00383*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in Physiology

REVIEW
published: 31 March 2015
doi: 10.3389/fphys.2015.00104

Mitochondrial BK_{Ca} channel

Enrique Balderas[1], *Jin Zhang*[2], *Enrico Stefani*[1,3,4,5] *and Ligia Toro*[1,2,4,5]*

[1] Department of Anesthesiology, University of California, Los Angeles, Los Angeles, CA, USA, [2] Deparment of Molecular and Medical Pharmacology, University of California, Los Angeles, Los Angeles, CA, USA, [3] Department of Physiology, University of California, Los Angeles, Los Angeles, CA, USA, [4] Brain Research Institute, University of California, Los Angeles, Los Angeles, CA, USA, [5] Cardiovascular Research Laboratory, University of California, Los Angeles, Los Angeles, CA, USA

Since its discovery in a glioma cell line 15 years ago, mitochondrial BK_{Ca} channel (mitoBK_{Ca}) has been studied in brain cells and cardiomyocytes sharing general biophysical properties such as high K^+ conductance (~300 pS), voltage-dependency and Ca^{2+}-sensitivity. Main advances in deciphering the molecular composition of mitoBK_{Ca} have included establishing that it is encoded by the *Kcnma1* gene, that a C-terminal splice insert confers mitoBK_{Ca} ability to be targeted to cardiac mitochondria, and evidence for its potential coassembly with β subunits. Notoriously, β1 subunit directly interacts with cytochrome c oxidase and mitoBK_{Ca} can be modulated by substrates of the respiratory chain. mitoBK_{Ca} channel has a central role in protecting the heart from ischemia, where pharmacological activation of the channel impacts the generation of reactive oxygen species and mitochondrial Ca^{2+} preventing cell death likely by impeding uncontrolled opening of the mitochondrial transition pore. Supporting this view, inhibition of mitoBK_{Ca} with Iberiotoxin, enhances cytochrome c release from glioma mitochondria. Many tantalizing questions remain open. Some of them are: how is mitoBK_{Ca} coupled to the respiratory chain? Does mitoBK_{Ca} play non-conduction roles in mitochondria physiology? Which are the functional partners of mitoBK_{Ca}? What are the roles of mitoBK_{Ca} in other cell types? Answers to these questions are essential to define the impact of mitoBK_{Ca} channel in mitochondria biology and disease.

Keywords: mitochondria, potassium channels, BK channels, MaxiK channels, subunit composition, permeability transition pore, ischemia reperfusion injury

OPEN ACCESS

Edited by:
Alex M. Dopico, The University of Tennessee Health Science Center, USA

Reviewed by:
Adam Szewczyk, Nencki Institute of Experimental Biology, Poland
Anna Bukiya, The University of Tennessee Health Science Center, USA

***Correspondence:**
Ligia Toro, Division of Molecular Medicine, Department of Anesthesiology, David Geffen School of Medicine at UCLA, BH-509A CHS, Box 957115, Los Angeles, CA 90095-7115, USA
ltoro@ucla.edu

Specialty section:
This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology

Received: *29 January 2015*
Accepted: *13 March 2015*
Published: *31 March 2015*

Citation:
Balderas E, Zhang J, Stefani E and Toro L (2015) Mitochondrial BK_{Ca} channel. Front. Physiol. 6:104. doi: 10.3389/fphys.2015.00104

Introduction

Mitochondria are key organelles defining cell fate and thus, much interest has developed in understanding the functional role of proteins present at its membranes. Mitochondria are shaped by an outer membrane, and an internal membrane that separates the intermembrane space and the matrix. The internal membrane is particularly important because it houses the respiratory chain protein complexes where reactive oxygen species and ATP are produced and also serves to delimit a Ca^{2+} storage space, making mitochondria not only producers of ATP but also regulators of Ca^{2+} and redox homeostasis. The list of proteins uncovered at the inner membrane keeps increasing, among them, K^+ selective channels. A recent review by Szabo and Zoratti (2014) summarizes the evidence for the presence/role of: ATP-sensitive (K_{ATP}), small-conductance calcium-activated (SK_{Ca}), intermediate-conductance calcium-activated (IK_{Ca}), large-conductance, voltage and calcium-activated (BK_{Ca}), voltage-gated 1.3 (Kv1.3), two-pore domain acid-sensitive type 3 (TASK-3), and pH-sensitive K^+channels. The subject of this review is the mammalian

mitochondrial BK_{Ca} channel (mitoBK_{Ca}) that has sparked much interest primarily because of its role in protecting the heart from ischemic insult, as first demonstrated by Xu et al. (2002).

General Design of BK_{Ca} Channels

BK_{Ca} channels at the plasma membrane are characterized by having a large conductance, and by sensing changes in membrane potential and intracellular calcium (for a recent review see Contreras et al., 2013). Structure-function studies have ascribed these properties to distinct domains of the 7 transmembrane (S0–S7) α subunit -encoded by the *Kcnma1* gene- that has an extracellular N-terminus and an intracellular C-terminus (**Figure 1A**). Four α subunits form a functional channel. The voltage sensing domain encompasses S0–S4 segments, the pore/gate domain includes S5–S6 and corresponding linker which lines the pore selectivity filter of the tetrameric channel, and the Ca^{2+}sensing domain is located at the C-terminus. Pore residues located extracellularly comprise the receptor for pore blockers, Charybdotoxin (ChTx) and Iberiotoxin (IbTx) (Gao and Garcia, 2003; Banerjee et al., 2013). The intracellular C-terminus, which occupies two thirds of the whole protein, contains two regions that can sense Ca^{2+} known as the regulators of K^+ conductance (RCK) 1 and 2. Mutagenesis studies have shown that RCK1 contains two critical aspartates (D362/D367) while RCK2 contains 5 consecutive aspartates in the "Ca^{2+} bowl" that together are sufficient for BK_{Ca} activation at physiological Ca^{2+} concentrations (Schreiber and Salkoff, 1997; Xia et al., 2002). However, recent crystal structures have only detected a single site of Ca^{2+} binding located in the "Ca^{2+} bowl" and utilizing two main-chain carbonyl oxygens of Q889 and D892 and side-chain carboxylate oxygens of D895 and D897 (underlined in ^{889}QFLDQDDDDPDT^{901}) (Yuan et al., 2010, 2012). In addition to Ca^{2+}, BK_{Ca} can also be activated by Mg^{2+} in the millimolar range. Interestingly, residues of distinct α subunits form part of the Mg^{2+} sensor, namely D99 and N172 from the voltage sensing domain of one subunit with E374 and E399 from the RCK1 domain of a different subunit (Shi et al., 2002; Yang et al., 2008).

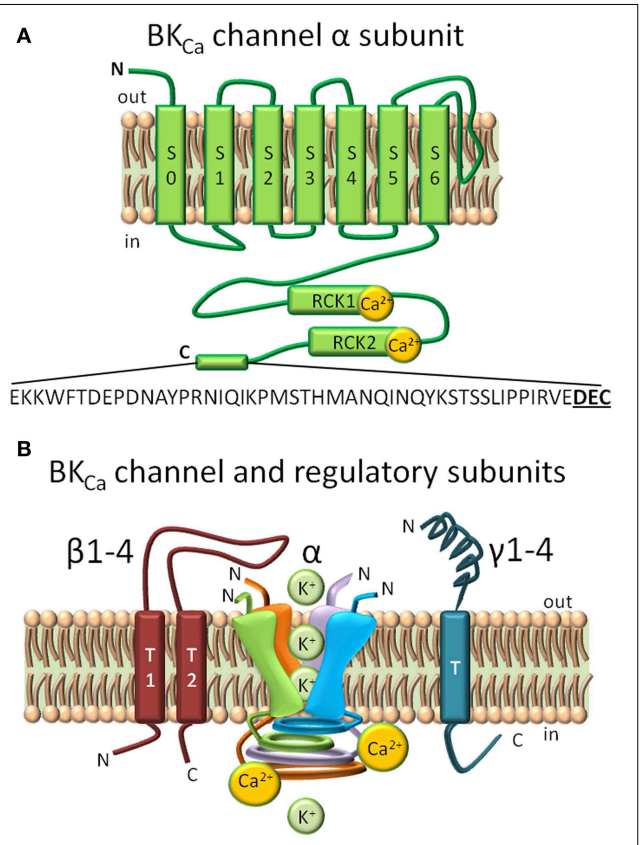


FIGURE 1 | Structural domains in BK_{Ca} channels and regulatory subunits. (A) BK_{Ca} is composed by 7 transmembrane domains (S0–S7) and a long intracellular C-terminus. S0–S4 form the voltage sensing domain, and S5–S6 conform the pore-gating domain. Ca^{2+} biding sites are highlighted in the Regulator of Potassium Conductance (RCK) 1 and RCK2 domains. A C-terminal 50 amino acid splice insert, DEC, is highlighted. **(B)** Regulatory BK_{Ca} subunits. Homotetramer model of the pore-forming α subunit, the two spanning domain regulatory β subunits (1–4), and single spanning domain γ (1–4) subunits. The loop of β4 subunit confers to BK_{Ca} α subunit its resistance to toxin inhibition (Meera et al., 2000).

The *Kcnma1* gene when transcribed can undergo extensive alternative splicing that give rise to multiple BK_{Ca} channel isoforms with varied functional characteristics including voltage/Ca^{2+} sensitivities, response to phosphorylation and arachidonic acid modulation, and subcellular localizations, including targeting to mitochondria as discussed later in this review (Saito et al., 1997; Tian et al., 2001; Zarei et al., 2004; Ma et al., 2007; Li et al., 2010; Singh et al., 2013).

BK_{Ca} channel functional heterogeneity is further increased by its association with modulatory β (1–4) or γ (Yan and Aldrich, 2010, 2012) subunits (**Figure 1B**) that are mostly tissue-specific and greatly modify functional and pharmacological characteristics like kinetics, Ca^{2+}/V sensitivities, and toxin blockade (Knaus et al., 1994; Wallner et al., 1999; Brenner et al., 2000; Meera et al., 2000; Uebele et al., 2000). Beta subunits (β1,β2,β4) can also act as modulators of channel density at the plasma membrane via endocytic processes (Toro et al., 2006; Zarei et al., 2007; Shruti et al., 2012; Cox et al., 2014). The relatively high tissue specificity of β subunits make them key in defining the function of BK_{Ca} channels in different organs, for example the abundance of β1 subunit in smooth muscle make them essential to maintain a healthy vascular tone.

Posttranslational modifications like lipidation and phosphorylation add another regulatory layer to BK_{Ca} function. For example, in the α subunit, internal myristoylation at N-terminal intracellular loops slows down the activation kinetics of BK_{Ca} channel and reduces its cell surface expression promoting endocytosis via a clathrin mediated mechanism (Alioua et al., 2011). Palmitoylation at S0–S1 linker also reduces cell surface expression, and at the spliced exon STREX (inserted at the C-terminus of BK_{Ca}) produces channels resistant to protein kinase C induced inhibition (Jeffries et al., 2010; Zhou et al., 2012). With respect to β subunits, juxtamembrane palmitoylation of the β4 subunit at its C-terminus promotes surface expression of BK_{Ca} α subunits but only when the latter contain a 50 amino acid C-terminal splice insert, named DEC (Chen et al., 2013). Most of the above

findings have been obtained in heterologous expression systems; the next challenge is to define the physiological or pathophysiological impact that these processes have in different organs of the body.

Discovery of BK_{Ca} Channel Activity at the Inner Mitochondrial Membrane and Biophysical Properties

The first evidence showing that BK_{Ca} channel with a conductance of about 300 pS (in 150 mM KCl) was present at the inner mitochondrial membrane was given by Siemen and coworkers in the late 90's; the channel was characterized using mitochondria devoid of external membranes (mitoplasts) of the glioma cell line, LN-229, and the patch clamp technique (Siemen et al., 1999). Since then, mitochondrial BK_{Ca} channels (mitoBK_{Ca}) with similar conductances ranging from 200 to 307 pS have been detected in other systems using channel reconstitution in lipid bilayers or by patch clamping mitoplasts. A single report shows a 564 pS channel in mitochondria of brain (**Table 1**).

We now know that mitoBK_{Ca} and plasma membrane BK_{Ca} channel pore-forming α subunits are encoded by the same gene (*Kcnma1*) (Singh et al., 2013) explaining why they share common basic biophysical properties including a large conductance, and being responsive to voltage and Ca^{2+}, although specific values may vary. A comparison of plasma membrane BK_{Ca} and mitoBK_{Ca} properties in a human glioma cell line (LN 229) shows that the conductance of the former was 199 ± 8 pS and of the latter was 278 ± 10 pS. Although both channels were voltage/Ca^{2+} dependent, their sensitivities were different. In the inside-out configuration, plasma membrane BK_{Ca} displayed a low sensitivity to voltage as it displayed a low open probability (Po) even at high potentials (Po < 0.1 at +80 mV and ∼0.4 at 100 mV) and 400 μM Ca^{2+} facing the cytosolic side of the channel. mitoBK_{Ca}, on the other hand, recorded on the on-mitoplast configuration and the same Ca^{2+} in the bath solution only needed a depolarization to −40 mV to reach a Po of ∼0.6 (Gu et al., 2014). Although in this configuration the precise Ca^{2+} concentration at the matrix side of the channel is difficult to establish, the authors showed that by decreasing Ca^{2+} in the bath to "zero" the channel Po decreased to ≤0.05 (see below for discussion of mitoBK_{Ca} orientation). Thus, assuming that both classes of channels could sense the same Ca^{2+} concentration, one possible explanation to these differences is that multiple BK_{Ca} isoforms exist, both at the plasma membrane and in mitochondria, which could result from a combination of factors including splice variation, association with auxiliary subunits or posttranslational modifications affecting how they respond to voltage and Ca^{2+}.

Along the above point of view, **Table 1** shows that different cell types appear to express mitoBK_{Ca} channels with varied voltage and Ca^{2+} sensitivities. For example, the cardiac mitoBK_{Ca} channel from guinea pig had a particularly high Po of ∼0.9 within a large voltage range (−60 to +60 mV) at 0.5 μM [Ca^{2+}] suggesting that its molecular composition (e.g., association with auxiliary subunits) may be substantially distinct from that expressed in glioma mitochondria which at 1 μM Ca^{2+} displays a Po of 0.5 at +41 mV (half activation potential, $V_{1/2}$ = 41 mV) (note that in both cases, experiments were performed in the on-mitoplast mode and Ca^{2+} was changed in the bath solution; thus, the

TABLE 1 | mitoBK_{Ca} biophysical properties in mammals.

Cell type/Organ/Method	Conductance, [K^+], mM Pipette/bath or cis/trans	$V_{½}$ or Open probability (Po) [Ca^{2+}], μM	Ca^{2+}, EC_{50}	References
Human glioma cell line (LN229) (on-mitoplast)	295 pS 150 K^+/150 K^+	At 8.7 Ca^{2+}, $V_{½}$ = −33 ±19 mV; At 1 Ca^{2+}, $V_{½}$ = 41 ± 23 mV	6.9 μM at −20 mV	Siemen et al., 1999
Guinea-pig ventricular myocytes (on-mitoplast)	307 pS 150 K^+/150 K^+	At 0.512 Ca^{2+}, Po ∼ 0.9 from −60 to +60 mV*	N/A	Xu et al., 2002
Rat ventricular myocytes (inside-out mitoplast)	270 pS 140 K^+/140 K^+	At 0.5 Ca^{2+}, Po = 0.0087 at +40 mV	N/A	Ohya et al., 2005
Human glioma cell line (LN229 and LN405) (on-mitoplast)	276 pS 150 K^+/150 K^+	At 200 Ca^{2+}, $V_{½}$ ∼ −42 mV*	N/A	Gu et al., 2007
Rat astrocytes (on-mitoplast)	295–296 pS 150 K^+/150 K^+	At 200 Ca^{2+}, $V_{½}$ ∼ −50 mV*	N/A	Cheng et al., 2008, 2011
Human endothelial cell line (EA.hy926) (on-mitoplast)	270 pS 150/150 K^+	At 100 Ca^{2+}, $V_{½}$ ∼ +20 mV*	N/A	Bednarczyk et al., 2013b
Rat whole brain Lipid bilayers	265 pS 50 K^+/450 K^+	At 0 Ca^{2+}, Po = 0.50 at +70 mV At 300 Ca^{2+}, Po = 0.77 at +70 mV	N/A	Skalska et al., 2009
Rat whole brain Lipid bilayers	211 pS 200 K^+/50 K^+	At "contaminant" Ca^{2+}, Po = 0.9 ± 0.01 at +40 mV, $V_{½}$ = 11 ± 1 mV	N/A	Fahanik-Babaei et al., 2011a
Rat whole brain Lipid bilayers	565 pS 200 K^+/50 K^+	At 100 Ca^{2+}, Po = 0.9 ± 0.05 at −40 to +40 mV. At 0 Ca^{2+}, Po = 0.8 at +20 mV and Po = 0.07 at −40 mV	N/A	Fahanik-Babaei et al., 2011b

** Estimated from published figure. On-mitoplast is also called mitoplast-attached configuration of the patch clamp technique. Abbreviations: $V_{½}$, half activation potential or potential where an open probability of 0.5 is achieved; EC_{50}, concentration of half maximal effect; N/A, not available.*

exact Ca^{2+} concentration in the matrix side is unknown). This variability is not exclusive of mitoBK$_{Ca}$ as different isoforms are also detected at the plasma membrane even within the same cell type. For example, BK$_{Ca}$ channels from coronary smooth muscle can display a predominant $V_{½}$ of −98 mV but also −66 mV, −38 mV, −16 mV and 21 mV (at 18 μM Ca^{2+}) (Tanaka et al., 1997) that could be explained by α + β1 channels with different β1 subunit stoichiometry; while skeletal muscle BK$_{Ca}$ single channels can have four-fold differences in $K_{0.5}$ for Ca^{2+} with an average of 14 ± 7 μM at +30 mV (McManus and Magleby, 1991).

Obviously, a detailed biophysical and molecular characterization of mitoBK$_{Ca}$is needed for each cell type to be able to understand the basis of their function.

Orientation of mitoBK$_{Ca}$; Is the Ca^{2+} Sensor Facing the Mitochondrial Matrix?

Siemen et al. (1999) showed in on-mitoplast patches that increasing Ca^{2+} in the bath increased channel Po; the EC_{50} for Ca^{2+} measured at +60 mV was ∼0.9 μM. The usage of a Ca^{2+} ionophore was dispensable, and thus, it was assumed that the normal Ca^{2+} import mitochondrial mechanisms were sufficient to increase matrix Ca^{2+} in the vicinity of mitoBK$_{Ca}$ Ca^{2+}sensor. In addition, mitoBK$_{Ca}$ was blocked by ChTx (the toxin binds plasma membrane BK$_{Ca}$ at the extracellular pore vestibule) applied to the patch pipette. ChTx displayed an EC_{50} of ∼1.5 nM and a Hill coefficient of 1.2 consistent with the sensitivity reported for plasma membrane BK$_{Ca}$ channels formed by α or α + β1 subunits (Meera et al., 2000). These results supported the idea that mitoBK$_{Ca}$ ChTx receptor (in the pore vestibule) is facing the mitochondrial intermembrane space and thus, the Ca^{2+} sensor (located in the opposite side of BK$_{Ca}$ protein) is facing the mitochondrial matrix.

Few years later Xu et al. (2002) investigated the presence of mitoBK$_{Ca}$ in ventricular mitoplasts from guinea pig also using on-mitoplast patches and isotonic 150 mM KCl. Ventricular myocytes contained mitochondria rich in mitoBK$_{Ca}$ whose conductance (∼300 pS) was similar to that observed in the glioma cells (295 pS) (**Table 1**). Ventricular mitoBK$_{Ca}$ activity was completely abolished by 200 nM ChTx applied to the patch pipette and the ensemble average patch currents augmented by increasing bath Ca^{2+} from 0.5 to 40 μM. In this case, the usage of a Ca^{2+} ionophore was also dispensable. Assuming that matrix Ca^{2+} was in equilibrium with the bath solution (aided by native Ca^{2+} import mechanisms), the results further supported the picture of mitoBK$_{Ca}$ with its ChTx receptor facing the intermitochondrial space and thus, its Ca^{2+} sensor facing the matrix side.

Other studies have supported the orientation of mitoBK$_{Ca}$ as being the pore vestibule facing the mitochondrial intermembrane space and the Ca^{2+} sensor in the matrix. Direct evidence using mitoplasts: (1) In the human glioma cell line LN-229, on-mitoplast patches showed an increased channel Po with matrix depolarization and exposing the external side of the mitoplast membrane to ChTx (using the whole-mitoplast or outside-out configurations and applying ChTx to the bath) reduced mitoBK$_{Ca}$ activity (Gu et al., 2007, 2014); (2) In rat astrocyte mitoplasts, IbTx added to the pipette in the on-mitoplast configuration reduced mitoBK$_{Ca}$ activity (Cheng et al., 2008), while depolarization of the inner side of the mitoplast (matrix side) promoted increased channel activity (Cheng et al., 2011). Indirect evidence using intact rat brain mitochondria: Ca^{2+}-induced depolarization was prevented by IbTx (50 nM) and ChTx (200 nM) (Skalska et al., 2009). Because the outer mitochondrial membrane is permeable to proteins up to 5 kDa and IbTx as well as ChTx are ∼4.2 kDa, both toxins are expected to traverse the outer membrane and reach the channel pore facing the intermembrane space; this arrangement would place the C-terminus facing the matrix.

The evidence supporting an opposite orientation, i.e., a C-terminus facing the intermembrane space is less clear. Recent recordings in inside-out mitoplast patches from a human astrocytoma cell line (U-87 MG) show a mitoBK$_{Ca}$ channel that was activated by hyperpolarization of the matrix side (positive potentials applied to the pipette interior) and blocked by IbTx also applied to the matrix side (bath solution) (Bednarczyk et al., 2013a). Assuming that this channel is encoded by the *Kcnma1* gene (encoding plasma membrane BK$_{Ca}$) (see below Singh et al., 2013), the above results would imply a pore vestibule facing the matrix and a C-terminus facing the intermembrane space. However, decreasing matrix Ca^{2+} decreased channel activity making the authors suggest that both IbTx and Ca^{2+} binding sites were probably facing the mitochondrial matrix. This hypothesis would support the existence of a mitoBK$_{Ca}$ channel unrelated to the plasma membrane BK$_{Ca}$, where the pore vestibule and the Ca^{2+} sensor are facing opposite sides of the plasma membrane (see **Figure 1**). It is evident that further studies on the orientation of mitoBK$_{Ca}$ channel at the inner mitochondrial membrane are needed.

mitoBK$_{Ca}$ Molecular Origin

mitoBK$_{Ca}$ molecular origin has been recently defined as the *Kcnma1* gene, which also encodes plasma membrane BK$_{Ca}$ (Singh et al., 2013). Utilizing an exon-scanning RT-PCR strategy of the mouse *Kcnma1* gene and ventriculocyte mRNAs (these cardiac cells were an excellent system for this quest since they are characterized by their lack of BK$_{Ca}$ channel activity at the plasma membrane) Singh et al., found transcript expression of 3 alternatively spliced exons, STREX, SV27 (27 amino acid insert) and the C-terminal 50 amino acid "DEC" exon. Notoriously, the amount of DEC exon transcripts equaled that of the total BK$_{Ca}$ predicting a mitoBK$_{Ca}$ tetrameric structure constituted by two subunits of BK$_{Ca}$ variant containing DEC insert. Indeed, expression of BK$_{Ca}$ constructs containing the DEC exon was sufficient for BK$_{Ca}$ targeting to mitochondria in adult ventriculocytes. Mass spectrometry analysis and functional data using BK$_{Ca}$ knockout mice further confirmed mitoBK$_{Ca}$ as being encoded by the *Kcnma1* gene.

Interestingly, the ability of DEC exon for targeting mitoBK$_{Ca}$ to mitochondria may vary depending on the cell type and/or its association with other subunits. In fact, expression in CHO cells of a BK$_{Ca}$-DEC variant (containing additional alternatively spliced exons including SV27) cloned from hair cells showed

prominent expression in Mitotracker labeled mitochondria but also at the cell periphery (Kathiresan et al., 2009). In contrast, expression of a DEC variant in COS-cells yielded signals confined to the endoplasmic reticulum (Ma et al., 2007). Thus, the DEC sequence may not be the only factor defining the targeting of BK_{Ca}-DEC to mitochondria in all cell types but cell-specific mechanisms may exist that facilitate its mitochondrial delivery.

mitoBK$_{Ca}$ and Regulatory Subunits

BK_{Ca} regulatory β subunits are expressed in mitochondria from heart, skeletal muscle, endothelial cells and brain as discussed below. Whether BK_{Ca} γ subunits exist in mitochondria is still unknown.

In the heart, β1 subunit was found in mitoplasts isolated from rat ventricular myocytes and a two hybrid system showed its direct interaction with cytochrome c oxidase subunit I (Ohya et al., 2005). These results demonstrated the localization of β1 in the mitochondrial inner membrane and associated with a component of the respiratory chain. Furthermore, functional experiments showed that in rat ventricular mitoplasts mitoBK$_{Ca}$ activity was enhanced by stimulation with 30 μM estradiol in the presence of 500 nM Ca^{2+} (Ohya et al., 2005). Under these conditions, estradiol is known to activate plasma membrane BK_{Ca} channels in complex with β1 subunits (Valverde et al., 1999). Interestingly, in mitoplasts of astrocytes the stimulatory effect of 30 μM β-estradiol on mitoBK$_{Ca}$ was transient resulting in a final inhibition of channel activity that was resilient to a second β-estradiol stimulus and was only partially restored by increasing Ca^{2+} in the matrix side of inside-out patches (Thiede et al., 2012).

In cultured pulmonary artery smooth muscle, β1 subunit was found to play a role in 11,12-epoxyeicosatrienoic acid induced depolarization of mitochondrial membrane potential as this effect was abrogated in the $\beta1^{-/-}$ animal (Loot et al., 2012).

In a human endothelial cell line, β2 was detected in lysates of mitochondria and mitoplasts. However, this subunit seems not to be forming complex with mitoBK$_{Ca}$ in this cell line because the reported channel activity (Bednarczyk et al., 2013b) does not show classical time-dependent inactivation conferred by β2 (Wallner et al., 1999; Benzinger et al., 2006).

In rat soleus muscle, β4 is visualized both at the plasma membrane and in mitochondria using immunocytochemistry; and by immunoblotting as a protein of ∼26 kDa using purified mitochondria (Skalska et al., 2008).

In the brain, β4 subunit is clearly localized to mitochondria of the gigantocellular reticular nucleus and in mitochondria of the pons. Because β4 could not be proteolyzed in intact mitochondria but only after detergent solubilization, it was speculated that it must be internal to the outer mitochondrial membrane. Western blot analysis of brain homogenates and mitochondrial fractions further confirmed the presence of β4 in mitochondria. Interestingly, β2 was also found in brain mitochondrial fractions but not β1 nor β3 subunits (Piwonska et al., 2008). β4 has also been observed in mitochondria of rat hippocampal neurons by immunochemistry and as a protein of ∼26 kDa in mitochondrial lysates of whole brain (Skalska et al., 2009). Thus, it is possible that β4 forms part of the mitoBK$_{Ca}$ complex at the inner mitochondrial membrane of various types of neurons.

β4 subunit expression in the glia appears to be disease dependent as it was not found expressed in glia of adult normal brain (Piwonska et al., 2008) but it has been detected in mitochondria of a human gliobastoma cell line (U-87 MG) where it comigrates with cytochrome c oxidase subunit I (Bednarczyk et al., 2013a). Yet, the functional impact that β4 may have on mitoBK$_{Ca}$ activity in glioma cells and in neurons needs to be established.

From the above studies it appears that mitochondrial β subunits show tissue specificity, a characteristic of their plasma membrane counterparts. However, more studies are needed to assess this hypothesis or to establish their functional role in mitochondria. Gene silencing models could be ideal for this task.

mitoBK$_{Ca}$ Channel and Protection from Ischemic Insult

mitoBK$_{Ca}$channel was first related to cardiac protection from global ischemia and reperfusion injury by Xu et al. (2002) using a BK_{Ca} opener, NS1619 (10–30 μM). The drug used to precondition the heart prior ischemia and reperfusion, improved left ventricular developed pressure and decreased infarct size. Both effects were abolished with 1 μM Paxilline, an inhibitor of BK_{Ca}. Several factors support the notion that NS1619 was opening BK_{Ca} located in mitochondria: (a) the fact that NS1619 could not be targeting plasma membrane BK_{Ca}, as adult cardiomyocytes are known for their lack of sarcolemmal BK_{Ca} expression/activity (Singh et al., 2013; Schmitt et al., 2014); (b) mitochondrial K^+ uptake was accelerated by NS1619 and decelerated by blocking BK_{Ca} with 100 nM IbTx; and (c) the protective effect of preconditioning the heart with NS1619 on reperfusion was not related to relaxation of the vasculature, where BK_{Ca} channel is abundant. Since then, other groups have confirmed and expanded these results (**Table 2**).

Stowe et al. (2006) confirmed an improved left ventricular developed pressure by 3 μM NS1619 preconditioning and measured mitochondrial Ca^{2+} and ROS production. *In situ* recording of these parameters during the ischemia/reperfusion protocol (in the left ventricle of the isolated, perfused heart) demonstrated that both parameters decreased by NS1619 preconditioning both during the ischemia period and ∼10 min after reperfusion started.

The cardioprotective effect of BK_{Ca} activation, in improving left ventricular developed pressure and reducing infarct size, has also been observed with BK_{Ca} opener NS11021 (1–3 μM) (Bentzen et al., 2009) and naringenin (4 μM) (Testai et al., 2013). Importantly, NS11021 is also effective when hearts or isolated cells are treated postischemia or postmetabolic inhibition and during reperfusion or re-energization, respectively (Bentzen et al., 2009; Borchert et al., 2013). These findings make BK_{Ca} an excellent target to improve cardiac function after an ischemic event as it occurs during heart infarct.

Interestingly, the protective effect of NS1619 may not be mediated by mitoBK$_{Ca}$ in all systems like in primary rat cortical neurons. In this type of neurons, preconditioning with NS1619 (150 μM) caused mitochondrial depolarization (consistent with

TABLE 2 | BK_{Ca} channel agonists and cardioprotection.

Model	Treatment	Baseline/Reperfusion (LVDP; mmHg)	Baseline/Reperfusion (CF; mL/min)	% Infarct size	References
Guinea pig	Control	104 ± 9/34 ± 6	43 ± 6/18 ± 2	∼50	Xu et al., 2002
	NS1619 (30 μM)	108 ± 3/**56 ± 5**	35 ± 5/19 ± 2	**∼20**	
	NS+Paxilline	103 ± 14/33 ± 10	48 ± 3/21 ± 5	∼55	
	Paxilline (1 μM)	113 ± 4/33 ± 5	51 ± 4/16 ± 1	∼60	
Guinea pig	Control	ND	ND	∼55	Stowe et al., 2006
	NS1619 (3 μM)			**∼25**	
	NS1619 (3 μM) + Paxilline (1 μM)			∼50	
	Paxilline (1 μM)			∼55	
Infant rabbit	Control	51 ± 2	4 ± 1	14 ± 5	Shi et al., 2007
	NS1619 (10 μM)	**65 ± 4**	5 ± 1	**10 ± 5**	
	Paxilline (1 μM)	55 ± 10	5 ± 1	13 ± 3	
	Paxilline (pretreatment)	53.4 ± 9			
Rat	Control	∼110/30 ± 3.3	ND	44.6±2	Bentzen et al., 2009
	Ischemic preconditioning	∼120/**∼65**		**7.9 ± 1.7**	
	NS11021 (1 μM)	∼110/**60.3 ± 7.2**		**20.6 ± 4.5**	
	NS11021 (3 μM)	∼110/**60.3 ± 7.2**		**11.4 ± 2**	
	NS 11021 (3 μM) + Paxilline (3 μM)	∼110/∼30		33.6 ± 5.6	
	NS11021 Postconditioning	∼110/**∼50**		**19.8 ± 3.3**	
Mouse	Control	∼120/∼60	ND	∼50	Singh et al., 2013
	NS1619 (10 μM)	∼120/**∼120**		**∼15**	

Positive effects are highlighted in bold. Experiments used the isolated heart preparation. LVDP, Left Ventricular Developed Pressure; CF, Coronary flow; ND, not determined.

K^+ influx) but this depolarization was not prevented by 5 min preincubation with 20 μM Paxilline (Gaspar et al., 2009). An alternative explanation to this negative result could be that Paxilline needs longer time to diffuse through the plasma membrane and reach mitoBK_{Ca} at the mitochondrial inner membrane.

Although BK_{Ca} channel activity is indeed inhibited by Paxilline (Zhou and Lingle, 2014), its exclusive use as pharmacological indicator of BK_{Ca} functional role in mitochondria or elsewhere needs to be taken with caution. This assertion is underscored by recent studies, where isoflurane preconditioning protected the heart from ischemic insult, showing that Paxilline (1 μM) abolished the anesthetic cardioprotective effect equally well in wild type and *Kcnma1*$^{-/-}$ mice (Wojtovich et al., 2011, 2013).

It is also important to highlight that the specific action of NS1619 on BK_{Ca} channel activation has been questioned (Szewczyk et al., 2010). Concentrations of NS1619 above 10 μM inhibited SERCA with a consequent Ca^{2+} overload in sarcoplasmic reticulum and posterior cytosolic contamination (Wrzosek, 2014). Other authors also reported non-specific effects of NS1619 at concentrations ≥10 μM such as inhibition of mitochondrial respiratory chain (∼15–30% inhibition at 10 μM) (Kicinska and Szewczyk, 2004; Cancherini et al., 2007), and H^+/K^+ leak with 50–100 μM of the drug (Aldakkak et al., 2010). Therefore, the use of low concentrations of NS1919 (i.e., ≤10 μM) in conjunction with the use of genetically modified models is desirable.

Conclusive evidence for the role of BK_{Ca} in cardioprotection has come from studies using BK_{Ca} knockout mouse models (*Kcnma1*$^{-/-}$) whose hearts are not protected from ischemic injury by NS1619 (5–10 μM) or NS11021 (500 nM) (Singh et al., 2013; Wojtovich et al., 2013), or by ischemic preconditioning (Soltysinska et al., 2014) as revealed by measurements of heart function and infarct size in isolated perfused hearts. Mitochondrial BK_{Ca} of cardiomyocytes (Singh et al., 2013; Soltysinska et al., 2014) as well as BK_{Ca} expressed in cardiac neurons (Wojtovich et al., 2013) contribute to the cardioprotective effects. A role of BK_{Ca} expressed in cardiac neurons is supported by the fact that an inhibitor of neural transmission prevents protection by NS1619 (Wojtovich et al., 2013). Supporting a role for mitoBK_{Ca} are measurements in isolated mitochondria at 10 min of reperfusion showing improved Ca^{2+} retention capacity with NS1619 preconditioning a property that was absent in the knockout animal (Singh et al., 2013). In addition, a model of anoxia/reoxygenation of isolated mitochondria (mimicking the isolated heart ischemia/reperfusion model) showed that the cardioprotective effect of preconditioning could be related to a mitoBK_{Ca}-mediated decrease of ROS production, as the production of ROS postanoxia was higher in the KO animal (Soltysinska et al., 2014).

mitoBK$_{Ca}$ and ROS Production

The knockout animal studies described above point to mitoBK$_{Ca}$ expression and ROS reduction as mitochondrial mechanisms playing a role in heart protection by ischemic preconditioning. The role of mitoBK$_{Ca}$ activation in the regulation of ROS production has also been examined in heart mitochondria under basal conditions and has been found to depend on the ROS producing conditions. In succinate energized mitochondria, where ROS production at site I is enhanced by reversed electron flow, putative stimulation of mitoBK$_{Ca}$ channel with 30 μM NS1619 produced a profound reduction of H_2O_2 production rate. Further, this decrease was partially overcome by pretreatment with 5 μM Paxilline (Heinen et al., 2007a). In contrast, when reverse electron flow was blocked by rotenone (i.e., succinate + rotenone), NS1619 increased H_2O_2 production rate and this effect was abolished by Paxilline (Heinen et al., 2007b). A decrease in ROS production by stimulating ROS production at complex I of the respiratory chain was also observed in brain mitochondria where activating mitoBK$_{Ca}$ channel either with 10 μM CGS7184 or 3 μM NS1619 reduced H_2O_2 production mediated by malate-glutamate or succinate. The CGS7184-mediated decrease in ROS production was abolished by 50 nM IbTx supporting the involvement of mitoBK$_{Ca}$ (Kulawiak et al., 2008). Experiments using the knockout models should provide definite proof of mitoBK$_{Ca}$ role in the maintenance of normal levels of ROS to impede cell damage.

mitoBK$_{Ca}$ Channel and Mitochondrial Transition Pore (mPTP)

Under stress conditions, opening of mPTP allows the passage of small molecules with osmotic consequences for the cell like swelling and rupture of the mitochondrial outer membrane that permits liberation of apoptotic factors including cytochrome *c* causing cell death. The key elements responsible to keep mPTP closed are the mitochondrial Ca^{2+} content and ROS levels. Interestingly, mitoBK$_{Ca}$ activity has been related to both ROS production as described above and to the regulation of mitochondrial Ca^{2+} content. Direct measurement of Ca^{2+} retention capacity (CRC) of cardiac mitochondria revealed that the protective effect of NS1619 preconditioning (increased CRC) was absent in the BK$_{Ca}$ KO animal (Singh et al., 2013). These results imply that opening of BK$_{Ca}$ to certain extent can protect mitochondria from uncontrolled mPTP opening. In line with this view, the proapoptotic protein Bax (Bcl-2 associated protein X) *directly* inhibits mitoBK$_{Ca}$ activity in astrocyte mitoplasts but does not by itself produce mPTP electrical activity; and thus, Bax has been proposed to produce mPTP opening via inhibition of mitoBK$_{Ca}$ (Cheng et al., 2011). In fact, inhibition of mitoBK$_{Ca}$ with IbTx reduces the amount of Ca^{2+} necessary to depolarize brain mitochondria (a measure of increased mPTP activity) (Cheng et al., 2008) and increases cytochrome c release (from GL261 glioma mitochondria), a landmark of mPTP opening and apoptosis (Cheng et al., 2011).

From the above studies, it is evident that there is a physiological coupling between mitoBK$_{Ca}$ and mPTP. Recent evidence indicates that mPTP is formed by dimers of the ATP-synthase (Giorgio et al., 2013). It would be interesting to test the hypothesis of a physical interaction between mitoBK$_{Ca}$ and the ATP-synthase. The functional coupling of mitoBK$_{Ca}$ with other components of the respiratory chain has been highlighted by the fact that mitoBK$_{Ca}$ activity is affected by substrates of the respiratory chain in human astrocytoma U-87 MG cells (Bednarczyk et al., 2013a). The precise mechanisms of this coupling remain to be elucidated.

Proposed Mechanisms of mitoBK$_{Ca}$ Channel Regulation of Common Pathological Conditions

In mitochondria isolated from brain of diabetic rats, a K^+ channel of 46 pS conductance that is inhibited by IbTx, a specific blocker of BK$_{Ca}$, has been identified. This channel is voltage dependent with an effective valence of ∼4.7 in contrast to ∼2.6 of mitoBK$_{Ca}$ from healthy brain (Noursadeghi et al., 2014). At present, it is difficult to ascribe a specific mechanism to this molecular switch. One possibility is that a subconductance of mitoBK$_{Ca}$ becomes much more stable under diabetic conditions inasmuch a ∼50 pS subconductance state of mitoBK$_{Ca}$ has been reported in cardiac mitoplasts (Xu et al., 2002).

Cancerous tumor cells are highly resistant to hypoxia suggesting the presence of a mechanism(s) that prevents their death. mitoBK$_{Ca}$ channels are expressed in cancerous cell lines (Siemen et al., 1999) and could participate in such mechanism as they are activated by hypoxia in mitoplasts derived from human glioma LN-229 cells and astrocytes (Gu et al., 2007, 2014; Cheng et al., 2008). Notably, hypoxia also reduces mPTP electrical activity in mitoplasts of liver mitochondria and delays mPTP opening in intact liver mitochondria as measured by Ca^{2+}-induced membrane depolarization (Cheng et al., 2008). How would mitoBK$_{Ca}$ sense oxygen levels during hypoxia? It is possible that chronic hypoxia-induced heme oxygenase-1 binds to the mitochondrial channel much like heme oxygenase-2 does with plasma membrane BK$_{Ca}$ (Williams et al., 2004) promoting CO production and activation of mitoBK$_{Ca}$.

Supporting the view that mitoBK$_{Ca}$ may regulate mitochondrial function as a redox sensor is the fact that its electrical activity can be inhibited by 300 nM hemin, a byproduct of hemoglobin (Augustynek et al., 2014) with oxidative properties that increases drastically (∼10 mM) during hemolysis like the one that occurs during hemorraghic stroke.

Concluding Remarks and Perspectives

As a general conclusion, we can state that BK$_{Ca}$ channel is present in the inner mitochondrial membrane of various cell types and in different species. The discovery that mitoBK$_{Ca}$ is encoded by the same gene as the plasma membrane BK$_{Ca}$ (*Kcnma1*) is just the beginning in our understanding of the molecular composition of mitoBK$_{Ca}$ isoforms in different tissues that seem to display a spectrum of biophysical characteristics just like the plasma membrane channel. A detailed biophysical characterization in

native mitochondria and in genetically engineered organelles should help in correlating distinct properties with molecular composition.

An immediate specific question to resolve is, for example, the orientation of mitoBK$_{Ca}$ in the inner mitochondrial membrane. Experiments in inside-out patches where matrix and intermembrane spaces can have defined Ca^{2+} concentrations should solve this problem. Moreover, they will allow defining with certainty $V_{1/2}$ and Ca^{2+} EC_{50} values for mitoBK$_{Ca}$ in each cell type. At present, most of the experiments have been done in the on-mitoplast configuration and this information is lacking. Other points to address are: whether the BK$_{Ca}$ γ subunit is present in mitochondria and if it forms part of mitoBK$_{Ca}$ complex, or if β subunits have alternative functions in mitochondria.

Many other questions remain to be solved such as the physiological/pathophysiological role of mitoBK$_{Ca}$ channel in different tissues, its subunit composition in different cell types, mechanisms of mitochondrial targeting, and interaction with mitochondrial protein complexes.

Acknowledgments

This work was supported by NIH R01 HL107418 (ES and LT), UC MEXUS-CONACYT Postdoctoral Fellowship FE 13-248 (EB) and AHA-WSA Postdoctoral Fellowship 15POST22490015 (EB).

References

Aldakkak, M., Stowe, D. F., Cheng, Q., Kwok, W. M., and Camara, A. K. (2010). Mitochondrial matrix K^+ flux independent of large-conductance Ca^{2+}-activated K^+ channel opening. *Am. J. Physiol. Cell. Physiol.* 298, C530–C541. doi: 10.1152/ajpcell.00468.2009

Alioua, A., Li, M., Wu, Y., Stefani, E., and Toro, L. (2011). Unconventional myristoylation of large-conductance $Ca^{(2)}$-activated K channel (Slo1) via serine/threonine residues regulates channel surface expression. *Proc. Natl. Acad. Sci. U.S.A.* 108, 10744–10749. doi: 10.1073/pnas.1008863108

Augustynek, B., Kudin, A. P., Bednarczyk, P., Szewczyk, A., and Kunz, W. S. (2014). Hemin inhibits the large conductance potassium channel in brain mitochondria: a putative novel mechanism of neurodegeneration. *Exp. Neurol.* 257, 70–75. doi: 10.1016/j.expneurol.2014.04.022

Banerjee, A., Lee, A., Campbell, E., and MacKinnon, R. (2013). Structure of a pore-blocking toxin in complex with a eukaryotic voltage-dependent $K^{(+)}$ channel. *Elife* 2:e00594. doi: 10.7554/eLife.00594

Bednarczyk, P., Koziel, A., Jarmuszkiewicz, W., and Szewczyk, A. (2013b). Large-conductance $Ca^{(2)(+)}$-activated potassium channel in mitochondria of endothelial EA.hy926 cells. *Am. J. Physiol. Heart Circ. Physiol.* 304, H1415–H1427. doi: 10.1152/ajpheart.00976.2012

Bednarczyk, P., Wieckowski, M. R., Broszkiewicz, M., Skowronek, K., Siemen, D., and Szewczyk, A. (2013a). Putative structural and functional coupling of the mitochondrial BK channel to the respiratory chain. *PLoS ONE* 8:e68125. doi: 10.1371/journal.pone.0068125

Bentzen, B. H., Osadchii, O., Jespersen, T., Hansen, R. S., Olesen, S. P., and Grunnet, M. (2009). Activation of big conductance $Ca^{(2+)}$-activated $K^{(+)}$ channels (BK) protects the heart against ischemia-reperfusion injury. *Pflug. Arch.* 457, 979–988. doi: 10.1007/s00424-008-0583-5

Benzinger, G. R., Xia, X. M., and Lingle, C. J. (2006). Direct observation of a preinactivated, open state in BK channels with beta2 subunits. *J. Gen. Physiol.* 127, 119–131. doi: 10.1085/jgp.200509425

Borchert, G. H., Hlavackova, M., and Kolar, F. (2013). Pharmacological activation of mitochondrial BK(Ca) channels protects isolated cardiomyocytes against simulated reperfusion-induced injury. *Exp. Biol. Med. (Maywood.)* 238, 233–241. doi: 10.1177/1535370212474596

Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y., and Aldrich, R. W. (2000). Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. *J. Biol. Chem.* 275, 6453–6461. doi: 10.1074/jbc.275.9.6453

Cancherini, D. V., Queliconi, B. B., and Kowaltowski, A. J. (2007). Pharmacological and physiological stimuli do not promote $Ca^{(2+)}$-sensitive K^+ channel activity in isolated heart mitochondria. *Cardiovasc. Res.* 73, 720–728. doi: 10.1016/j.cardiores.2006.11.035

Chen, L., Bi, D., Tian, L., McClafferty, H., Steeb, F., Ruth, P., et al. (2013). Palmitoylation of the beta4-subunit regulates surface expression of large conductance calcium-activated potassium channel splice variants. *J. Biol. Chem.* 288, 13136–13144. doi: 10.1074/jbc.M113.461830

Cheng, Y., Gu, X. Q., Bednarczyk, P., Wiedemann, F. R., Haddad, G. G., and Siemen, D. (2008). Hypoxia increases activity of the BK-channel in the inner mitochondrial membrane and reduces activity of the permeability transition pore. *Cell. Physiol. Biochem.* 22, 127–136. doi: 10.1159/000149790

Cheng, Y., Gulbins, E., and Siemen, D. (2011). Activation of the permeability transition pore by Bax via inhibition of the mitochondrial BK channel. *Cell. Physiol. Biochem.* 27, 191–200. doi: 10.1159/000327944

Contreras, G. F., Castillo, K., Enrique, N., Carrasquel-Ursulaez, W., Castillo, J. P., Milesi, V., et al. (2013). A BK (Slo1) channel journey from molecule to physiology. *Channels (Austin.)* 7, 442–458. doi: 10.4161/chan.26242

Cox, N., Toro, B., Pacheco-Otalora, L. F., Garrido-Sanabria, E. R., and Zarei, M. M. (2014). An endoplasmic reticulum trafficking signal regulates surface expression of beta4 subunit of a voltage- and $Ca^{(2)(+)}$-activated $K^{(+)}$ channel. *Brain Res.* 1553, 12–23. doi: 10.1016/j.brainres.2014.01.028

Fahanik-Babaei, J., Eliassi, A., Jafari, A., Sauve, R., Salari, S., and Saghiri, R. (2011a). Electro-pharmacological profile of a mitochondrial inner membrane big-potassium channel from rat brain. *Biochim. Biophys. Acta* 1808, 454–460. doi: 10.1016/j.bbamem.2010.10.005

Fahanik-Babaei, J., Eliassi, A., and Saghiri, R. (2011b). How many types of large conductance $Ca^{(+)(2)}$-activated potassium channels exist in brain mitochondrial inner membrane: evidence for a new mitochondrial large conductance $Ca^{(2)(+)}$-activated potassium channel in brain mitochondria. *Neuroscience* 199, 125–132. doi: 10.1016/j.neuroscience.2011.09.055

Gao, Y. D., and Garcia, M. L. (2003). Interaction of agitoxin2, charybdotoxin, and iberiotoxin with potassium channels: selectivity between voltage-gated and Maxi-K channels. *Proteins* 52, 146–154. doi: 10.1002/prot.10341

Gaspar, T., Domoki, F., Lenti, L., Katakam, P. V., Snipes, J. A., Bari, F., et al. (2009). Immediate neuronal preconditioning by NS1619. *Brain Res.* 1285, 196–207. doi: 10.1016/j.brainres.2009.06.008

Giorgio, V., von Stockum, S., Antoniel, M., Fabbro, A., Fogolari, F., Forte, M., et al. (2013). Dimers of mitochondrial ATP synthase form the permeability transition pore. *Proc. Natl. Acad. Sci. U.S.A.* 110, 5887–5892. doi: 10.1073/pnas.1217823110

Gu, X. Q., Pamenter, M. E., Siemen, D., Sun, X., and Haddad, G. G. (2014). Mitochondrial but not plasmalemmal BK channels are hypoxia-sensitive in human glioma. *Glia* 62, 504–513. doi: 10.1002/glia.22620

Gu, X. Q., Siemen, D., Parvez, S., Cheng, Y., Xue, J., Zhou, D., et al. (2007). Hypoxia increases BK channel activity in the inner mitochondrial membrane. *Biochem. Biophys. Res. Commun.* 358, 311–316. doi: 10.1016/j.bbrc.2007.04.110

Heinen, A., Aldakkak, M., Stowe, D. F., Rhodes, S. S., Riess, M. L., Varadarajan, S. G., et al. (2007a). Reverse electron flow-induced ROS production is attenuated by activation of mitochondrial Ca^{2+}-sensitive K^+ channels. *Am. J. Physiol. Heart Circ. Physiol.* 293, H1400–H1407. doi: 10.1152/ajpheart.00198.2007

Heinen, A., Camara, A. K., Aldakkak, M., Rhodes, S. S., Riess, M. L., and Stowe, D. F. (2007b). Mitochondrial Ca^{2+}-induced K^+ influx increases respiration and enhances ROS production while maintaining membrane potential. *Am. J. Physiol. Cell. Physiol.* 292, C148–C156. doi: 10.1152/ajpcell.00215.2006

Jeffries, O., Geiger, N., Rowe, I. C., Tian, L., McClafferty, H., Chen, L., et al. (2010). Palmitoylation of the S0-S1 linker regulates cell surface expression of

voltage- and calcium-activated potassium (BK) channels. *J. Biol. Chem.* 285, 33307–33314. doi: 10.1074/jbc.M110.153940

Kathiresan, T., Harvey, M., Orchard, S., Sakai, Y., and Sokolowski, B. (2009). A protein interaction network for the large conductance $Ca^{(2+)}$-activated $K^{(+)}$ channel in the mouse cochlea. *Mol. Cell. Proteomics.* 8, 1972–1987. doi: 10.1074/mcp.M800495-MCP200

Kicinska, A., and Szewczyk, A. (2004). Large-conductance potassium cation channel opener NS1619 inhibits cardiac mitochondria respiratory chain. *Toxicol. Mech. Methods* 14, 59–61. doi: 10.1080/15376520490257482

Knaus, H. G., Folander, K., Garcia-Calvo, M., Garcia, M. L., Kaczorowski, G. J., Smith, M., et al. (1994). Primary sequence and immunological characterization of β-subunit of high conductance Ca^{2+}-activated K^+ channel from smooth muscle. *J. Biol. Chem.* 269, 17274–17278.

Kulawiak, B., Kudin, A. P., Szewczyk, A., and Kunz, W. S. (2008). BK channel openers inhibit ROS production of isolated rat brain mitochondria. *Exp. Neurol.* 212, 543–547. doi: 10.1016/j.expneurol.2008.05.004

Li, J., Al-Khalili, O., Ramosevac, S., Eaton, D. C., and Denson, D. D. (2010). Protein-protein interaction between cPLA2 and splice variants of alpha-subunit of BK channels. *Am. J. Physiol. Cell. Physiol.* 298, C251–C262. doi: 10.1152/ajpcell.00221.2009

Loot, A. E., Moneke, I., Keseru, B., Oelze, M., Syzonenko, T., Daiber, A., et al. (2012). 11,12-EET stimulates the association of BK channel alpha and beta(1) subunits in mitochondria to induce pulmonary vasoconstriction. *PLoS ONE* 7:e46065. doi: 10.1371/journal.pone.0046065

Ma, D., Nakata, T., Zhang, G., Hoshi, T., Li, M., and Shikano, S. (2007). Differential trafficking of carboxyl isoforms of Ca^{2+}-gated (Slo1) potassium channels. *FEBS Lett.* 581, 1000–1008. doi: 10.1016/j.febslet.2007.01.077

McManus, O. B., and Magleby, K. L. (1991). Accounting for the Ca^{2+}-dependent kinetics of single large-conductance Ca^{2+}-activated K^+ channels in rat skeletal muscle. *J. Physiol.* 443, 739–777. doi: 10.1113/jphysiol.1991.sp018861

Meera, P., Wallner, M., and Toro, L. (2000). A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca^{2+}-activated K^+ channel resistant to charybdotoxin and iberiotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5562–5567. doi: 10.1073/pnas.100118597

Noursadeghi, E., Jafari, A., Saghiri, R., Sauve, R., and Eliassi, A. (2014). Impairment of brain mitochondrial charybdotoxin- and ATP-insensitive BK channel activities in diabetes. *Neuromol. Med.* 16, 862–871. doi: 10.1007/s12017-014-8334-6

Ohya, S., Kuwata, Y., Sakamoto, K., Muraki, K., and Imaizumi, Y. (2005). Cardioprotective effects of estradiol include the activation of large-conductance $Ca^{(2+)}$-activated $K^{(+)}$ channels in cardiac mitochondria. *Am. J. Physiol. Heart Circ. Physiol.* 289, H1635–H1642. doi: 10.1152/ajpheart.00016.2005

Piwonska, M., Wilczek, E., Szewczyk, A., and Wilczynski, G. M. (2008). Differential distribution of Ca^{2+}-activated potassium channel beta4 subunit in rat brain: immunolocalization in neuronal mitochondria. *Neuroscience* 153, 446–460. doi: 10.1016/j.neuroscience.2008.01.050

Saito, M., Nelson, C., Salkoff, L., and Lingle, C. J. (1997). A cysteine-rich domain defined by a novel exon in a slo variant in rat adrenal chromaffin cells and PC12 cells. *J. Biol. Chem.* 272, 11710–11717. doi: 10.1074/jbc.272.18.11710

Schmitt, N., Grunnet, M., and Olesen, S. P. (2014). Cardiac potassium channel subtypes: new roles in repolarization and arrhythmia. *Physiol. Rev.* 94, 609–653. doi: 10.1152/physrev.00022.2013

Schreiber, M., and Salkoff, L. (1997). A novel calcium-sensing domain in the BK channel. *Biophys. J.* 73, 1355–1363. doi: 10.1016/S0006-3495(97)78168-2

Shi, J., Krishnamoorthy, G., Yang, Y., Hu, L., Chaturvedi, N., Harilal, D., et al. (2002). Mechanism of magnesium activation of calcium-activated potassium channels. *Nature* 418, 876–880. doi: 10.1038/nature00941

Shi, Y., Jiang, M. T., Su, J., Hutchins, W., Konorev, E., and Baker, J. E. (2007). Mitochondrial big conductance KCa channel and cardioprotection in infant rabbit heart. *J. Cardiovasc. Pharmacol.* 50, 497–502. doi: 10.1097/FJC.0b013e318137991d

Shruti, S., Urban-Ciecko, J., Fitzpatrick, J. A., Brenner, R., Bruchez, M. P., and Barth, A. L. (2012). The brain-specific Beta4 subunit downregulates BK channel cell surface expression. *PLoS ONE* 7:e33429. doi: 10.1371/journal.pone.0033429

Siemen, D., Loupatatzis, C., Borecky, J., Gulbins, E., and Lang, F. (1999). Ca^{2+}-Activated K Channel of the BK-Type in the inner mitochondrial membrane of a human glioma cell line. *Biochim. Biophys. Res. Comm.* 257, 549–554. doi: 10.1006/bbrc.1999.0496

Singh, H., Lu, R., Bopassa, J. C., Meredith, A. L., Stefani, E., and Toro, L. (2013). mitoBKCa is encoded by the Kcnma1 gene, and a splicing sequence defines its mitochondrial location. *Proc. Natl. Acad. Sci. U.S.A.* 110, 10836–10841. doi: 10.1073/pnas.1302028110

Skalska, J., Bednarczyk, P., Piwonska, M., Kulawiak, B., Wilczynski, G., Dolowy, K., et al. (2009). Calcium ions regulate $K^{(+)}$ uptake into brain mitochondria: the evidence for a novel potassium channel. *Int. J. Mol. Sci.* 10, 1104–1120. doi: 10.3390/ijms10031104

Skalska, J., Piwonska, M., Wyroba, E., Surmacz, L., Wieczorek, R., Koszela-Piotrowska, I., et al. (2008). A novel potassium channel in skeletal muscle mitochondria. *Biochim. Biophys. Acta* 1777, 651–659. doi: 10.1016/j.bbabio.2008.05.007

Soltysinska, E., Bentzen, B. H., Barthmes, M., Hattel, H., Thrush, A. B., Harper, M. E., et al. (2014). KCNMA1 encoded cardiac BK channels afford protection against ischemia-reperfusion injury. *PLoS ONE* 9:e103402. doi: 10.1371/journal.pone.0103402

Stowe, D. F., Aldakkak, M., Camara, A. K., Riess, M. L., Heinen, A., Varadarajan, S. G., et al. (2006). Cardiac mitochondrial preconditioning by Big Ca^{2+}-sensitive K^+ channel opening requires superoxide radical generation. *Am. J. Physiol. Heart Circ. Physiol.* 290, H434–H440. doi: 10.1152/ajpheart.00763.2005

Szabo, I., and Zoratti, M. (2014). Mitochondrial channels: ion fluxes and more. *Physiol. Rev.* 94, 519–608. doi: 10.1152/physrev.00021.2013

Szewczyk, A., Kajma, A., Malinska, D., Wrzosek, A., Bednarczyk, P., Zablocka, B., et al. (2010). Pharmacology of mitochondrial potassium channels: dark side of the field. *FEBS Lett.* 584, 2063–2069. doi: 10.1016/j.febslet.2010.02.048

Tanaka, Y., Meera, P., Song, M., Knaus, H.-G., and Toro, L. (1997). Molecular constituents of maxi K_{Ca} channels in human coronary smooth muscle. Predominant α + β subunit complexes. *J. Physiol.* 502, 545–557. doi: 10.1111/j.1469-7793.1997.545bj.x

Testai, L., Martelli, A., Marino, A., D'Antongiovanni, V., Ciregia, F., Giusti, L., et al. (2013). The activation of mitochondrial BK potassium channels contributes to the protective effects of naringenin against myocardial ischemia/reperfusion injury. *Biochem. Pharmacol.* 85, 1634–1643. doi: 10.1016/j.bcp.2013.03.018

Thiede, A., Gellerich, F. N., Schonfeld, P., and Siemen, D. (2012). Complex effects of 17beta-estradiol on mitochondrial function. *Biochim. Biophys. Acta* 1817, 1747–1753. doi: 10.1016/j.bbabio.2012.02.034

Tian, L., Duncan, R. R., Hammond, M. S., Coghill, L. S., Wen, H., Rusinova, R., et al. (2001). Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J. Biol. Chem.* 276, 7717–7720. doi: 10.1074/jbc.C000741200

Toro, B., Cox, N., Wilson, R. J., Garrido-Sanabria, E., Stefani, E., Toro, L., et al. (2006). KCNMB1 regulates surface expression of a voltage and Ca^{2+}-activated K^+ channel via endocytic trafficking signals. *Neuroscience* 142, 661–669. doi: 10.1016/j.neuroscience.2006.06.061

Uebele, V. N., Lagrutta, A., Wade, T., Figueroa, D. J., Liu, Y., McKenna, E., et al. (2000). Cloning and functional expression of 2 families of {beta}-subunits of the large conductance calcium-activated K^+ channel. *J. Biol. Chem.* 275, 23211–23218. doi: 10.1074/jbc.M910187199

Valverde, M. A., Rojas, P., Amigo, J., Cosmelli, D., Orio, P., Bahamonde, M. I., et al. (1999). Acute activation of maxi-K channels (hSlo) by estradiol binding to the beta subunit [In Process Citation]. *Science* 285, 1929–1931. doi: 10.1126/science.285.5435.1929

Wallner, M., Meera, P., and Toro, L. (1999). Molecular basis of fast inactivation in voltage and Ca^{2+}-activated K^+ channels: a transmembrane β–subunit homolog. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4137–4142. doi: 10.1073/pnas.96.7.4137

Williams, S. E., Wootton, P., Mason, H. S., Bould, J., Iles, D. E., Riccardi, D., et al. (2004). Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. *Science* 306, 2093–2097. doi: 10.1126/science.1105010

Wojtovich, A. P., Nadtochiy, S. M., Urciuoli, W. R., Smith, C. O., Grunnet, M., Nehrke, K., et al. (2013). A non-cardiomyocyte autonomous mechanism of cardioprotection involving the SLO1 BK channel. *PeerJ.* 1:e48. doi: 10.7717/peerj.48

Wojtovich, A. P., Sherman, T. A., Nadtochiy, S. M., Urciuoli, W. R., Brookes, P. S., and Nehrke, K. (2011). SLO-2 is cytoprotective and contributes to mitochondrial potassium transport. *PLoS ONE* 6:e28287. doi: 10.1371/journal.pone.0028287

Wrzosek, A. (2014). The potassium channel opener NS1619 modulates calcium homeostasis in muscle cells by inhibiting SERCA. *Cell Calcium* 56, 14–24. doi: 10.1016/j.ceca.2014.03.005

Xia, X. M., Zeng, X., and Lingle, C. J. (2002). Multiple regulatory sites in large-conductance calcium-activated potassium channels. *Nature* 418, 880–884. doi: 10.1038/nature00956

Xu, W., Liu, Y., Wang, S., McDonald, T., Van Eyk, J. E., Sidor, A., et al. (2002). Cytoprotective role of Ca^{2+}- activated K^+ channels in the cardiac inner mitochondrial membrane. *Science* 298, 1029–1033. doi: 10.1126/science.1074360

Yan, J., and Aldrich, R. W. (2010). LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. *Nature* 466, 513–516. doi: 10.1038/nature09162

Yan, J., and Aldrich, R. W. (2012). BK potassium channel modulation by leucine-rich repeat-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7917–7922. doi: 10.1073/pnas.1205435109

Yang, H., Shi, J., Zhang, G., Yang, J., Delaloye, K., and Cui, J. (2008). Activation of Slo1 BK channels by Mg^{2+} coordinated between the voltage sensor and RCK1 domains. *Nat. Struct. Mol. Biol.* 15, 1152–1159. doi: 10.1038/nsmb.1507

Yuan, P., Leonetti, M. D., Hsiung, Y., and MacKinnon, R. (2012). Open structure of the Ca^{2+} gating ring in the high-conductance Ca^{2+}-activated K^+ channel. *Nature* 481, 94–97. doi: 10.1038/nature10670

Yuan, P., Leonetti, M. D., Pico, A. R., Hsiung, Y., and MacKinnon, R. (2010). Structure of the human BK channel Ca^{2+}-activation apparatus at 3.0 a resolution. *Science* 329, 182–186. doi: 10.1126/science.1190414

Zarei, M. M., Eghbali, M., Alioua, A., Song, M., Knaus, H. G., Stefani, E., et al. (2004). An endoplasmic reticulum trafficking signal prevents surface expression of a voltage- and Ca^{2+}-activated K^+ channel splice variant. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10072–10077. doi: 10.1073/pnas.0302919101

Zarei, M. M., Song, M., Wilson, R. J., Cox, N., Colom, L., Knaus, H.-G., et al. (2007). Endocytic trafficking signals in KCNMB2 regulate surface expression of a voltage and Ca^{2+}-Activated K^+ channel. *Neuroscience* 147, 80–89. doi: 10.1016/j.neuroscience.2007.04.019

Zhou, X., Wulfsen, I., Korth, M., McClafferty, H., Lukowski, R., Shipston, M. J., et al. (2012). Palmitoylation and membrane association of the stress axis regulated insert (STREX) controls BK channel regulation by protein kinase C. *J. Biol. Chem.* 287, 32161–32171. doi: 10.1074/jbc.M112.386359

Zhou, Y., and Lingle, C. J. (2014). Paxilline inhibits BK channels by an almost exclusively closed-channel block mechanism. *J. Gen. Physiol.* 144, 415–440. doi: 10.1085/jgp.201411259

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 09 December 2014
doi: 10.3389/fphys.2014.00476

The large conductance Ca^{2+} -activated K^+ (BKCa) channel regulates cell proliferation in SH-SY5Y neuroblastoma cells by activating the staurosporine-sensitive protein kinases

Angela Curci†, Antonietta Mele†, Giulia Maria Camerino, Maria Maddalena Dinardo and Domenico Tricarico*

Department of Pharmacy-Drug Science, University of Bari "Aldo Moro", Bari, Italy

Edited by:
Thomas M. Weiger, University of Salzburg, Austria

Reviewed by:
Núria Comes, University of Barcelona, Spain
Harpreet Singh, Drexel University College of Medicine, USA
Ramon A. Lorca, Washington University in St. Louis, USA

***Correspondence:**
Domenico Tricarico, Department of Pharmacy-Drug Science, University of Bari "Aldo Moro", Via Orabona n°4, Bari 70125, Italy
e-mail: domenico.tricarico@uniba.it

† *These authors have contributed equally to this work.*

Here we investigated on the role of the calcium activated K^+-channels(BKCa) on the regulation of the neuronal viability. Recordings of the K^+-channel current were performed using patch-clamp technique in human neuroblastoma cells (SH-SY5Y) in parallel with measurements of the cell viability in the absence or presence of the BKCa channel blockers iberiotoxin(IbTX) and tetraethylammonium (TEA) and the BKCa channel opener NS1619. Protein kinase C/A (PKC, PKA) activities in the cell lysate were investigated in the presence/absence of drugs. The whole-cell K^+-current showed a slope conductance calculated at negative membrane potentials of 126.3 pS and 1.717 nS($n = 46$) following depolarization. The intercept of the I/V curve was −33 mV. IbTX(10^{-8} – 4×10^{-7} M) reduced the K^+-current at +30 mV with an IC_{50} of 1.85 $\times 10^{-7}$ M and an Imax of −46% (slope = 2.198) ($n = 21$). NS1619(10–100 $\times$ 10^{-6} M) enhanced the K^+-current of +141% ($n = 6$), at −10 mV(Vm). TEA(10^{-5}–10^{-3} M) reduced the K^+-current with an IC_{50} of 3.54 $\times$ 10^{-5} M and an Imax of −90% (slope = 0.95) ($n = 5$). A concentration-dependent increase of cell proliferation was observed with TEA showing a maximal proliferative effect(MPE) of +38% (10^{-4} M). IbTX showed an MPE of +42% at 10^{-8} M concentration, reducing it at higher concentrations. The MPE of the NS1619(100 $\times$ 10^{-6} M) was +42%. The PKC inhibitor staurosporine (0.2–2 $\times$ 10^{-6} M) antagonized the proliferative actions of IbTX and TEA. IbTX (10 $\times$ 10^{-9} M), TEA (100 $\times$ 10^{-6} M), and the NS1619 significantly enhanced the PKC and PKA activities in the cell lysate with respect to the controls. These results suggest that BKCa channel regulates proliferation of the SH-SY5Y cells through PKC and PKA protein kinases.

Keywords: maxi-calcium activated K^+-channels, cell proliferation, voltage dependent K^+-channels, SH-SY5Y neuroblastoma cells, protein kinases, staurosporine

INTRODUCTION

The calcium activated K^+-channels (BKCa) channels are ubiquitously present in most human cells and play an essential role in the regulation of basic cellular processes such as electrical excitability of cell membrane, vascular tone, neurotransmitter release (Zhang et al., 2003; Tricarico et al., 2005; Lee and Cui, 2010).

BKCa channels are composed by the alpha subunit encoded by one gene (slo1/KCNMA1) assembled as tetramer and transmembrane beta subunits (beta1-4) encoded by KCNMB1-4 genes. The alpha subunit of BKCa channels may assemble with beta-subunits with 1:1 stoichiometry enhancing the calcium sensitivity, favoring the trafficking into the membrane and modulating the pharmacological responses (Kyle and Braun, 2014). The alpha, alpha+beta 1, alpha+beta 2/3, or beta 4 mimics the skeletal muscle, vascular smooth muscle and neuronal BKCa channels, respectively (Orio and Latorre, 2005; Lee and Cui, 2010). Furthermore, splicing isoforms of the alpha subunit gene are expressed in the tissues including skeletal muscle affecting physiological properties and pharmacological response of the native channels (Dinardo et al., 2012). More recently gamma subunits have been described (Toro et al., 2014). The gamma subunits are auxiliary leucine-rich repeat (LRR) -containing protein 26 (LRRC26) that following interaction with the BKCa channel lead to channel activation at negative voltages without rising in the intracellular calcium concentration. Several gamma subunits have been identified in excitable and non-excitable tissues modulating the gating of a BKCa channel by enhancing the allosteric coupling between voltage-sensor activation and the channel's closed-open transition (Yan and Aldrich, 2010, 2012).

Activation of BKCa channels has been reported to be involved in the regulation of cell viability and apoptosis besides its electrophysiological function. Emerging evidences suggest that the BKCa channel plays a role in cell viability in different cell types including osteoblasts, vascular smooth muscle cells and in cell lines expressing the recombinant channel subunits (Henney et al., 2009; Jia et al., 2013). It has been recently shown that high glucose enhances HEK293 cell viability by inhibition of cloned BKCa channel subunits hslo + beta 1 (Chang et al., 2011). The BKCa channel openers NS1619 or tamoxifen significantly induced apoptosis reducing cell viability in cells expressing the

combination of the hslo + beta 1 subunits under hyperglycemia conditions indicating that cloned BKCa channel regulates apoptosis and proliferation of HEK293 cell. These findings suggest that this effect may have a role in the diabetic vascular dysfunction associated with a vascular wall hypertrophy (Chang et al., 2011). In line with this observation, we recently demonstrated that the hslo subunit regulates the cell viability in response to changes of the external K^+ ion concentration (Tricarico et al., 2013). The cell viability after 24 h of incubation under hyperkalemia was enhanced by 82 ± 6 and 33 ± 7% in hslo-HEK293 cells and HEK293 cells, respectively. The BKCa channel blockers iberiotoxin (IbTx), charybdotoxin (ChTX), and tetraethylammonium (TEA) enhanced cell viability in hslo-HEK293 and the BKCa openers prevented the enhancement of the cell viability induced by hyperkalemia or IbTx in hslo-HEK293. In contrast, under hypokalemia cell viability was reduced by −22 ± 4 and −23 ± 6% in hslo-HEK293 and HEK293 cells, respectively, thereby suggesting that the BKCa channel regulates cell viability under hyperkalemia but not hypokalemia conditions. These findings may have relevance in the neuromuscular disorders associated with abnormal K^+ ion homeostasis including periodic paralysis and myotonia. Hyperkalemia condition associated with hypertrophic phenotype is indeed often observed in myotonia (Adrian and Bryant, 1974; Cannon, 1996).

The role of BKCa channels in the proliferation process in the tumor cells is controversial. Some studies have suggested that BKCa channels contributed to the high proliferative or invasive potential in a number of malignant cell lines, such as non-metastatic (MCF-7) breast cancer cells (Ouadid-Ahidouch and Ahidouch, 2008), brain-specific metastatic (MDA-MB-361) breast cancer cells (Khaitan et al., 2009), human prostate cancer (Bloch et al., 2007), colorectal carcinogenesis (Koehl et al., 2010), and glioma (Weaver et al., 2006; Sontheimer, 2008).

Some others concluded that BKCa channels are not required for the proliferation in glioma (Abdullaev et al., 2010) or breast cancer cells because the BKCa channel blockers charybdotoxin or iberiotoxin did not affect cell proliferation (Roger et al., 2004).

In contrast, BKCa channels have been reported to exhibit antiproliferative and anti-tumorogenic properties in osteosarcoma cells, ovarian cancer cells, glioma cells and in human MDA-MB-231 breast cancer cells. The bisphosonates Zoledronic acid used in the osteoporosis associated with bone metastasis and the BKCa channel opener NS1619, induced apoptosis through the opening of BKCa channels, while hslo gene silencing or channel blockers induced cell proliferation (Cambien et al., 2008; Han et al., 2008; Debska-Vielhaber et al., 2009; Ma et al., 2012).

We therefore investigated on the role of BKCa channels in the proliferation process in SH-SY5Y neuroblastoma cells. This cell type shows a high activity of BKCa channels, but their specific contribution to the proliferation process is not known (Park et al., 2010).

Moreover, very little is known about the non-conducting functions of BKCa channels, in particular which signaling cascades they modify. In this work, the ion channel characterizations were performed using patch–clamp techniques in SH-SY5Y neuroblastoma cells. Cell proliferation was investigated by evaluating the mitochondrial succinic dehydrogenases activity, cell diameter and volume changes. The capability of the staurosporine (STS), a well-known not selective protein kinase C inhibitor, to antagonize the drug-induced cell proliferation was also investigated. The involvement of the protein kinase C (PKC) and protein kinase A (PKA) in the drug-induced cell proliferation was investigated using an enzyme-linked immuno-absorbent assay (ELISA) assay in the cell lysates.

Our findings may have relevance either in the neuromuscular disorders where this mechanism may play a role in the cell repair and in the proliferative disorders. PKC and PKA enzymes other than recognized pathways involved in the cell proliferation may have a role in the repair processes.

MATERIALS AND METHODS

DRUGS AND SOLUTIONS

In whole cell patch-clamp experiments, the pipette (intracellular) solutions contained (10^{-3} M): 132 K^+-glutamate, 1 ethylene glycol bis (β-aminoethyl ether)-N, N, N, N-tetraacetic acid (EGTA), 10 NaCl, 2 $MgCl_2$, 10 HEPES, 1 Na_2ATP, 0.3 Na_2GTP, pH = 7.2 with KOH. The bath solution contained (10^{-3} M): 142 NaCl, 2.8 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 11 glucose, 10 HEPES, pH = 7.4 with NaOH. $CaCl_2$ was added to the pipette solutions to give free Ca^{2+} ion concentration of 1.6×10^{-6} M in whole cell experiments. The calculation of the free Ca^{2+} ion concentration in the pipette was performed using the Maxchelator software (Stanford University, USA).

The BKCA opener under investigation was NS1619. The selective and impermeant BKCA blocker investigated here was iberiotoxin (IbTX), the unselective blocker was tetraethylammonium (TEA). The Kir blocker used was Ba^{2+} ions. The drugs and toxins were purchased from Sigma (SIGMA Chemical Co., Mi, Italy). Stock solutions of the drugs under investigation were prepared dissolving the drugs in dimethylsulphoxide (DMSO) at concentrations of 118.6×10^{-3} M. Microliter amounts of the stock solutions were then added to the bath solutions as needed. DMSO did not exceed 0.07% in the bath, at this concentration the solvent does not normally affects the K^+-current or cell proliferation. Cell viability experiments were performed in Dulbecco's Modified Eagle's Medium (DMEM) (+) solution enriched with 1X antibiotics (1%), L-glutamine (1%), and fetal serum albumin (FBS) (10%).

PATCH-CLAMP EXPERIMENTS

The K^+-currents and drug actions on the channel currents were investigated in the human neuroblastoma cell line SH-SY5Y during voltage steps, in the range of potentials going from −150 mV(Vm) to +110/+150 mV (Vm), $HP = -60$ mV (Vm), in the presence of internal Ca^{2+} ions, in asymmetrical K^+ ion concentrations (int K^+: 132×10^{-3} M; ext K^+: 2.8×10^{-3} M) using whole-cell patch-clamp technique. The resulting K^+-current was a leak subtracted and normalized to capacitance. Drug effects were investigated in a physiological range of potentials from −10 mV (Vm) to + 30 mV (Vm) for all drugs. The K^+-current was recorded at 20°C and sampled at 1 kHz (filter = 2 kHz) using an Axopatch-1D amplifier equipped with a CV-4 headstage (Axon Instruments, Foster City, CA). The channel currents were identified on the basis of their voltage dependence

and response to toxins and drugs. The leak currents were measured in the presence of saturating concentration of Ba^{2+} (5×10^{-3} M) and TEA (5×10^{-3} M) which caused a full block of Kir, Kv, and BKCA channels.

Current analysis was performed using pClamp 10 software package (Axon Instruments). The criteria for accepting the data entering were based on the stability of the seal evaluated by observing the noise levels not exceeding 0.6 pA at 2 kHz. Pipettes resistance was 9 ± 0.2 MΩ (Number of pipettes = 150).

The cells were exposed to the drug solutions for 2 min. before recordings. Increasing concentrations of drug solutions were applied to the cells by the fast perfusion system (AutoMate, Sci. Berkeley, California 94710, USA). Each application of drug solution was followed by a washout period of 1 min to allow recovering of channel currents to control values. No more than three different drug concentrations were applied to the same cell, with one compound per cell tested at a time. Due to the not reversibility of the IbTX action following washout during the time of observation, only one concentration per cell and plate was tested at a time for this drug. Seal resistance was continuously monitored during patch solutions exchange.

CELL VIABILITY: MITOCHONDRIAL SUCCINIC DEHYDROGENASES ACTIVITY ASSAY

Cell viability was evaluated by measuring the succinic dehydrogenases activity in the cell suspension using the cell counting Kit-8 (CCK-8) (Enzo Life Sciences International, Inc., USA) which utilizes highly water-soluble tetrazolium salt. WST-8 2- (2- methoxy -4-nitrophenyl) -3-(4- nitrophenyl)- 5-(2,4-disulfophenyl)-2H- tetrazolium, monosodium salt produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. It is reduced by mitochondrial dehydrogenases in cells to give a yellow colored product (formazan), which is soluble in the tissue culture medium. The detection sensitivity of CCK-8 is higher than other tetrazolium salts. The changes of the cell vitality were expressed as % changes of cell viability induced by drugs and toxin with respect to the controls.

CELL VIABILITY: CELL VOLUME ASSAY

Measures of cell volume were based on the relationship existing between voltage changes and cell volume changes. Precise cell volumes are drawn into a sensor and the measurements are based on the impedentiometric principle. As cells flow through the aperture in the sensor, resistance increases. This increase in resistance causes a subsequent increase in voltage. Voltage changes are recorded as spikes with each passing cell and it is proportional to the cell volume. The spikes of the same size are bucketed into a histogram and counted. This histogram gives the quantitative data on cell morphology that can be used to examine the quality and health of your cell culture. The Scepter™ 2.0 cell counter (MERK-Millipore, USA) is compatible with 60 and 40 μm sensors, in our experiments we used the 60 μm sensor for particles between 6 and 36 μm.

PROTEIN KINASE ACTIVITY ASSAY

The PKA and PKC activities assay used in our experiments are based on a solid phase ELISA that utilizes a specific synthetic peptide as a substrate for PKA or PKC and a polyclonal antibody that recognizes the phosphorylated form of the substrate (Abcam, Cambridge, UK). The assay is designed for analysis of PKA or PKC activity in the solution phase. For statistical results, the assays were run in triplicate. The data from the crude protein preparations were compared with the data obtained from the standard calibration curves performed using the purified enzymes.

DATA ANALYSIS AND STATISTICS

The data were collected and analyzed using Excel software (Microsoft Office 2010). The data are expressed as mean ± S.E. unless otherwise specified.

In the case of the channel blockers IbTX and TEA molecules and Ba^{2+} ions the data could be fitted with the following equation:

$$(I\text{drug} - I\text{control}/I\text{control} - I\text{max}) \times 100 = (1 + \text{IC50}/[\text{Drug}])n/I\text{max}$$

I drug is the K^+-current measured in the presence of the molecules under study and normalized to the maximal currents recorded in the same patches; IC50 is the concentrations of the drugs needed to inhibit the current by 50%; Drug is the concentration of the drug tested; I max is the maximal current recorded in the patches at 110/150 mV (Vm); I control is the current recorded in the absence of drugs; and n is the slope factor of the curve. The algorithms of the fitting procedures used are based on a Marquardt least-squares fitting routine. Data analysis and plot were performed using SigmaPlot software (Systat Software, Inc., San Jose, CA).

The % change of the cell viability induced by the drugs and toxin, was calculated in respect to the controls (absence of blockers) using the following equations:

$$\%\text{change of the cell viability} = ([\text{drugs}]/\text{Controls}) \times 100.$$

The Scepter™ Software Pro was used for the calculation of the cell diameter and volume in the cell population (MERK-Millipore, USA). Data can be exported and further analyzed using excel software (Microsoft Office 2010).

The % activation of kinases activity (Relative PKC or PKA activity drug/Relative PKC activity CTRL) × 100.

The % inhibition of kinases activity (Relative PKC activity drug/Relative PKC activity CTRL) × 100 – 100.

Differences between mean were evaluated using the student t-test, at $p < 0.05$ level of significance.

RESULTS

In our experiments the whole cell K^+-current recorded in asymmetrical K^+ ion concentrations (int K^+: 132×10^3 M; ext K^+: 2.8×10^{-3} M) and internal free Ca^{2+} ions of 1.6×10^{-6} M concentration showed a sigmoid I/V relationship in the range of membrane potentials from −150 to +70 mV; a decay of the current was observed at membrane potentials > +70 mV which is consistent with the presence of the inactivation process. The slope conductance calculated in the range of membrane potentials from −150 to −30 mV was 126.3 ± 11 pS, and it was

1.717 ± 101 nS ($n = 46$) in the range of membrane potentials from −10 to +50 mV (**Figures 1A–C**). The intercept of the I/V curve on the voltage membrane axis was −33 mV which is consistent with the depolarized resting potential characterizing the SH-SY5Y neuronal cell line (Yang and Brackenbury, 2013).

The concentration-response relationships were investigated at −10, +10, and +30 mV of voltage membrane which are physiological membrane potentials for this cell line at which BKCA channel should be operative. The application of the BKCa channel blocker IbTX (4×10^{-7} M) induced a significant reduction of the outward K^+-current on −53% at +30 mV (Vm) which was not-reversible following washout of the toxin solution (**Figure 2A**). IbTX ($10^{-10} - 4 \times 10^{-5}$ M) induced a concentration-dependent reduction of the K^+-current with an IC_{50} of 1.85×10^{-7} M and an Imax of −46% (slope = 2.198) at +30 mV(Vm) ($n = 21$) (**Figure 2B**).

The application of the unselective K^+ channel blocker TEA (10^{-5}–10^{-3} M) induced a concentration-dependent reduction of the outward K^+- current which was reversible following washout of the drug solution. A full reduction of the K^+-current on −100% at +30 mV (Vm) was observed in the presence of TEA at 10^{-3} M concentration (**Figure 2C**). Concentration-response analysis showed that TEA (10^{-7}–10^{-1} M) reduced the K^+-current with an IC_{50} of 3.54×10^{-5} M and an Imax of −90% (slope = 0.95) ($n = 5$) (**Figure 2D**). No significant effects of IbTX and TEA were observed on the K^+-current at negative membrane potentials.

The BKCa channel opener NS1619(10–100×10^{-6} M) enhanced the K^+-current in the range of potentials from −10 mV to +10 mV. This drug enhanced the K^+-current of +41.7% ($n = 6$) and +141% ($n = 6$) respectively at 10×10^{-6} M and 100×10^{-6} M, at −10 mV(Vm), while leading to the a mild reduction of the K^+-current of −23.15% at +30 mV(Vm) suggesting a possible interaction of this drug with Kv channels other than BKCa channels (**Figure 3**).

A concentration-dependent increase of the cell proliferation was observed following 6 h of incubation time of the cells in the presence of TEA showing a maximal proliferative effect (MPE) of +38% at 10^{-4} M concentration as determined by mitochondrial succinic dehydrogenase activity assay. IbTX caused an MPE of +42% at a 10^{-8} M concentration, but reducing its efficacy at higher concentrations (**Figure 4**). This may be related to the fact that the toxin at higher concentration *per se* may lead to unspecific actions on cell viability unrelated to the BKCa channel blocking mechanism.

The BKCa channel opener NS1619 also induced cell proliferation showing an MPE of +42% at 10^{-4} M concentration. The co-incubation of the cells with NS1619+IbTx or TEA failed to prevent the enhancement of the cell proliferation induced by IbTX or TEA.

The co-incubation of the cells for 6 h with IbTX or TEA + STS (0.2×10^{-6} M) fully antagonized the proliferative actions of the IbTX and TEA (**Figure 4**). The STS at a 0.2×10^{-6} M concentration did not significantly affect the cell viability, while at a 2×10^{-6} M concentration, reduced the cell viability causing proteolysis with respect to the controls according to its apoptotic action.

The effects of the IbTX and TEA on the cell volume were also investigated. Cell volume changes are indeed more strikingly related to surface ion channel activity. In our experimental conditions the most frequently observed cell population showed a diameter size in the range of 13–16 μm (**Figure 5A**). We found

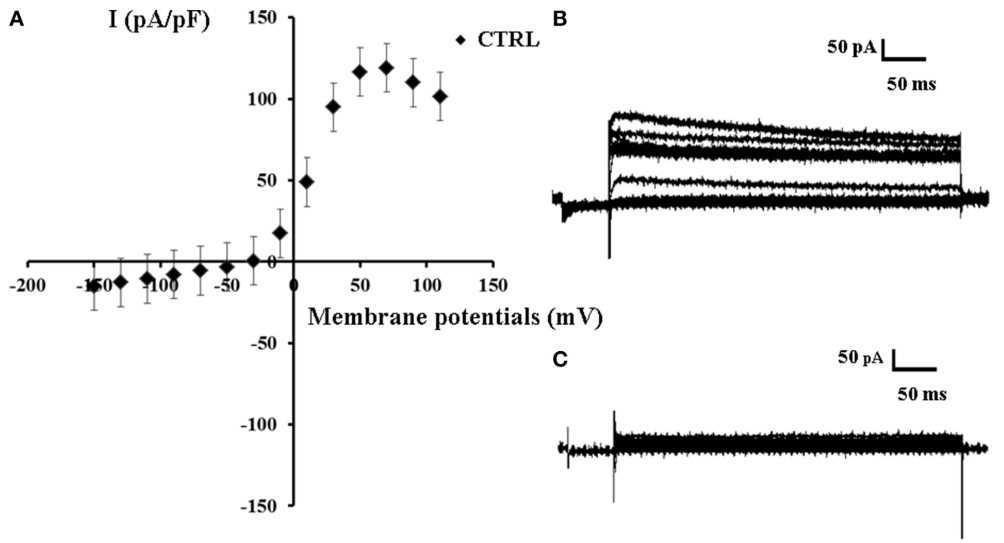


FIGURE 1 | Current voltage-relationship of K^+ -current recorded in SH-SY5Y neuronal cell line. (A) A sigmoid I/V relationship of the mean K^+ ions currents ($n = 46$) recorded in the control condition (CTRL) in asymmetrical K^+ ions concentrations (int K^+: 132×10^{-3} M; ext K^+: 2.8×10^{-3} M), in the presence of internal 1.6×10^{-6} M concentration of free Ca^{2+} ions, in the range of potentials going from −150 to +110 mV, *HP* = −60 mV (Vm), using whole-cell patch-clamp technique. The K^+-current was leak subtracted and normalized to capacitance. The intercept of the I/V curve on the voltage membrane axis was −33 mV. **(B)** Sample traces of the K^+ ions current recorded in the control conditions. **(C)** Sample trace of the leak current recorded in asymmetrical K^+ ion concentrations in the presence of Ba^{2+} ions (5×10^{-3} M) and TEA (5×10^{-3} M) in the bath solutions.

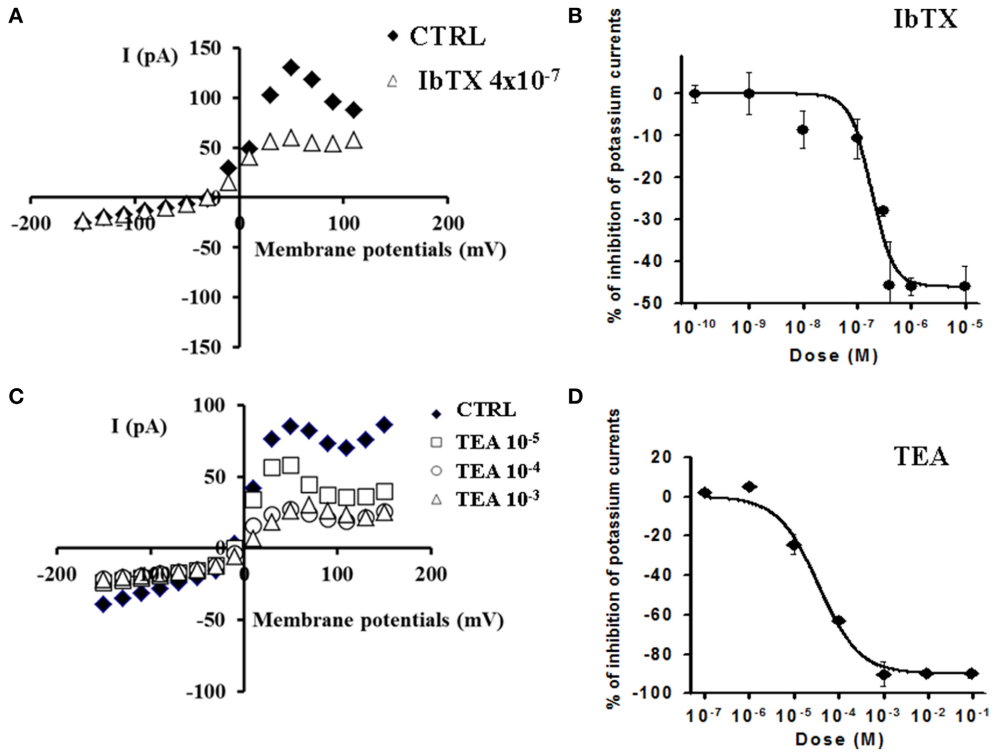


FIGURE 2 | Effects of the BKCa channel blockers IbTX and TEA on K^+-current recorded in SH-SY5Y neuronal cell line. The effects of IbTX and TEA were investigated on the K^+-current recorded in asymmetrical K^+ ions concentrations (int K^+: 132×10^{-3} M; ext K^+: 2.8×10^{-3} M), in the presence of internal 1.6×10^{-6} M concentration of free Ca^{2+} ions, in the range of potentials going from −150 to +110 mV, *HP* = −60 mV using whole cell patch clamp technique. The whole cell K^+-current was a leak subtracted and normalized to capacitance. **(A)** I/V relationship in the absence or presence of IbTX from a single patch. IbTX (4×10^{-7} M) reduced the outward K^+-current of the −53% at +30 mV (Vm) in this patch. **(B)** IbTX (10^{-10}–10^{-5} M) induced a concentration-dependent reduction of the K^+-current at +30 mV (Vm). **(C)** I/V relationship in the absence or presence of increasing concentrations of unselective K^+ channel blocker TEA from a single patch. **(D)** TEA (10^{-5}–10^{-3} M) induced a concentration-dependent reduction of the outward K^+- current. A full reduction of the K^+-current on −100% at +30 mV (Vm) was observed in the presence of TEA at 10^{-3} M concentration.

that after 6 h of incubation time the number of cells showing a diameter size in the range of 13–16 μm was significantly enhanced by IbTX and TEA (**Figure 5B**). IbTX (4×10^{-7} M) equally enhanced the number of cells showing a diameter size in the range of 6–36 μm suggesting that the observed proliferation is mostly due to an increased number of cells with normal morphology (**Figure 5B**). IbTX at 4×10^{-7} M concentration induced at comparable values of cell proliferation of +22 and +18% (diam. range: 13–16 μm) as determined by the mitochondrial succinic dehydrogenase and the cell volume assays, respectively, suggesting that the proliferative effect of this drug is mediated by a common mechanism affecting either cell volume and the mitochondrial succinic dehydrogenase enzyme. TEA instead caused a significant enhancement of the number of cells showing a diameter size in the range of 6–36 μm in respect to that of the control cells (diam. range: 13–16 μm). This suggests the presence of an abnormal cell population following TEA treatment with a diameter size different from control cells. Moreover, TEA at 10^{-3} M concentration induced a different quantitative enhancement of the cell proliferation of +30 and +19% (diam.: range: 6–36 μm) as determined by the mitochondrial succinic dehydrogenase and the cell volume assays, respectively, suggesting that the two mechanisms may be unrelated to this drug.

The basal PKC activity measured by ELISA assay after 6 h of incubation time was higher than PKA activity in the cells lysate in the control condition. The BKCA channel blockers IbTX (10×10^{-9} M) and TEA (100×10^{-6} M) enhanced the PKC activity, respectively, by 224.12 and 184.31%, with respect to the controls; the BKCa channel opener NS1619 (50×10^{-6} M) also enhanced PKC activity by 203.53% while as expected the STS (0.2×10^{-6} M) reduced it by −52.12% (**Figure 6A**).

The BKCa channel blockers IbTX and TEA also enhanced the PKA activity, respectively, by 241.75 and 199.96% with respect to the controls; the BKCA channel opener NS1619 enhanced PKA activity by 193.23%, while STS did not affect it (**Figure 6B**).

DISCUSSION

In the present work we investigated on the role of the BKCa channels in the cell proliferation of the human neuroblastoma cell line SH-SY5Y. In asymmetrical K^+ ions concentrations, these cells show an elevated K^+-currents sustained by Kv and BKCa channels, with a minor contribution of the Kirs currents to the total K^+-currents. The I/V relationship showed an S shaped form going from −150 to +100 mV (Vm) with a reduction of the current amplitude at voltages >100 mV possibly related to inactivation processes characterizing Kv channels. As expected, these

cells were depolarized as compared to the native excitable cells such as muscle fibers or neurons (Yang and Brackenbury, 2013; Urrego et al., 2014). In our experiments, the BKCA channels contributed significantly to the total voltage dependent current component; indeed the IbTX sensitive BKCa channel current was about 50% of the total currents recorded following depolarization in the presence of 1.6×10^{-6} M concentrations of internal free Ca^{2+} ions.

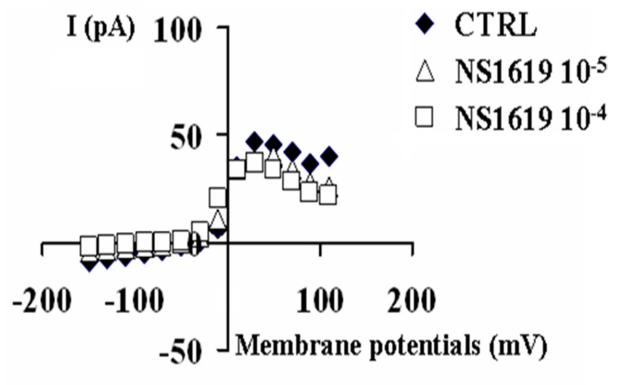


FIGURE 3 | Effects of the BKCa channel opener NS1619 on K^+-current recorded in SH-SY5Y neuronal cell line. The effects of the NS1619 (10×10^{-6} M and 100×10^{-6} M) were investigated on the K^+-current recorded in asymmetrical K^+ ions concentrations (int K^+: 132×10^{-3} M; ext K^+: 2.8×10^{-3} M), in the presence of internal 1.6×10^{-6} M concentration of free Ca^{2+} ions, in the range of potentials going from −150 to +110 mV, $HP = -60$ mV using whole cell patch clamp technique. The K^+-current was leak subtracted and normalized to capacitance. The I/V relationship was constructed in the absence (CTRL) or presence of different concentrations of NS1619. In the this experiment, the application of 10×10^{-6} M and 100×10^{-6} M concentrations of this drug enhanced the K^+-channel current, at −10 mV (Vm), respectively by +41.7 and +171% with respect to the control.

The BKCa channel blocker IbTX and the unselective K^+ channel blocker TEA induced a maximal cell proliferation of about 40%, suggesting that BKCa channel and Kv channels similarly contribute to cell proliferation in this cell line. The toxin at a higher concentration of 4×10^{-7} markedly reduced channel currents of −47%, enhanced cell proliferation by +25% and cell volume of +22% of normal cells. TEA caused a full Kv channel block, enhanced cell proliferation of about +30–40% and cell volume of 17%. These findings are in agreement with the fact that specific BK or Kv channel blockers are expected to increase cell volume and proliferation while specific channel openers are expected to reduce cell volume (Lang and Hoffmann, 2013).

In our experiments a significant cell proliferation was also observed in the presence of low concentrations of IbTX (10^{-7}–10^{-8} M) that caused a partial reduction of the K^+-current of about −10%. Currently, we can hypothesize that this effect may be unrelated to the conduction properties of the channel but rather may involve protein-protein interactions that lead to activation of intracellular signaling (Urrego et al., 2014). This finding appears to be in agreement with the idea that BKCa channels contribute to the high proliferative or invasive potential in a number of malignant cell lines (Weaver et al., 2006; Bloch et al., 2007; Ouadid-Ahidouch and Ahidouch, 2008; Sontheimer, 2008; Khaitan et al., 2009; Koehl et al., 2010). MaxiK channel overexpression has been correlated with the malignancy of human gliomas, which has been associated with an abnormal overactive gBKCa channel (Toro et al., 2014).

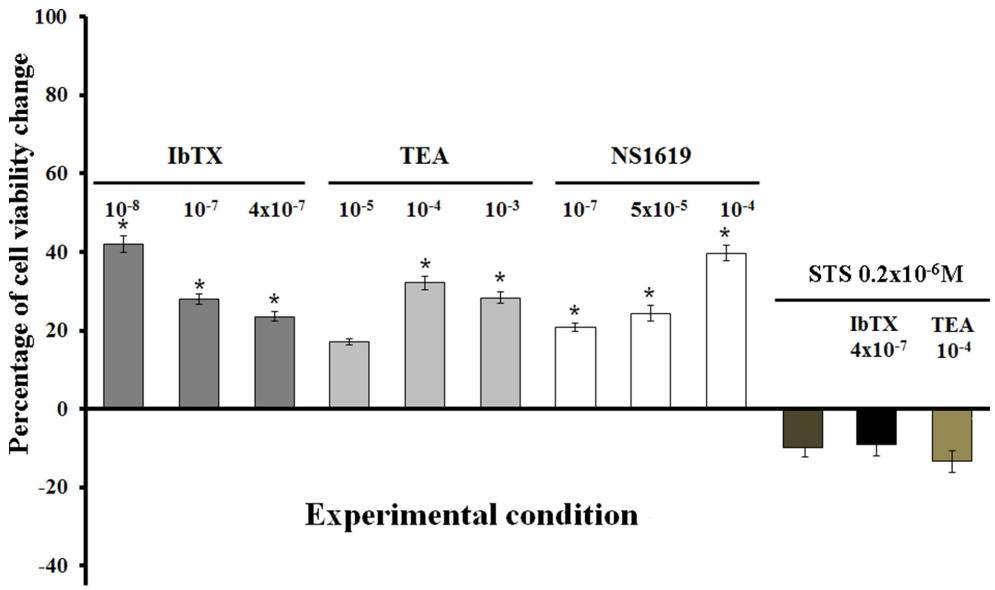


FIGURE 4 | Effects of the channel blockers IbTX and TEA and BKCa channel opener NS1619 on the viability of SH-SY5Y cells. Concentration-dependent increase of the cell proliferation observed with TEA as determined by mitochondrial succinic dehydrogenase activity assay. An inverse relationship was observed with IbTX showing a maximal proliferative effect on 10^{-8} M concentration, but reducing cell proliferation at high concentrations. The NS1619 enhanced cell proliferation showing a maximal proliferative effect of +117%. The co-incubation of the cells with IbTX/TEA+STS(2×10^{-6} M) fully antagonized the proliferative actions of the IbTX and TEA, also leading to cell death in respect to the controls. *Data significantly different with respect to the controls for $p < 0.05$ as determined by student *t*-test.

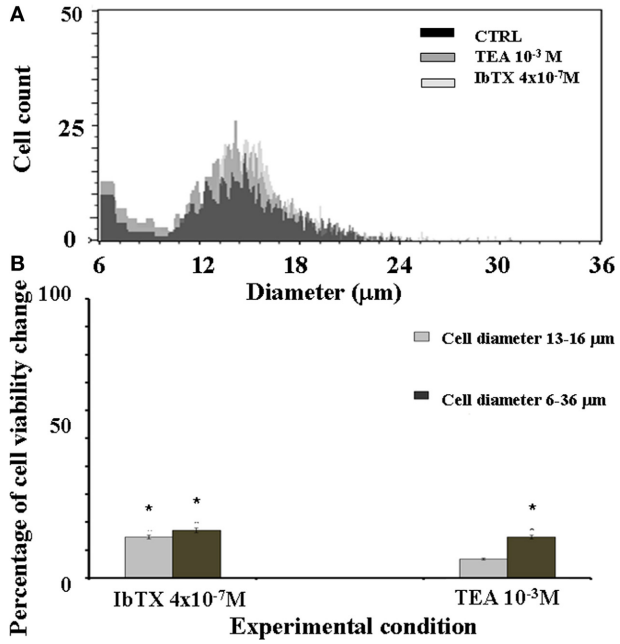


FIGURE 5 | SH-SY5Y neuronal cell distribution in the presence or absence of the BKCa channel blockers IbTX and TEA. The effects of the IbTX and TEA on the cell volume were investigated. The cellular distribution followed a Gaussian type distribution **(A)** The most frequently observed cell population in our experimental condition showed a diameter size in the range of 13–16 μm as determined by cell volume assay. The mean cell diameter did not significantly differ between treatments, the values were 14.89 ± 1 in the controls (CTRL) and were 15.02 ± 2 and 14.5 ± 1, respectively, in the cells treated with IbTX and TEA **(B)** After 6 h of incubation time the number of the cells showing a diameter size in the range of 13–16 μm was significantly enhanced by IbTX and TEA. *Data significantly different with respect to the controls for $p < 0.05$ as determined by student *t*-test.

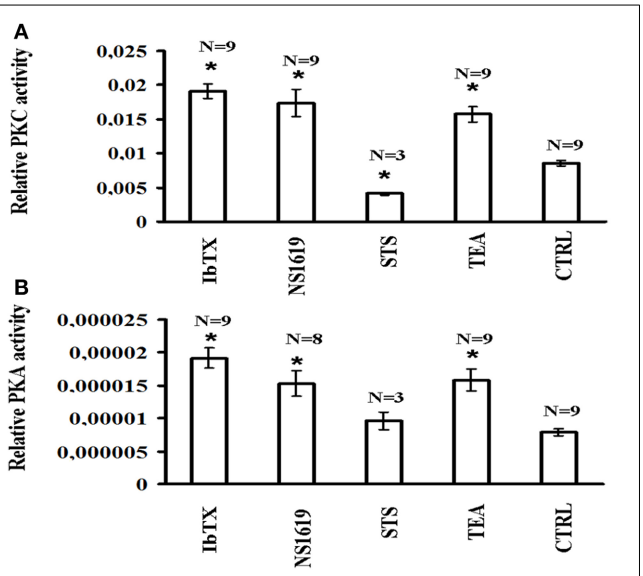


FIGURE 6 | PKC and PKA activities in SH-SY5Y neuronal cell after 6 h incubation in the presence of the channel blockers IbTX and TEA and opener NS1619. The effects of the BKCa channel modulators on the protein kinase C (PKC) and protein kinase A (PKA) were investigated in the cell lysate. The cells were incubated with the drugs under investigation for 6 h and the cell lysate were analyzed using an ELISA assay for PKC and PKA activities **(A)** IbTX (10×10^{-9} M), TEA (100×10^{-6} M), and NS1619 (50×10^{-6} M) significantly enhanced PKC activity, while the STS (0.2×10^{-6} M) reduced it with respect to the controls **(B)** TEA, IbTX, and NS1819 enhanced PKA activity, while STS was without effect with respect to the controls. *Data significantly different with respect to the controls for $p < 0.05$ as determined by student *t*-test.

In HEK293 cells transfected with the recombinant channel subunits, the NS1619 prevented cell proliferation induced by the BKCa channel blockers reducing cell viability, in our experiments NS1619 enhanced proliferation of the SH-SY5Ycells (Chang et al., 2011; Tricarico et al., 2013). This apparent discrepancy can be explained, taking into account the different molecular composition and properties of the recombinant vs. the native BKCa channel subtypes functionally expressed in the cells. NS1619 may also exert unspecific actions, for instance against L-type Ca^{2+} channels (Park et al., 2007).

The cell proliferation induced by the IbTX and TEA was prevented by staurosporine suggesting that this phenomenon is mediated by PKC or other staurosporine-sensitive protein kinases. We tested this hypothesis investigating the PKC and PKA activities using an ELISA assay in the lysates of cells incubated for 6 h with BKCa and Kv channel modulators. We found a marked enhancement of the PKC activity in the cells following IbTX, NS1619, and TEA, while staurosporine significantly reduced the PKC activity without significantly affecting the PKA activity in the cell lysates.

In our experiments the involvement of the surface BKCa channel in cell proliferation was investigated by performing patch-clamp experiments and cell proliferation assays in the presence of IbTX which is a specific and almost impermeable BKCa channel blocker. The partial channel block and the proliferation induced by IbTX at a low concentration was accompanied by the enhancement of the number cells showing a normal morphology without the appearance of any abnormal cell population as determined by the cell volume assay performed in our experiments. The fact that IbTX at a 4×10^{-7} M concentration induced at comparable values of cell proliferation of +22 and +18% (diam. range: 13–16 μm) using the mitochondrial succinic dehydrogenase and the cell volume assays, respectively, suggested that the proliferative effect of this drug is mediated by a common target affecting either cell volume and the mitochondrial succinic dehydrogenase enzyme. Because IbTX is a relatively impermeant toxin, the main target of this action can be the surface BKCa channel. But it should be stressed that the contribution of the nuclear BKCa channels in the neuronal cell proliferation cannot be excluded (Li et al., 2014).

In conclusion, BKCa channel plays an essential role in the proliferation of the native human neuroblastoma cell line SH-SY5Y and this effect is mediated by PKC and PKA enzymes, other protein kinases sensitive to staurosporine may be also involved. Other than in cell proliferation, BKCa has been involved in cell migration. It should be stressed that BKCa channels are not currently considered an oncogene *per se*, but instead can modulate cell

migration and invasion and act like a facilitator as it has been reported in glioma cells (Lisheng et al., 2014).

The BKCa/PKC/PKA pathway may play a role in the cell repair processes. PKC and PKA enzymes other than recognized pathways involved in the proliferative disorders (Parker et al., 2014), their induction/activation was recently associated with the cell repair processes. The PKC isoforms particularly the alpha type, regulates physiological processes such as phagocytosis, endocytosis and desmosome downregulation; exert a cytoprotecting role against some forms of tumors (Larsen et al., 2000; Cheeseman et al., 2006; Boyle et al., 2014; McHarg et al., 2014). These mechanisms promote cell plasticity function favoring cell survival. The induction/activation of the PKA subtypes is also involved in the cell cytoprotection in the neurite plasmalemma repair (Zuzek et al., 2013).

REFERENCES

Abdullaev, I. F., Rudkouskaya, A., Mongin, A. A., and Kuo, Y. H. (2010). Calcium-activated potassium channels BKCA and IK1 are functionally expressed in human gliomas but do not regulate cell proliferation. *PLoS ONE* 5:e12304. doi: 10.1371/journal.pone.0012304

Adrian, R. H., and Bryant, S. H. (1974). On the repetitive discharge in myotonic muscle fibres. *J Physiol.* 240, 505–515.

Bloch, M., Ousingsawat, J., Simon, R., Schraml, P., Gasser, T. C., Mihatsch, M. J., et al. (2007). KCNMA1 gene amplification promotes tumor cell proliferation in human prostate cancer. *Oncogene* 26, 2525–2534. doi: 10.1038/sj.onc.1210036

Boyle, G. M., D'Souza, M. M., Pierce, C. J., Adams, R. A., Cantor, A. S., Johns, J. P., et al. (2014). Intra-lesional injection of the novel PKC activator EBC-46 rapidly ablates tumors in mouse models. *PLoS ONE* 9:e108887. doi: 10.1371/journal.pone.0108887

Cambien, B., Rezzonico, R., Vitale, S., Rouzaire-Dubois, B., Dubois, J. M., Barthel, R., et al. (2008). Silencing of hSlo potassium channels in human osteosarcoma cells promotes tumorigenesis. *Int. J. Cancer* 123, 365–371. doi: 10.1002/ijc.23511

Cannon, S. C. (1996). Sodium channel defects in myotonia and periodic paralysis. *Annu. Rev. Neurosci.* 19, 141–164. doi: 10.1146/annurev.ne.19.030196.001041

Chang, H., Ma, Y. G., Wang, Y. Y., Song, Z., Li, Q., Yang, N., et al. (2011). High glucose alters apoptosis and proliferation in HEK293 cells by inhibition of cloned BKCA Ca channel. *J. Cell. Physiol.* 226, 1660–1675. doi: 10.1002/jcp.22497

Cheeseman, K. L., Ueyama, T., Michaud, T. M., Kashiwagi, K., Wang, D., Flax, L. A., et al. (2006). Targeting of protein kinase C-epsilon during Fcgamma receptor-dependent phagocytosis requires the epsilonC1B domain and phospholipase C-gamma1. *Mol. Biol. Cell* 17, 799–813. doi: 10.1091/mbc.E04-12-1100

Debska-Vielhaber, G., Godlewski, M. M., Kicinska, A., Skalska, J., Kulawiak, B., Piwonska, M., et al. (2009). Large-conductance K^+ channel openers induce death of human glioma cells. *J. Physiol. Pharmacol.* 60, 27–36.

Dinardo, M. M., Camerino, G., Mele, A., Latorre, R., Conte Camerino, D., and Tricarico, D. (2012). Splicing of the rSlo gene affects the molecular composition and drug response of Ca^{2+}-activated K^+ channels in skeletal muscle. *PLoS ONE* 7:e40235. doi: 10.1371/journal.pone.0040235

Han, X., Xi, L., Wang, H., Huang, X., Ma, X., Han, Z., et al. (2008). The potassium ion channel opener NS1619 inhibits proliferation and induces apoptosis in A2780 ovarian cancer cells. *Biochem. Biophys. Res. Commun.* 375, 205–209. doi: 10.1016/j.bbrc.2008.07.161

Henney, N. C., Li, B., Elford, C., Reviriego, P., Campbell, A. K., Wann, K. T., et al. (2009). A large-conductance (BKCA) potassium channel subtype affects both growth and mineralization of human osteoblasts. *Am. J. Physiol. Cell Physiol.* 297, C1397–C1408. doi: 10.1152/ajpcell.00311.2009

Jia, X., Yang, J., Song, W., Li, P., Wang, X., Guan, C., et al. (2013). Involvement of large conductance Ca(2+)-activated K (+) channel in laminar shear stress induced inhibition of vascular smooth muscle cell proliferation. *Pflugers Arch.* 465, 2012-2032. doi: 10.1007/s00424-012-1182-z

Khaitan, D., Sankpal, U. T., Weksler, B., Meister, E. A., Romero, I. A., Couraud, P. O., et al. (2009). Role of KCNMA1 gene in breast cancer invasion and metastasis to brain. *BMC Cancer* 9:258. doi: 10.1186/1471-2407-9-258

Koehl, G. E., Spitzner, M., Ousingsawat, J., Schreiber, R., Geissler, E. K., and Kunzelmann, K. (2010). Rapamycin inhibits oncogenic intestinal ion channels and neoplasia in APC(Min/+) mice. *Oncogene* 29, 1553–1560. doi: 10.1038/onc.2009.435

Kyle, B. D., and Braun, A. P (2014). The regulation of BKCA channel activity by pre- and post-translational modifications. *Front. Physiol.* 5:316. doi: 10.3389/fphys.2014.00316

Lang, F., and Hoffmann, E. K. (2013). CrossTalk proposal: Cell volume changes are an essential step in the cell death machinery. *J. Physiol.* 591, 6119–6121. doi: 10.1113/jphysiol.2013.258632

Larsen, E. C., DiGennaro, J. A., Saito, N., Mehta, S., Loegering, D. J., Mazurkiewicz, J. E., et al. (2000). Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells. *J. Immunol.* 165, 2809–2817. doi: 10.4049/jimmunol.165.5.2809

Lee, U. S., and Cui, J. (2010). BKCA channel activation: structural and functional insights. *Trends Neurosci.* 33, 415–423. doi: 10.1016/j.tins.2010.06.004

Li, B., Jie, W., Huang, L., Wei, P., Li, S., Luo, Z., et al. (2014). Nuclear BKCA channels regulate gene expression via the control of nuclear calcium signaling. *Nat. Neurosci.* 17, 1055–1063. doi: 10.1038/nn.3744

Lisheng, G. E., Hoa, N. T., Wilson, Z., Arismendi-Morillo, G., Kong, X., Tajhya, R. B., et al. (2014). Big Potassium (BK) ion channels in biology, disease and possible targets for cancer immunotherapy. *Int. Immunopharmacol.* 22, 427–443. doi: 10.1016/j.intimp.2014.06.040

Ma, Y.-G., Liu, W.-C., Dong, S., Du, C., Wang, X.-J., Li, J. S., et al. (2012). Activation of BKCACa channels in zoledronic acid-induced apoptosis of MDA-MB-231 breast cancer cells. *PLoS ONE* 7:e37451. doi: 10.1371/journal.pone.0037451

McHarg, S., Hopkins, G., Lim, L., and Garrod, D. (2014). Down-regulation of desmosomes in cultured cells: the roles of PKC, microtubules and lysosomal/proteasomal degradation. *PLoS ONE* 9:e108570. doi: 10.1371/journal.pone.0108570

Orio, P., and Latorre, R. (2005). Differential effects of beta1 and beta2 subunits on BKCA channel activity. *J. Gen. Physiol.* 125, 395–411. doi: 10.1085/jgp.200409236

Ouadid-Ahidouch, H., and Ahidouch, A. (2008). K^+ channel expression in human breast cancer cells: involvement in cell cycle regulation and carcinogenesis. *J. Membr. Biol.* 221, 1–6. doi: 10.1007/s00232-007-9080-6

Park, J. H., Park, S. J., Chung, M. K., Jung, K. H., Choi, M. R., Kim, Y., et al. (2010). High expression of large-conductance Ca2+-activated K+ channel in the CD133+ subpopulation of SH-SY5Y neuroblastoma cells. *Biochem. Biophys. Res. Commun.* 396, 637–642. doi: 10.1016/j.bbrc.2010.04.142

Park, W. S., Kang, S. H., Son, Y. K., Kim, N., Ko, J. H., Kim, H. K., et al. (2007). The mitochondrial Ca2+-activated K+ channel activator, NS 1619 inhibits L-type Ca2+ channels in rat ventricular myocytes. *Biochem Biophys Res Commun.* 362, 31–36. doi: 10.1016/j.bbrc.2007.07.057

Parker, P. J., Justilien, V., Riou, P., Linch, M., and Fields, A. P. (2014). Atypical protein kinase Cι as a human oncogene and therapeutic target. *Biochem. Pharmacol.* 88, 1–11. doi: 10.1016/j.bcp.2013.10.023

Roger, S., Potier, M., Vandier, C., Le Guennec, J. Y., and Besson, P. (2004). Description and role in proliferation of iberiotoxin-sensitive currents in different human mammary epithelial normal and cancerous cells. *Biochim. Biophys. Acta* 1667, 190–199. doi: 10.1016/j.bbamem.2004.10.002

Sontheimer, H. (2008). An unexpected role for ion channels in brain tumor metastasis. *Exp. Biol. Med. (Maywood)* 233, 779–791. doi: 10.3181/0711-MR-308

Toro, L., Li, M., Zhang, Z., Singh, H., Wu, Y., and Stefani, E. (2014). MaxiK channel and cell signalling. *Pflugers Arch.* 466, 875–886. doi: 10.1007/s00424-013-1359-0

Tricarico, D., Mele, A., Calzolaro, S., Cannone, G., Camerino, G. M., Dinardo, M. M., et al. (2013). Emerging role of calcium-activated potassium channel in the regulation of cell viability following potassium ions challenge in HEK293 cells and pharmacological modulation. *PLoS ONE* 8:e69551. doi: 10.1371/journal.pone.0069551

Tricarico, D., Mele, A., and Conte Camerino, D. (2005). Phenotype-dependent functional and pharmacological properties of BKCA channels in skeletal muscle: effects of microgravity. *Neurobiol. Dis.* 20, 296–302. doi: 10.1016/j.nbd.2005.03.011

Urrego, D., Tomczak, A. P., Zahed, F., Stühmer, W., and Pardo, L. A. (2014). Potassium channels in cell cycle and cell proliferation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369:20130094. doi: 10.1098/rstb.2013.0094

Weaver, A. K., Bomben, V. C., and Sontheimer, H. (2006). Expression and function of calcium-activated potassium channels in human glioma cells. *Glia* 54, 223–233. doi: 10.1002/glia.20364

Yan, J., and Aldrich, R. W. (2010). LRRC26 auxiliary protein allows BKCa channel activation at resting voltage without calcium. *Nature* 466, 513–516. doi: 10.1038/nature09162

Yan, J., and Aldrich, R. W. (2012). BKCa potassium channel modulation by leucine-rich repeat-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7917–7922. doi: 10.1073/pnas.1205435109

Yang, M., and Brackenbury, W. J. (2013). Membrane potential and cancer progression. *Front. Physiol.* 4:185. doi: 10.3389/fphys.2013.00185

Zhang, X. F., Gopalakrishnan, M., and Shieh, C. C. (2003). Modulation of action potential firing by iberiotoxin and NS161 in rat dorsal root ganglion neurons. *Neuroscience* 122, 1003–1011. doi: 10.1016/j.neuroscience.2003.08.035

Zuzek, A., Fan, J. D., Spaeth, C. S., and Bittner, G. D. (2013). Sealing of transected neurites of rat B104 cells requires a diacylglycerol PKC-dependent pathway and a PKA-dependent pathway. *Cell. Mol. Neurobiol.* 33, 31–46. doi: 10.1007/s10571-012-9868-5

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 July 2014; accepted: 20 November 2014; published online: 09 December 2014.

*Citation: Curci A, Mele A, Camerino GM, Dinardo MM and Tricarico D (2014) The large conductance Ca^{2+} -activated K^{+} (BKCa) channel regulates cell proliferation in SH-SY5Y neuroblastoma cells by activating the staurosporine-sensitive protein kinases. Front. Physiol. **5**:476. doi: 10.3389/fphys.2014.00476*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

REVIEW ARTICLE
published: 02 December 2014
doi: 10.3389/fphys.2014.00466

Ethanol modulation of mammalian BK channels in excitable tissues: molecular targets and their possible contribution to alcohol-induced altered behavior

Alex M. Dopico[1]*, Anna N. Bukiya[1] and Gilles E. Martin[2]

[1] Department of Pharmacology, College of Medicine, The University of Tennessee Health Science Center, Memphis, TN, USA
[2] Department of Psychiatry, The University of Massachusetts Medical School, Worcester, MA, USA

Edited by:
Ali Mobasheri, University of Surrey, UK

Reviewed by:
Richard Barrett-Jolley, University of Liverpool, UK
Susanne Schmid, University of Western Ontario, Canada

***Correspondence:**
Alex M. Dopico, Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, 874 Union Ave., Memphis, TN 38163, USA
e-mail: adopico@uthsc.edu

In most tissues, the function of Ca^{2+}- and voltage-gated K^+ (BK) channels is modified in response to ethanol concentrations reached in human blood during alcohol intoxication. In general, modification of BK current from ethanol-naïve preparations in response to brief ethanol exposure results from changes in channel open probability without modification of unitary conductance or change in BK protein levels in the membrane. Protracted and/or repeated ethanol exposure, however, may evoke changes in BK expression. The final ethanol effect on BK open probability leading to either BK current potentiation or BK current reduction is determined by an orchestration of molecular factors, including levels of activating ligand (Ca_i^{2+}), BK subunit composition and post-translational modifications, and the channel's lipid microenvironment. These factors seem to allosterically regulate a direct interaction between ethanol and a recognition pocket of discrete dimensions recently mapped to the channel-forming (slo1) subunit. Type of ethanol exposure also plays a role in the final BK response to the drug: in several central nervous system regions (e.g., striatum, primary sensory neurons, and supraoptic nucleus), acute exposure to ethanol reduces neuronal excitability by enhancing BK activity. In contrast, protracted or repetitive ethanol administration may alter BK subunit composition and membrane expression, rendering the BK complex insensitive to further ethanol exposure. In neurohypophyseal axon terminals, ethanol potentiation of BK channel activity leads to a reduction in neuropeptide release. In vascular smooth muscle, however, ethanol inhibition of BK current leads to cell contraction and vascular constriction.

Keywords: slo1 proteins, BK beta subunits, membrane lipids, ethanol-recognition site, n-alkanols, alcohol tolerance, ion channels

INTRODUCTION

Ca^{2+}-activated K^+ channels are defined by their high selectivity for K^+ over other monovalents and enhanced activity upon increases in intracellular Ca^{2+} (Ca_i^{2+}). Based on unitary conductance (γ), Ca^{2+}-activated K^+ channels have been classified into large (BK), intermediate (IK) and small conductance (SK) channels. These phenotypes also present differential sensitivity to Ca_i^{2+}, membrane voltage and distinct peptide blockers (Latorre et al., 1989; Stocker, 2004; Salkoff et al., 2006). The term BK channel, however, more properly applies to not only Ca^{2+}-activated K^+ channels of large conductance, i.e., BK α subunits or slo1 channels, which are products of the *Slo1*, *KCNMA1* gene or its orthologs (see **Table 1** for nomenclature), but also to the channel-forming protein products of *Slo2* and *Slo3*, which are primarily gated not by Ca_i^{2+} but by Na^+/Cl^- and H^+, respectively (Schreiber et al., 1998; Dryer, 2003; Xia et al., 2004; Salkoff et al., 2006). In this mini-review, however, we will use "BK channel" to designate a functional channel that results from tetrameric association of slo1 proteins and may include additional regulatory subunits that contribute to the current phenotype (see below).

In most neurons, K^+ efflux due to Ca^{2+}-activated K^+ channel activity effectively drives the membrane potential toward more negative values leading to reduced excitability (Wong and Prince, 1981; MacDermott and Weight, 1982; Brown et al., 1983; Sah, 1996). This led to the early speculation that Ca^{2+}-activated K^+ "conductances" could be modified by alcohols and other sedative/hypnotic agents in central nervous system (CNS) neurons (Krnjevic, 1972; Nicoll and Madison, 1982). Early studies on modulation of Ca^{2+}-activated K^+ currents by ethanol were

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP, action potential; BK, Ca^{2+} and voltage-gated, large conductance K^+; cAMP, cyclic adenosine monophosphate; Ca_i^{2+}, cytosolic Ca^{2+}; Ca^{2+}-activated K^+; CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; CNS, central nervous system; CTD, cytosolic tail domain; DRG, dorsal root ganglia; EC_{max}, maximal effective concentration; EC_{50}, half-maximal effective concentration; EPSP, excitatory post-synaptic potential; GH3, GH4/C1, rat pituitary epithelial-like tumor cell lines; HEK, human embryonic kidney (cell line); IP3, Inositol trisphosphate; MSN, medium spiny neurons; MthK, potassium channel from *Methanobacterium thermoautotrophicum;* NMDA, N-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase C; Po, single channel open probability; PP, phosphatase; RCK, regulator of conductance for K^+; RyR, ryanodine receptor; S, transmembrane segment in ion channel core; SK, potassium channels of small conductance; TM, transmembrane domain.

Table 1 | Nomenclature of large-conductance K^+ channel proteins and genes used in this review.

BK	Protein complex forming an ion channel with a phenotype that combines high-conductance for K^+ with voltage- and Ca^{2+}-gating, disregarding subunit composition. Also cited in literature as BK_{Ca} or MaxiK channels.
KCNMA1	Mammalian gene that encodes the BK channel-forming slo1 protein, so called BK α subunit.
KCNMB1-4	Genes that encode the regulatory BK β subunits, these subunits being unable to form functional channels by themselves. Four β subunits have been identified (β1-4), each of the four types resulting from its corresponding gene (*KCNMB1-4*).
Slo1	Same as *KCNMA1.*
Slo	General term to define any non-mammalian ortholog of *Slo1.*
xslo1	BK channel-forming α subunit, where "x" denotes the species of origin (e.g., hslo1 from human, mslo1 from mouse, etc.) For consistency with previously published work, an exception was made for cbv1, which denotes slo1 proteins cloned from rat cerebral blood vessel (artery) myocytes.
slo2	High-conductance K^+ channel protein gated by Na_i^+ or Cl_i^-.
slo3	High-conductance for K^+ channel protein gated by H_i^+/OH_i^-.

conducted on non-neuronal preparations and/or using alcohol concentrations well above circulating ethanol levels that are usually lethal in alcohol-naïve mammals (>100 mM), as reviewed elsewhere (Dopico et al., 1999a). A few early studies, however, did show that 5–20 mM ethanol (legal intoxication in the US is defined by 10–17.4 mM ethanol in blood) applied to hippocampal CA1 or CA3 neurons and granule cells, and cerebellar Purkinje cells enhanced a Ca^{2+}-dependent after-hyperpolarization while increasing overall K^+ conductance (Carlen et al., 1982, 1985; Niesen et al., 1988). Likewise, ethanol concentrations as low as 5 mM activate a Ca^{2+}-activated K^+ conductance in *Helix aspersa* right parietal ganglion (Madsen and Edeson, 1990). From these early studies, however, it was not possible to discern the Ca^{2+}-activated K^+ channel type affected by ethanol. In addition, these and later studies conducted in intact cells could not address whether ethanol effect on Ca^{2+}-activated K^+ current resulted from drug action on the Ca^{2+}-activated K^+ current itself or, rather, was secondary to ethanol modulation of Ca^{2+}-sources that controlled Ca_i^{2+}-activated K^+ channel activity.

BK channels received particular attention as functional targets of ethanol in the CNS as they are usually expressed and play major roles in all three neuronal compartments: somata, axon terminals and dendrites. Moreover, the channel's sensitivity to both voltage and Ca_i^{2+} places it at the nexus of many cellular pathways associated with neuronal plasticity. BK channel pluripotency is further underscored by a recent study showing its presence in the neuronal nuclear membrane where it controls Ca^{2+} flux and gene expression (Li et al., 2014). At the presynaptic membrane, BK channels control the release of neurotransmitters by dampening the depolarization evoked by incoming action potentials (APs) (Raffaelli et al., 2004; Wang, 2008). On the post-synaptic side, BK channels contribute to AP shaping (Faber and Sah, 2002, 2003) and patterning (Jin et al., 2000; Zhang et al., 2003; Brenner et al., 2005; Meredith et al., 2006), and modulate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and N-methyl-D-aspartic acid (NMDA)-mediated excitatory post-synaptic potentials (EPSPs) (Isaacson and Murphy, 2001; Liu et al., 2011). The BK channel also controls dendritic excitability (Golding et al., 1999; Wessel et al., 1999; Rancz and Häusser, 2006; Benhassine and Berger, 2009), as well as retrograde propagation of somatic APs to the dendrites (Wessel et al., 1999; Ji and Martin, 2012).

By the mid to late nineties, using isolated neurohypophyseal axon terminals and pituitary epithelial-like tumor cell lines (GH3 cells) from the rat, two groups communicated the selective activation of BK channels by acute exposure to clinically relevant ethanol concentrations: half-maximal effective concentration (EC_{50}) ≈ 22 mM; maximal effective concentration (EC_{max}) ≤ 100 mM (Dopico et al., 1996; Jakab et al., 1997). Experimental conditions from these two studies demonstrated that ethanol action was due to drug targeting of the BK channel complex itself and/or its immediate proteolipid environment. Since then, activation of native BK channels by brief exposure to clinically relevant ethanol levels has been extended to both excitable and non-excitable tissues (Brodie et al., 2007; Martin et al., 2008; Pietrzykowski et al., 2008; Bukiya et al., 2009; Wynne et al., 2009; Velázquez-Marrero et al., 2011; Bettinger et al., 2012; Handlechner et al., 2013; Liu et al., 2013; Davis et al., 2014; Malysz et al., 2014). In parallel, several groups have documented ethanol-SK channel functional interactions and their relevance to alcohol-induced modified behaviors. Literature on ethanol and SK channels has been reviewed elsewhere (Brodie et al., 2007; Mulholland et al., 2009) and is not dealt with in this review, which focuses on modulation of BK channels from mammalian systems in response to acute ethanol administration. In particular, we concentrate on the many molecular entities and mechanisms that determine the final BK current response to brief (acute) ethanol exposure, and the consequences of such modulation on the physiology of excitable tissues. Neuronal and behavioral adaptations involving BK channels or neuronally-expressed genes coding for BK channel subunits to repetitive or protracted ethanol exposure have been well documented in both mammals and non-mammals (Mulholland et al., 2009; Treistman and Martin, 2009; McIntire, 2010; Ghezzi and Atkinson, 2011) and comprehensively treated in this volume by Bettinger and Davies (2014).

ETHANOL EFFECT ON BK CURRENTS IN ALCOHOL-NAïVE SYSTEMS: PHENOMENOLOGY AND MODIFICATIONS IN CHANNEL GATING

Following brief exposure (<5 min) of native BK channels to clinically relevant ethanol concentrations (10–100 mM), steady-state ionic current potentiation, refractoriness and reduction have all been observed, this heterogeneity being reported even between different compartments of a same neuronal type (Dopico et al.,

1999b; Martin et al., 2004; Wynne et al., 2009). The variety of molecular factors that contribute to such heterogeneity are extensively discussed in a separate section below. However, some generalizations from studies of acute ethanol action on native channels and recombinant BK proteins expressed in natural membranes or following channel reconstitution into artificial planar bilayers can be made. In the vast majority of cases, provided that the channel consists of homomeric slo1 or heteromeric slo1 + β4 subunits and is evaluated at Ca_i^{2+} within nM to low μM, a few min exposure to ethanol potentiates steady-state current (Brodie et al., 2007; Mulholland et al., 2009). This potentiation occurs in absence of changes in K^+ permeability (Dopico et al., 1996, 1998; Jakab et al., 1997; Gruß et al., 2001; Martin et al., 2004) or selectivity over Na^+ (Dopico et al., 1996, 1998) and BK membrane expression (Dopico et al., 1998) but results from ethanol-induced increase in channel open probability (Po). In neurohypophyseal terminals, this increase is consistently observed provided that alcohol-naïve preparations are briefly exposed to the drug (a few min) (Dopico et al., 1996) and totally disappears after 12 min of constant ethanol exposure (Pietrzykowski et al., 2004). The mechanisms leading to this rapid desensitization to ethanol exposure remain to be fully addressed (see next Sections on Molecular Targets). However, when neurohypophyseal explants are subject to 24 h-long ethanol exposure, decreased BK current density has been linked to a reduction in BK channel clustering in the cell membrane and internalization of the channel α (slo1) subunit (Pietrzykowski et al., 2004).

Following brief exposure to alcohol-naïve systems, ethanol-induced maximal increase in BK is reached at 75–100 mM, with an EC_{50} = 20–25 mM (reviewed in Brodie et al., 2007), the latter being close to blood alcohol levels considered legal intoxication in most US states (0.08g/dl = 17.4 mM) (Diamond, 1992; Thombs et al., 2003). While these ethanol concentrations are significantly higher than those of other BK channel modulators (Weiger et al., 2002), different studies ruled out that an osmotic load to the membrane and/or channel complex was a major contributor to ethanol action on BK currents (Dopico et al., 1996, 1998; Jakab et al., 1997). Because ethanol acute action on BK channels studied in cell-free systems like isolated membrane patches or following reconstitution into planar lipid bilayers of simple composition mimics drug action in intact cells (reviewed in Brodie et al., 2007; Mulholland et al., 2009), it is possible to conclude that acute ethanol modulation of BK current in alcohol-naïve systems is largely independent of the continuous presence of cytosolic signals, internal organelles, complex membrane cytoarchitecture, and ethanol metabolism. It should be noted, however, that acetaldehyde applied to the intracellular surface of GH3 cell membrane patches was able to reduce ethanol-induced activation of BK channels (Handlechner et al., 2013), raising the hypothesis that an ethanol metabolite in excitable tissues contributes to the overall effect of ethanol on native BK currents.

Increased BK Po by ethanol itself results from several modifications in both open and closed-times distributions that lead to minor increase in mean open time and major decrease in mean closed time, the latter primarily due to drug-induced destabilization of channel long-closed states (Dopico et al., 1996, 1998; Chu et al., 1998; Crowley et al., 2003). A 10-state model of slo1 (mslo1, from mouse brain; mbr5 variant) channel gating reveals that ethanol modifies Ca^{2+}-dependent parameters, such as the channel open conformation-Ca^{2+} dissociation (K_O) and closed conformation-Ca^{2+} dissociation (K_C) constants. In contrast, Ca^{2+}-independent parameters, such the equivalent gating charge associated with the open-to-closed equilibrium (Q) and the open-to-closed equilibrium constant in absence of Ca^{2+} and transmembrane voltage (L_0 or "intrinsic gating") remain unchanged. Moreover, slo1 becomes ethanol-resistant when gated by voltage/Mg_i^{2+} in absence of activating Ca_i^{2+}, with fully effective activatory concentrations of ethanol (100 mM) failing to modify mslo1 Po. Consistently, combination of amino acid substitutions (5D5N) in the Ca^{2+}-bowl and in the high affinity regulator of conductance for K^+ (RCK) 1 domain (D362A, D367A), which render both high affinity Ca_i^{2+}-recognition sites non-functional, results in a channel that is ethanol-resistant. However, substitutions that hamper each site result in slo1 channels that retain ethanol sensitivity. These data indicate that ethanol action on BK channels requires activating Ca_i^{2+}. Moreover, as far as Ca_i^{2+} is able to interact with one of its physiological recognition sites, the BK channel is activated by ethanol (Liu et al., 2008). The structural basis of ethanol activation of slo1 channels and its relation to Ca_i^{2+} is provided in a separate section.

The Ca_i^{2+}-dependence of ethanol action, however, further conditions drug action on slo1 channels: ethanol potentiation of Po and macroscopic current progressively diminishes as Ca_i^{2+} increases until ethanol becomes an inhibitor of BK activity; for homomeric slo1 channels, whether mslo mbr5 or cbv1 (from rat cerebral artery myocytes), the "cross-over" from ethanol-induced activation to ethanol-induced inhibition occurs at ∼20 μM Ca_i^{2+} (Liu et al., 2008; Bukiya et al., 2009). Remarkably, this cross-over can be shifted by modulators that fine-tune the overall Ca_i^{2+} sensitivity of the native BK channel, accessory β1 subunits in particular (see separate section). An empirically-derived single channel kinetic model reveals that ethanol-induced inhibition of slo1 Po is related to the drug-induced facilitation of channel dwelling into Ca_i^{2+}-driven low Po modes (Liu et al., 2008), an action that can be conceptualized into ethanol-induced facilitation of Ca_i^{2+}-driven BK channel "desensitization" (Dopico and Lovinger, 2009).

In synthesis, exposure of BK channels to clinically-relevant ethanol concentrations in alcohol naïve, excitable cells under physiological, resting conditions usually results in BK current potentiation, yet refractoriness or inhibition may occur. This heterogeneous response is determined by several molecular entities, which are individually discussed in separate sections below. Ethanol action on BK ionic current results from modification of Po, this action being dependent on the ion that activates the channel under physiological conditions, that is, Ca_i^{2+}.

CHANGES IN PHYSIOLOGY OR BEHAVIOR RELATED TO MODIFICATION OF BK CURRENTS BY ACUTE ETHANOL EXPOSURE

Regulation of BK Po and thus, steady-state ionic current by ethanol exposure has been implicated in alcohol-induced modification of physiology and behavior (reviewed in Brodie et al., 2007; Mulholland et al., 2009; Treistman and Martin, 2009;

McIntire, 2010; Ghezzi and Atkinson, 2011). Early studies concentrated in neurosecretory cells given the central role of BK channels in controlling AP firing and hormone/neurotransmitter release (see above). In rats, ethanol-induced potentiation of BK currents, together with drug-induced inhibition of voltage-dependent Ca^{2+} channels (Wang et al., 1994) has been recognized as a central mechanism in ethanol-induced inhibition of vasopressin and oxytocin release by supraoptic axon terminals (Dopico et al., 1995; Knott et al., 2002). Likewise, ethanol-induced BK channel activation in GH3 and GH4/C1 rat pituitary tumor cells would likely lead to inhibition of hormone release by alcohol (Jakab et al., 1997, 2006). In spite of BK current potentiation, ethanol actually increases growth hormone secretion by GH3-GH4/C1 cells, which has been attributed to increased Ca_i^{2+} (Stojilkovic et al., 2005; Jakab et al., 2006; Brodie et al., 2007) and to cell swelling itself being able to evoke hormone release (Strbak, 2006). Indeed, ethanol has been proven to increase Ca_i^{2+} and cause cell swelling in GH3-GH4/C1 cells (Jakab et al., 2006).

In the rat and mouse striatum, ethanol potentiation of BK currents has been demonstrated to reduce nucleus accumbens medium spiny neurons (MSN) AP firing rate and thus, decrease neuronal excitability (Martin et al., 2004, 2008), the consequences of this ethanol action being linked to ethanol-induced perturbation of motor behavior and alcohol preference (see below and also review by Treistman and Martin, 2009). A decrease in AP frequency in response to 40 mM ethanol has been reported in dorsal root ganglia (DRG) neurons that show positive staining for isolectin B4, a marker for nociceptive neurons. Ethanol also shortens AP duration and increases AP mean threshold, these ethanol actions being blunted by selective blockade of BK channels by iberiotoxin (Gruß et al., 2001). Thus, authors of this study proposed that ethanol actions leading to reduced firing activity and decreased excitability of distinct DRG neurons might contribute to ethanol's analgesic effect in the peripheral nervous system.

In *Caenorhabditis elegans*, ethanol activates BK channels *in vivo*. Notably, the behavioral phenotype of slo1 gain-of-function mutants resembles that of ethanol-intoxicated worms (Davies et al., 2003; Bettinger and Davies, 2014). In *Drosophila melanogaster*, BK channels have been shown to play a central role in the development of drug tolerance to ethanol-induced sedation and dependence (Ghezzi et al., 2004, 2010; Cowmeadow et al., 2005, 2006). The literature on the role of slo channels in alcohol-altered behavior is discussed by Bettinger and Davies in this volume (2014). In conclusion, neuronal BK channels are considered as one of the central players in behavioral responses to ethanol observed across non-vertebrate and vertebrate species.

Ethanol-induced BK channel activation has been proposed as a mechanism for the neuroprotective effect of ethanol preconditioning against post-ischemic neuronal injury in mice (Wang et al., 2010). In contrast to BK channel activation, ethanol-induced BK channel inhibition in both rats and mice has been demonstrated to play a central role in ethanol-induced cerebral artery constriction (Liu et al., 2004; Bukiya et al., 2009). Likewise, this drug action has been hypothesized to also contribute to ethanol-induced aortic constriction (Walters et al., 2000). Exposure of human endothelial umbilical vein cells to 10–50 mM ethanol, however, leads to BK current potentiation, an ethanol action that leads to increased NO production and cell proliferation with eventual bolstering of endothelial function (Kuhlmann et al., 2004). Finally, a recent study shows that BK channel activation plays a critical role in alcohol-induced relaxation of guinea pig urinary bladder smooth muscle (Malysz et al., 2014).

In synthesis, in most neuronal tissues from mammals ethanol-induced activation of BK channels leads to decreased cell excitability whereas in vascular smooth muscle, ethanol-induced inhibition of BK channels leads to arterial constriction.

MOLECULAR TARGETS AND MECHANISMS THAT DETERMINE THE FINAL RESPONSE IN BK CHANNEL ACTIVITY TO ACUTE ETHANOL

Ethanol modulation of BK channel activity has been consistently reported in membrane patches that expressed either native or recombinant channel proteins and after reconstitution of channel subunits into artificial planar lipid bilayers of simple composition (Chu et al., 1998; Crowley et al., 2003, 2005; Yuan et al., 2008, 2011; Bukiya et al., 2011). Thus, functional targets of ethanol action are limited to the channel subunit themselves, their surrounding lipids and any possible interface. In a most reductionist approach, ethanol potentiation of hslo1 channels (from human brain) was observed with homomeric recombinant channel reconstituted into a single species phosphoglyceride, i.e., 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) (Crowley et al., 2003) indicating that this extremely simple proteolipid system must include an ethanol sensor(s). A summary of the different molecular factors and mechanisms that determine the Bk channel response to acute ethanol in alcohol-naïve systems in given in **Figure 1**.

a) Identification of a Protein Pocket of Discrete Dimensions in the Slo1 Protein that Interacts with Ethanol thus Leading to Increased Channel Activity

Slo1 channel proteins are conceptualized as "modular proteins," i.e., with rather defined motifs each serving a defined channel function. Thus, slo1 proteins share with other members of the six transmembrane (TM6) voltage-gated superfamily of ion channels a TM "core," which includes the voltage-sensing domain and the ion permeation pore-gate domain (Toro et al., 1998; Wang and Sigworth, 2009; Lee and Cui, 2010). In addition, BK channels include an additional segment (S) termed "0" leading to an exofacial N-end (Toro et al., 1998) and a long cytosolic tail domain (CTD), which includes two RCK domains largely responsible for sensing changes in physiological levels of Ca_i^{2+} (Latorre and Brauchi, 2006; Lee and Cui, 2010; Hoshi et al., 2013). Remarkably, purely voltage-gated TM6 K^+ (K_V) channels are resistant to potentiation by $\leq$100 mM ethanol, these channels lacking the Ca_i^{2+}-sensing CTD that is found in slo1 proteins. As mentioned above, ethanol activation of slo1 channels has been linked to modulation of Ca^{2+}-driven gating. Moreover, when studied in the same expression system, Ca_i^{2+}-sensitive slo1 is activated by ethanol while Na^+-gated slo2 and H^+-gated slo3, no matter the concentration of activating ion, remain ethanol-resistant. In addition, the S0-lacking but Ca_i^{2+}-sensitive TM2 K^+ channel from *Methanobacterium thermoautotrophicum*

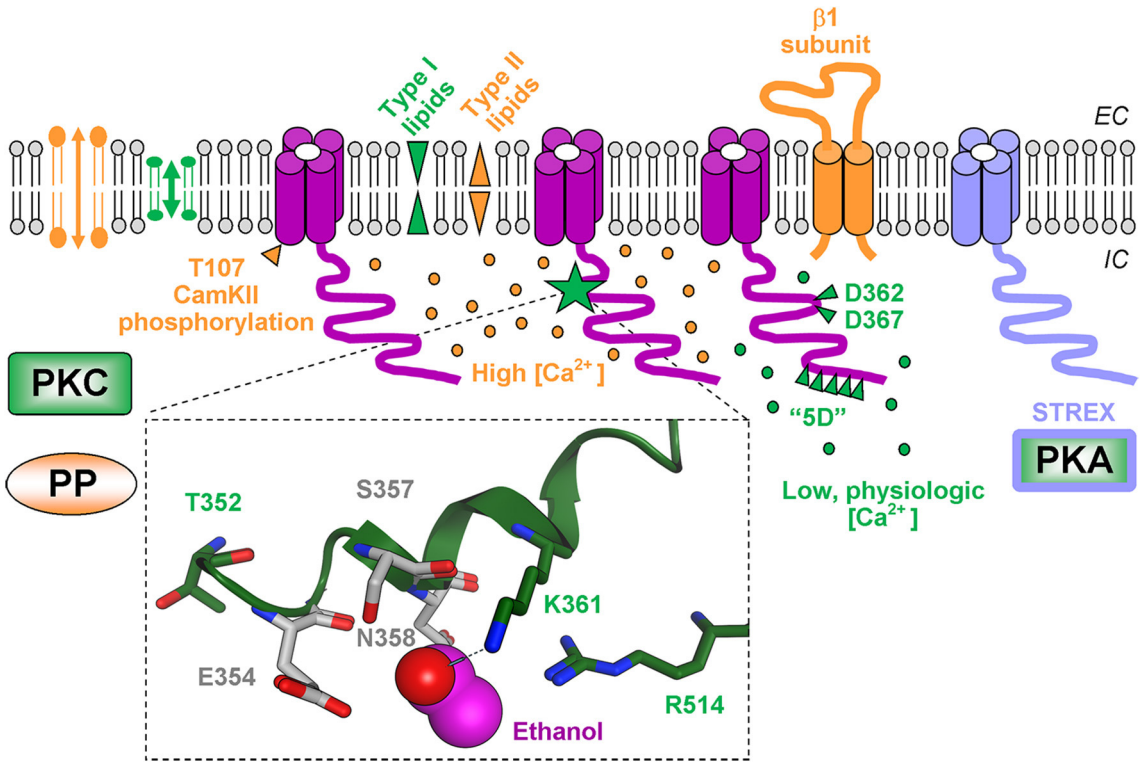


FIGURE 1 | Molecular determinants of ethanol final effect on BK channel activity following brief (up to several minutes) ethanol delivery to alcohol-naïve systems. Functional BK channels are shown as tetramers. For clarity, however, the cytosolic tail domain (CTD) of only one α subunit within each tetrameric complex is displayed. Molecular components that favor ethanol-induced BK channel activation are shown in green whereas factors that counteract ethanol-induced BK channel activation are in orange. Insert depicts a recently identified ethanol-sensing site in the slo1 CTD. Ethanol molecule is depicted in pink; hydrogen bond between ethanol and K361 is highlighted by a light-blue dash line. Oxygen atoms are shown in red, nitrogen atoms are in blue.

(MthK) retains ethanol-sensitivity (Liu et al., 2013). Thus, it has been hypothesized that Ca_i^{2+}-sensing domains within the CTD, whether attached to a TM2 or TM6 core, are responsible for ethanol-sensing. Next, amino acid sequences of Ca_i^{2+}-sensing (e.g., cytosolic) regions of mslo1 and MthK were aligned to render regions that share sequence similarity (Bukiya et al., 2014). Computational modeling, point-mutagenesis and patch-clamp studies on mslo1 expressed in *Xenopus* oocytes revealed details of the ethanol-sensing site. The latter consists of several key elements (**Figure 1**): (1) K361 forms hydrogen-bond with ethanol molecule; (2) R514 provides net positive charge in the vicinity of the ethanol-K361 interaction point; (3) E354, S357, and N358 are located in close vicinity to ethanol, allowing access of ethanol to its K361 bonding partner. In a more recent work substitution of T352 with Ile resulted in elimination of ethanol-induced potentiation of BK current (Davis et al., 2014). T352 is located in the vicinity of the site recently described by Bukiya et al. (2014). Computational modeling shows that T352 points away from the ethanol-sensing pocket. Thus, T352I is unlikely to provide steric hindrance for hydrogen-bonding between ethanol and K361, the latter being critical for channel activation by ethanol. This hydrogen bonding, however, is hampered as the ethanol molecule cannot be positioned within the ethanol-sensing pocket when T352 is substituted by Ile (Bukiya and Dopico, unpublished). The critical role of T352 in ethanol sensing could be explained by the strategic position of this amino acid at the N-terminus of an α-helix within the slo1 CTD; polar/charged amino acids at the N- or C-terminus may neutralize the dipole moment associated with α-helix back-bone. Thus, in the T352I-substituted CTD, a neutral Ile could potentially disrupt the electrostatic interaction that likely exists between the polar Thr and the α-helix dipole. Eventually, the ethanol molecule cannot be positioned inside the ethanol-sensing pocket *possibly* due to repulsive electrostatic force(s) introduced by modification in the α-helix dipole moment. This explanation is in line with earlier speculation on the critical role of electrostatic interactions in the binding of polar molecules (including ethanol) to α-helical structures (Dwyer and Bradley, 2000).

Identification of ethanol-sensing site allows us to explain why BK channels fail to respond to ethanol in virtual absence of Ca_i^{2+}. Crystallographic data demonstrate that CTD conformation in Ca_i^{2+}-free environment (Wu et al., 2010) differs from that in presence of Ca_i^{2+} (Yuan et al., 2010). As a result, in Ca_i^{2+}-free environment ethanol is no longer able to establish a hydrogen bond with K361 due to steric hindrance and repositioning of R514 away from the ethanol-sensing site (Bukiya et al., 2014). In addition to explaining the Ca_i^{2+}-dependence of ethanol action on BK channels, the identification of the ethanol-sensing site allows

us to explain the "cut-off" phenomenon reported for 1-alkanol effects of BK channels originally described over a decade ago. This phenomenon shows BK current potentiation by propanol, butanol, pentanol, hexanol and heptanol, but refractoriness to octanol and nonanol (Chu and Treistman, 1997). Indeed, recent data show that the ethanol-sensing site in the slo1 CTS can accommodate 1-alkanols (propanol-heptanol) that activate BK channels but is unable to fit 1-alkanols that are ineffective (octanol and nonanol) (Bukiya et al., 2014). Thus, 20 years after the first report on the ethanol sensitivity of BK channels (Dopico et al., 1994), an ethanol-recognition site of discrete dimensions and drug-receptor interacting bonds responsible for ethanol activation of this channel have been identified in the channel-forming slo1 protein CTD (Bukiya et al., 2014). This ethanol-recognition pocket is close but does not significantly overlap with the slo1 protein CTD sites that sense Ca_i^{2+}. Thus, ethanol and Ca_i^{2+} constitute heterotropic ligands of the BK channel.

b) Slo Isoforms and their Regulation by Epigenetic Mechanisms following Protracted Ethanol Exposure

Studies on recombinant homomeric slo1 channels cloned from a wide variety of mammalian species (human, rat and mouse brain) consistently document increased Po upon brief exposure to ethanol in alcohol-naïve systems (reviewed in Brodie et al., 2007; Mulholland et al., 2009). A notable exception is the bslo1 channel (cloned from bovine aortic smooth muscle (Dopico, 2003; Liu et al., 2003), this difference most likely being determined by Ca^{2+}/Calmodulin-dependent protein kinase II (CamKII) phosphorylation of bslo1 at a residue that is not found in most slo1 isoforms (see separate section below).

Although slo1 proteins are products of a single gene (see above), pre-mRNA alternative splicing is a major source for diversity of BK channel proteins (Johnson et al., 2011; Kyle and Braun, 2014). In particular, BK-STREX is a stress-induced splice variant of BK channels that presents a phenotype associated with enhanced repetitive firing in neurosecretory cells (Xie and McCobb, 1998). The pituitary hormone-releasing cell lines GH3, GH4/C1, and GH4/C1-STREX have been used as models to address ethanol action on three BK channel subtypes that differ in slo1 subunits. In outside-out patches, however, 30 mM ethanol added to bath solution increases the steady-state activity of all three BK channel variants (Brodie et al., 2007).

In contrast to the rather homogeneous ethanol responses described in the previous paragraph, ethanol responses of BK channels vary greatly following "chronic" (hours) ethanol exposure, which involves "adaptation" of slo1 isoforms at a variety of levels. In two mammalian brain regions important in alcohol abuse and addiction, the supraoptic nucleus and the striatum, BK currents develop "tolerance" to ethanol (Knott et al., 2002; Pietrzykowski et al., 2004). A detailed study in the neurohypophyseal system shows that BK channel tolerance to ethanol exposure initially manifests itself as a slow-developing de-clustering within groups of channels and their subsequent internalization from the plasma membrane. After 24-h ethanol exposure, BK channels in the membrane are less clustered and less dense within those clusters (Pietrzykowski et al., 2004). Importantly, remaining BK channels display an almost complete lack of sensitivity to ethanol when acutely challenged again following withdrawal of the drug. The time-course of the acute ethanol response of native BK channels has been replicated in a study using hslo1 channels reconstituted into artificial lipid bilayers (Yuan et al., 2008). Collectively, these results indicate that, as interpreted for the immediate drug response of the naïve system, the time-dependent component of the channel response to acute ethanol is mainly determined by the channel-forming subunit itself and/or its immediate proteolipid environment.

To understand how the BK channels that were not internalized following several hrs-long ethanol exposure lost their ethanol sensitivity, analysis of BK channel properties in primary striatal cultured and HEK293 cells reveals that slo1 subunit expression is drastically altered by ethanol exposure. While the slo1 subunit is mostly the product of mRNAs coding for an ethanol-sensitive isoform in alcohol-naïve neurons, following chronic exposure it rapidly switches to an alcohol-insensitive variant called STREX (Pietrzykowski et al., 2008; Velázquez-Marrero et al., 2011). Of eight slo1 variants identified in primary striatal cultures, chronic ethanol led to elimination of variants more sensitive to ethanol while sparing those exhibiting much lower sensitivity to the drug (Pietrzykowski et al., 2008). This loss of ethanol-sensitive isoforms occurs because chronic ethanol exposure up-regulates a particular microRNA (miR9), which is a key factor controlling the expression of mRNA splice variants of slo1 channels. To further understand this phenomenon, authors focused on the slo1 channel mRNA 3′ untranslated region (UTR), which is known for its regulation of mRNA stability and being a target of miRNAs. The slo1 channel contains 3 distinct 3′ UTRs regions, each exhibiting different miRNA-binding patterns. Furthermore, the 3′ UTR containing a miR9-binding site is "stitched" to mRNA transcripts encoding slo1 isoforms with a high sensitivity to ethanol. Thus, it seems that chronic ethanol increases the probability of interaction between miR9 and its binding site located on specific 3′ UTRs by upregulating miR9. As a consequence of this interaction, mRNAs associated with these 3' UTRs are degraded, eventually shifting the ratio of ethanol-sensitive/ethanol-tolerant variants leading to alcohol-resistance. Collectively, these data point to a central role for miR9 in ethanol action on striatal neurons and strongly suggest that increase in miR9 might contribute to development of tolerance to protracted ethanol challenge.

c) BK β Subunits

In most mammalian tissues, slo1 channels are associated with a variety of regulatory proteins, including the so called BK β subunits (types 1–4, encoded by *KCNMB1-4*, respectively). Remarkably, BK β types present a rather selective expression, with β1 and β4 being primarily abundant in smooth muscle cells and central neurons, respectively (Orio et al., 2002). These subunits substantially alter the ethanol effect on BK channels. For instance, the presence of β1 or β4 subunits may reduce acute ethanol potentiation of hslo1 after co-expression in human embryonic kidney (HEK) cells *via* an unknown mechanism that seems to be Ca_i^{2+}-independent (Feinberg-Zadek and Treistman, 2007). At physiological Ca_i^{2+}, however, the apparent Ca_i^{2+}-sensitivity of slo1 channels is drastically increased by β1 subunits with β4 failing

to do so (Brenner et al., 2000). After cloning slo1 (cbv1) and β1 subunits from rat cerebrovascular myocytes, Dopico et al., found that β1 subunits shift the "crossover" for ethanol-induced channel activation to inhibition toward lower Ca_i^{2+} ($\leq 3\,\mu M$) (Bukiya et al., 2009). In contrast, β4 fails to modify such crossover when co-expressed with slo1 (Liu et al., 2008). BK β1 tuning of ethanol action results in ethanol inhibition of recombinant BK channels at low micromolar Ca_i^{2+} (Bukiya et al., 2009), as found with the native cerebrovascular channel (Liu et al., 2004). Consistently with a key role for β1 in blunting slo1 channel activation by ethanol and favoring drug-induced inhibition, ethanol fails to activate native cerebral artery BK channels in *KCNMB1* knockout mice (Bukiya et al., 2009). Whether using native BK channels in freshly isolated mouse cerebral artery myocytes or recombinant BK proteins cloned from rat cerebral artery myocytes, β1 subunits inhibit BK channels at physiological Ca_i^{2+} provided that critical levels of cholesterol are kept in the plasmalemma (Bukiya et al., 2009, 2011) (see Section on Membrane Lipids below). These studies led to the idea that Ca_i^{2+}, membrane cholesterol and BK β1 subunits conform a functional triad that determines the slo1 channel response to brief ethanol exposure (Bukiya et al., 2011).

When considering rat supraoptic magnocellular neurons, ethanol causes robust and mild activation of BK channels in nerve terminals and somata, respectively (Dopico et al., 1999b; Wynne et al., 2009). Likewise, in rat nucleus accumbens MSN, ethanol evokes robust and mild channel activation in somata and dendrites, respectively (Martin et al., 2004). Thus, in both supraoptic magnocellular and nucleus accumbens MSN neurons, BK isochannels from two domains within a same neuronal type display phenotypes consistent with differential expression of accessory channel β subunits within each domain. Moreover, single-channel recordings of BK activity in the MSN somatic region reveal biophysical properties consistent with co-expression of slo1 and β4 subunits. In contrast, similar electrophysiological recordings in the dendritic region, unveil a phenotype that is consistent with slo1 and β1 co-expression. Remarkably, while MSN somatic BK Po is significantly enhanced by 10–50 mM ethanol, their dendritic counterparts are ethanol-resistant. These data underscore the role of BK β1 in blunting ethanol-induced potentiation of BK channels (Martin et al., 2008). This study documents that homomeric slo1 and heteromeric hslo1 + β4 show similar ethanol sensitivity, as communicated by Liu et al. (2008) with mslo ± β4, yet in contrast to data from Feinberg-Zadek and Treistman (2007). Homomeric slo1 channels, however, rapidly develop tolerance to ethanol. In contrast, the ethanol-induced potentiation of hslo1 +β4 heteromers persists during the whole exposure to the drug, whether evaluated in heterologous expression systems or in freshly dissociated nucleus accumbens MSNs (Martin et al., 2008). Collectively, the studies described in this section indicate that BK β subunits can drastically influence both the BK channel's response to acute ethanol exposure in alcohol-naïve systems and the channel's response to ethanol following protracted drug administration. A summary of molecular entities and mechanisms participating in the BK channel response to protracted ethanol exposure is given in **Figure 2**.

d) Phosphorylation of BK Channels and/or Channel-associated Proteins

Ethanol-induced slo1 channel activation is also controlled by phosphorylation/dephosphorylation processes. This phenomenon was first reported in GH3 cells where potentiation of BK channel activity by 30 mM ethanol was blocked by protein kinase C (PKC) inhibition and favored by phosphatase (PP) inhibitors (Jakab et al., 1997). In GH4/C1 and GH4/C1-STREX cells, BK steady-state activity is increased only in some of the membrane patches under investigation, this variability being attributed to post-translational modification of slo1 proteins. PKC blockers diminish ethanol potentiation of BK channel activity in GH4/C1 cells but have no effect on GH4/C1-STREX cells. BK-STREX channel activation by ethanol, however, is protein kinase A (PKA)-dependent (reviewed in Brodie et al., 2007).

As mentioned in a previous section, bovine aortic BK channels (Walters et al., 2000) are inhibited by ethanol. It is highly likely that part of this drug effect is explained by the abundant expression of β1 subunits in vascular smooth muscle, as these subunits are responsible for blunting ethanol-induced potentiation and favoring inhibition of BK channel activity (see above). However, in contrast to other slo1 channels cloned from mammalian tissues, bslo1 (cloned from bovine aorta) is also inhibited by 10–100 mM ethanol (Dopico, 2003; Liu et al., 2003). This slo1 isoform distinctly includes a T107 in the S0-S1 intracellular loop. Incremental CaMKII-mediated phosphorylation of channel subunits at position 107 in the BK tetramer progressively increases channel Po and gradually switches the channel's ethanol responses from robust activation to inhibition. Thus, CaMKII phosphorylation of bslo1 T107 works as a "molecular dimmer switch," this mechanism being able to override ethanol allosteric coupling to channel activation by physiological levels of Ca_i^{2+}. Notably, T107 is a region that is missing in K_V channels other than BK. Moreover, T107 equivalent position in mslo1, hslo1 and cbv1 is occupied by non-phosphorylatable residues, all these proteins forming homotetramers that are ethanol-sensitive (see above).

In a very recent study, Velàzquez-Marrero et al. (2014) examined the influence of protein kinase A (PKA), CaMKII, and PP on ethanol actions on slo1 ± β4 channels in HEK 293 cells and nucleus accumbens MSNs. Data show that the presence of β4 drastically alters the effects of PKA, CaMKII, and PP, echoing a study in HEK293 cells showing that this auxiliary subunit alters cAMP-mediated activation of BK channels (Petrik and Brenner, 2007). Interestingly, slo1 channel's rapid tolerance to ethanol is reversed following PP inhibition. In addition, slo1 + β4 channels develop ethanol tolerance in presence of CaMKIIN, a specific CaMKII inhibitor (Velàzquez-Marrero et al., 2014). Thus, while early studies focused on addressing the modulation of acute ethanol action on BK channel-forming proteins by kinases and phosphatases in alcohol-naïve systems, more recent studies are beginning to unveil a complex interplay between phosphorylation/dephosphorylation processes, slo1 proteins and the different types of accessory β subunits.

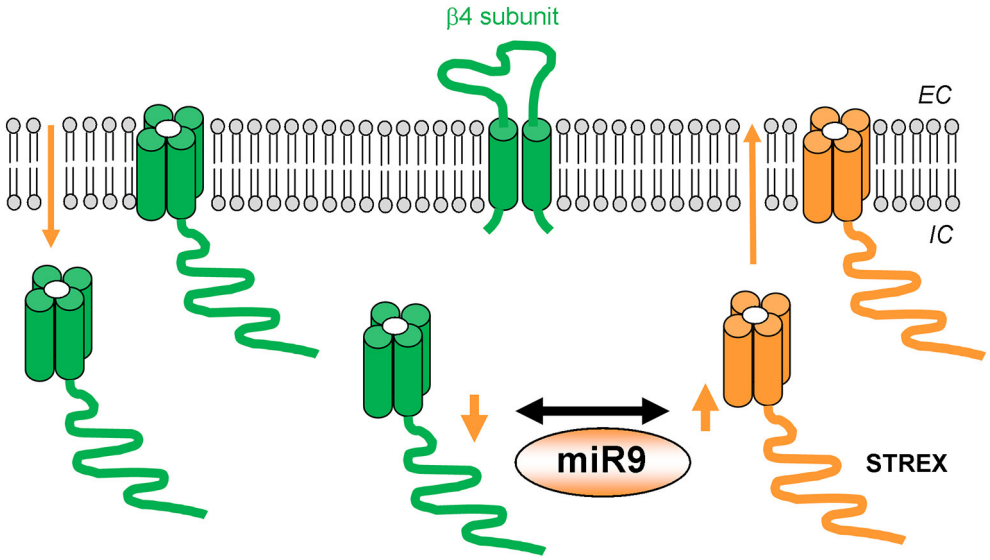


FIGURE 2 | Molecular factors that contribute to BK channel resistance to ethanol action following protracted (hours-long) alcohol exposure. BK channel α isoforms and β4 subunits, which sustain ethanol-induced channel activation, are shown in green. BK channel α isoforms and mechanisms leading to ethanol "tolerance" are shown in orange.

e) Membrane Lipids

Several studies documented a critical role for membrane lipids in tuning ethanol's final effect on BK Po. A consistent finding is a role for lipid "effective shape," independently of lipid head net charge: for instance, ethanol-induced activation of hslo1 channels incorporated into planar lipid bilayers is favored by type I lipids, that is, those with a polar head cross sectional area larger than the tail area (e.g., phosphatidylserine), which introduce "positive monolayer curvature." Conversely such ethanol action is blunted by type II lipids, that is, those with a polar head cross sectional area smaller than the hydrophobic tails/rings (e.g., phosphatidyglycerol, cholesterol) (Crowley et al., 2005). Considering that ethanol can be likened to a type I molecule, authors speculated that the reduced modulation of ethanol action by cholesterol in a POPE bilayer was due to a reduced action of a type II lipid (cholesterol) in a type II lipid environment (POPE). Cholesterol antagonism of slo1 channel activation by ethanol, however, has been attributed to a variety of factors. For example, cholesterol insertion in a bilayer favors liquid-order phase formation, which might facilitate ethanol partition in the bilayer and access to the channel target (discussed in Crowley et al., 2003). However, cholesterol is likely to modify ethanol action on dwell-times distribution and thus Po. Two non-mutually exclusive explanations for this antagonism on Po include opposite modification of physical bilayer properties by each modulator and direct protein-ligand interactions between modulator and the slo1 protein (reviewed in Dopico et al., 2012). Heteromeric BK channels composed of pore-forming cbv1 and β1 subunits cloned from rat cerebral artery myocytes are resistant to 50 mM ethanol when evaluated in cholesterol-free bilayers. Inclusion of 23 mol% cholesterol into the lipid mixture results in ethanol-induced BK channel inhibition (Bukiya et al., 2011). Although the molecular underpinnings of ethanol-cholesterol interactions in β1 subunit-containing BK channels remain unknown, there is a common theme from studies in artificial bilayers: cholesterol presence shifts the ethanol-exposed system toward lower Po, whether turning refractoriness into channel inhibition in the case of β1 subunit-containing BK channels or by diminishing ethanol-induced activation of homomeric slo1 channels. Remarkably, ethanol-cholesterol antagonism on slo1 channels could not be observed when cholesterol was substituted by *ent*-cholesterol, that is, its "mirror image" enantiomer (Yuan et al., 2011), suggesting that cholesterol tuning of the ethanol effect involves specific cholesterol-protein interactions. Indeed, both ethanol-recognition (see above) and cholesterol-recognition sites have been mapped to the slo1 CTD. The latter seem to include seven CRAC domains, with CRAC4 (the domain adjacent to the inner membrane leaflet where cholesterol is abundant) playing a major role (Singh et al., 2012). While cholesterol-recognition and ethanol-recognition sites on slo1 are nearby, they do not share key residues that are involved in recognition of each ligand.

Data on cholesterol-ethanol interactions on BK channels acquire particular relevance because these channels cluster in cholesterol-enriched rafts, and changes in cholesterol levels and distribution have been reported in cell membranes following chronic ethanol exposure (discussed in Crowley et al., 2003; Yuan et al., 2008). Indeed, work with lipid bilayer provides clear evidence that the membrane lipid composition influences tolerance to ethanol exposure. Thus, acute ethanol tolerance is observed in stable (20:1) phosphatidylcholine–dioleoylphosphatidylethanolamine (PC–DOPE) but not in sphingomyelin–DOPE bilayers (Yuan et al., 2008). It has been hypothesized that changes in the channel's lipid environment selectively alter ethanol access to sites in the channel protein that mediate opposing effects (potentiation vs. inhibition) on BK steady-state activity (Yuan et al., 2008).

An intriguing finding is that the ethanol response and adaptation of BK channels is sensitive to the bilayer thickness (Yuan et al., 2008), which can have particular importance in light of evidence for the presence of lipid rafts and location of BK channels within these domains (Weaver et al., 2007). Altering the thickness of the bilayer by adjusting the acyl chain length of the component lipids affects the time course of the acute response to alcohol and can turn ethanol-induced potentiation into inhibition. Hslo1 channels embedded in a thin bilayer are strongly potentiated by the drug whereas channels placed in a thicker bilayer are inhibited (Yuan et al., 2008). Insights into the mechanisms by which bilayer thickness affects BK function and pharmacology are becoming more accessible from our growing understanding of channel protein structure. In BK, the linker that connects the S6 gate to the RCK domains forms a passive spring with the gating ring and is involved in Ca_i^{2+}-dependent activation (Niu et al., 2004), the latter being required for ethanol potentiation of slo1 channels (Liu et al., 2008, 2013). A simple mechanical model to explain modulation of channel function by bilayer thickness has been hypothesized, in which lateral stress within the lipid bilayer in combination with forces generated by local hydrophobic mismatch between membrane lipids and the slo1 protein play a major role (Yuan et al., 2007). In synthesis, membrane lipid modulation of ethanol action on BK channel proteins may potentially result from lipid-induced modification of ethanol partition into the bilayer and access to ethanol's channel target site(s), modulation of bilayer physical properties by ethanol and lipid resulting in modification of channel gating, binding of ethanol and lipid species to distinct BK channel complex protein sites, which also would lead to gating modification, or any combination of these possibilities. Recognition sites in BK proteins have been only identified for a few lipid species (Dopico and Bukiya, 2014), and their role in ethanol modulation of channel function remains to be determined.

f) Coupling to Nearby Ion Channels

As mentioned above, BK channels cluster in membrane rafts that co-segregate signaling molecules and ion channels in addition to BK themselves. Thus, in most excitable tissues, BK channels constitute functional complexes, as first reported for BK and voltage-dependent Ca^{2+} channels (Marrion and Tavalin, 1998). Ethanol modulation of other ion channels may impact on the levels of Ca_i^{2+} faced in the vicinity of the BK channels, activating Ca_i^{2+} representing a key factor for ethanol to modulate BK currents (see above). In GH4/C1 cells, ethanol increases overall Ca_i^{2+} in absence of extracellular Ca^{2+}, an ethanol action that may contribute to drug modulation of BK channels (Jakab et al., 2006). Cross-talking between BK channels and nearby ion channels has been well studied in cerebrovascular smooth muscle where BK channel activity negatively feeds back on contraction driven by voltage-dependent Ca^{2+} influx. Contraction is also favored by IP3-sensitive, internal Ca^{2+}-release channels that generate "Ca^{2+}-waves." In contrast, Ca^{2+}-release via ryanodine-sensitive receptors (RyR) generates localized, "Ca^{2+}-sparks," which are located in close vicinity of and activate the BK channel, favoring vascular smooth muscle dilation (Jaggar et al., 1998; Narayanan et al., 2012). In cerebral artery smooth muscle cells, 50 mM ethanol fails to significantly modify Ca^{2+}-waves and voltage-dependent Ca^{2+} currents. In sharp contrast, 50 mM blunts Ca^{2+} spark frequency and amplitude, a major mechanism thought to contribute to ethanol inhibition of BK currents, this ethanol action being responsible for cerebrovascular constriction (Liu et al., 2004). A recent study documents that both Ca^{2+} sparks and recombinant RyR2 (the type prevalent in rat cerebral artery myocytes; Vaithianathan et al., 2010) are inhibited by ethanol with an $IC_{50} \sim 10$ mM (Ye et al., 2014).

Conversely, ethanol modulation of BK currents may alter function of nearby ion channel proteins. In neurons, BK activation could also alter the refractory period of nearby voltage-dependent channels, leading to an actual increase in neuronal excitability (Warbington et al., 1996; Van Goor et al., 2001). This mechanism has been advanced to explain slo-mediated tolerance to the sedative/hypnotic effect of alcohol in *drosophila* (Ghezzi and Atkinson, 2011). Studies of ethanol action on the fly have been comprehensively reviewed in this volume by Bettinger and Davies (2014). In synthesis, ethanol actions on BK currents are usually a composite that results from drug action on BK channel complex themselves and on other ion channel proteins that modulate BK channel activity, making it extremely difficult to extrapolate ethanol effects on BK channels reported in specific cells or cell domains to another.

CONCLUDING REMARKS AND FUTURE CHALLENGES

Data summarized and discussed in this review make evident that a multiplicity of molecular target and mechanisms conditions the final response of BK currents to acute ethanol exposure in alcohol-naïve systems, with current potentiation, refractoriness and inhibition all being reported, including within different domains of a given neuronal type. In addition, functional association between BK channels with other ion channels within a cell domain may determine that a given ethanol action on BK channels results in opposite effects. Ethanol activation of BK channels clearly reduces excitability in nucleus accumbens MSN, yet such drug action may actually increase excitability in *Drosophila* neurons as the refractory period of other voltage-gated conductances may be affected. Along the same lines, BK current potentiation and voltage-gated Ca^{2+}-channel inhibition contribute to decrease neuropeptide release from neurohypophyseal axon terminals, yet BK channel activation in growth-hormone release cells cannot overcome drug action on intracellular channels and signaling, resulting in increased hormone release by alcohol. Thus, ethanol actions on BK channels in one system cannot be simply extrapolated to another. Molecular multiplicity leads to different ethanol responses even within different domains within a given neuronal type, as reported for supraoptic neurons and nucleus accumbens MSN. On a practical note, identification of the molecular entities and mechanisms that determine ethanol final effect on BK currents is critical for any therapeutic intervention to prevent or revert modification of BK channel-regulated physiology by acute ethanol exposure.

In light of the BK channel's sensitivity to ethanol intoxicating concentrations and the channel expression in regions central to the development of dependence to drugs of abuse (including ethanol itself), a number of studies have probed BK channel's role

in behavioral tolerance. Moreover, several of the key elements that determine the final ethanol response of BK channels in alcohol-naïve systems (e.g., slo1 channel isoforms, BK β subunits, membrane lipids) also play a key role in a modified system response to protracted ethanol exposure. Plastic changes at the molecular, cellular and neurocircuitry levels very likely result in behavioral manifestations of ethanol misuse and consumption. Indeed, in a 2-bottle choice drinking paradigm, BK β1 and β4 subunits have opposite effects on voluntary alcohol intake of dependent rodents, with the former and the latter respectively accelerating and attenuating the escalation (Kreifeldt et al., 2013). Finally, it has been advanced that presence of enhanced acute behavioral tolerance to alcohol in humans can serve as a marker for the likelihood of future development of alcoholism (Schuckit, 1985a,b, 1994; Heath et al., 1999). Therefore, understanding the adaptations in neuronal BK currents and the underlying molecular mechanisms that sustain ethanol tolerance and dependence is of fundamental significance to gain insights on the bases of alcohol vulnerability, and even develop a molecular target identification-designed therapy for treating alcohol misuse.

ACKNOWLEDGMENTS

Supported by HL104631 and AA11560 (Alex M. Dopico); Alcoholic Beverage Medical Research Foundation grant for New Investigator and NIH Support Opportunity for Addiction Research for New Investigators R03 AA020184 (Anna N. Bukiya), and R01 AA020501 (Gilles E. Martin).

REFERENCES

Benhassine, N., and Berger, T. (2009). Large-conductance calcium-dependent potassium channels prevent dendritic excitability in neocortical pyramidal neurons. *Pflugers Arch*. 457, 1133–1145. doi: 10.1007/s00424-008-0569-3

Bettinger, J. C., and Davies, A. G. (2014). The role of the BK channel in ethanol response behaviors: evidence from model organism and human studies. *Front. Physiol.* 5:346. doi: 10.3389/fphys.2014.00346

Bettinger, J. C., Leung, K., Bolling, M. H., Goldsmith, A. D., and Davies, A. G. (2012). Lipid environment modulates the development of acute tolerance to ethanol in *Caenorhabditis elegans*. *PLoS ONE* 7:e35192. doi: 10.1371/journal.pone.0035192

Brenner, R., Chen, Q. H., Vilaythong, A., Toney, G. M., Noebels, J. L., and Aldrich, R. W. (2005). BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat. Neurosci*. 8, 1752–1759. doi: 10.1038/nn1573

Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y., and Aldrich, R. W. (2000). Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, *hKCNMB3* and *hKCNMB4*. *J. Biol. Chem*. 275, 6453–6461. doi: 10.1074/jbc.275.9.6453

Brodie, M. S., Scholz, A., Weiger, T. M., and Dopico, A. M. (2007). Ethanol interactions with calcium-dependent potassium channels. *Alcohol. Clin. Exp. Res*. 31, 1625–1632. doi: 10.1111/j.1530-0277.2007.00469.x

Brown, D. A., Constanti, A., and Adams, P. R. (1983). Ca-activated potassium current in vertebrate sympathetic neurons. *Cell Calcium* 4, 407–420. doi: 10.1016/0143-4160(83)90017-9

Bukiya, A. N., Kuntamallappanavar, G., Edwards, J., Singh, A. K., Shivakumar, B., and Dopico, A. M. (2014). An alcohol-sensing site in the calcium- and voltage-gated, large conductance potassium (BK) channel. *Proc. Natl. Acad. Sci. U.S.A.* 111, 9313–9318. doi: 10.1073/pnas.1317363111

Bukiya, A. N., Liu, J., and Dopico, A. M. (2009). The BK channel accessory beta1 subunit determines alcohol-induced cerebrovascular constriction. *FEBS Lett*. 583, 2779–2784. doi: 10.1016/j.febslet.2009.07.019

Bukiya, A. N., Vaithianathan, T., Kuntamallappanavar, G., Asuncion-Chin, M., and Dopico, A. M. (2011). Smooth muscle cholesterol enables BK β1 subunit-mediated channel inhibition and subsequent vasoconstriction evoked by alcohol. *Arterioscler. Thromb. Vasc. Biol*. 31, 2410–2423. doi: 10.1161/ATVBAHA.111.233965

Carlen, P. L., Gurevich, N., and Durand, D. (1982). Ethanol in low doses augments calcium-mediated mechanisms measured intracellularly in hippocampal neurons. *Science* 215, 306–309. doi: 10.1126/science.7053581

Carlen, P. L., Gurevich, N., Davies, M. F., Blaxter, T. J., and O'Beirne, M. (1985). Enhanced neuronal K^+ conductance: a possible common mechanism for sedative-hypnotic drug action. *Can J. Physiol. Pharmacol*. 63, 831–837.

Chu, B., Dopico, A. M., Lemos, J. R., and Treistman, S. N. (1998). Ethanol potentiation of calcium-activated potassium channels reconstituted into planar lipid bilayers. *Mol. Pharmacol*. 54, 397–406.

Chu, B., and Treistman, S. N. (1997). Modulation of two cloned potassium channels by 1-alkanols demonstrates different cutoffs. *Alcohol. Clin. Exp. Res*. 21, 1103–1107.

Cowmeadow, R. B., Krishnan, H. R., and Atkinson, N. S. (2005). The slowpoke gene is necessary for rapid ethanol tolerance in *Drosophila*. *Alcohol. Clin. Exp. Res*. 29, 1777–1786. doi: 10.1097/01.alc.0000183232.56788.62

Cowmeadow, R. B., Krishnan, H. R., Ghezzi, A., Al'Hasan, Y. M., Wang, Y. Z., and Atkinson, N. S. (2006). Ethanol tolerance caused by slowpoke induction in *Drosophila*. *Alcohol. Clin. Exp. Res*. 30, 745–753. doi: 10.1111/j.1530-0277.2006.00087.x

Crowley, J. J., Treistman, S. N., and Dopico, A. M. (2003). Cholesterol antagonizes ethanol potentiation of human brain BK_{Ca} channels reconstituted into phospholipid bilayers. *Mol. Pharmacol*. 64, 365–372. doi: 10.1124/mol.64.2.365

Crowley, J. J., Treistman, S. N., and Dopico, A. M. (2005). Distinct structural features of phospholipids differentially determine ethanol sensitivity and basal function of BK channels. *Mol. Pharmacol*. 68, 4–10.

Davies, A. G., Pierce-Shimomura, J. T., Kim, H., VanHoven, M. K., Thiele, T. R., Bonci, A., et al. (2003). A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. *Cell* 115, 655–666. doi: 10.1016/S0092-8674(03)00979-6

Davis, S. J., Scott, L. L., Hu, K., and Pierce-Shimomura, J. T. (2014). Conserved single residue in the BK potassium channel required for activation by alcohol and intoxication in *C. elegans*. *J. Neurosci*. 34, 9562–9573. doi: 10.1523/JNEUROSCI.0838-14.2014

Diamond, I. (1992). "Alcoholism and alcohol abuse," in *Cecil Textbook of Medicine*, eds J. B. Wyngaarden, L. H. Jr. Smith, and F. Plum (Philadelphia: Saunders), 44–47.

Dopico, A. M. (2003). Ethanol sensitivity of $BK_{(Ca)}$ channels from arterial smooth muscle does not require the presence of the beta 1-subunit. *Am. J. Physiol. Cell. Physiol*. 284, C1468–C1480. doi: 10.1152/ajpcell.00421.2002

Dopico, A. M., Anantharam, V., and Treistman, S. N. (1998). Ethanol increases the activity of $Ca^{(++)}$-dependent K^+ (mslo) channels: functional interaction with cytosolic Ca^{++}. *J. Pharmacol. Exp. Ther*. 284, 258–268.

Dopico, A. M., and Bukiya, A. N. (2014). Lipid regulation of BK channel function. *Front. Physiol*. 5:312. doi: 10.3389/fphys.2014.00312

Dopico, A. M., Bukiya, A. N., and Singh, A. K. (2012). Large conductance, calcium- and voltage-gated potassium (BK) channels: regulation by cholesterol. *Pharmacol. Ther*. 135, 133–150. doi: 10.1016/j.pharmthera.2012.05.002

Dopico, A. M., Chu, B., Lemos, J. R., and Treistman, S. N. (1999a). Alcohol modulation of calcium-activated potassium channels. *Neurochem. Int*. 35, 103–106. doi: 10.1016/S0197-0186(99)00051-0

Dopico, A. M., Lemos, J. R., and Treistman, S. N. (1994). Ethanol activates large conductance, Ca^{2+}-activated K^+ channels in neurohypophysial terminals. *Soc. Neurosci. Abstr*. 20, 1521.

Dopico, A. M., Lemos, J. R., and Treistman, S. N. (1995). "Alcohol and the release of vasopressin and oxytocin," in *Drug and Alcohol Abuse Reviews-Alcohol and Hormones*, ed R. Watson (Totowa, NJ: Humana Press), 209–226.

Dopico, A. M., Lemos, J. R., and Treistman, S. N. (1996). Ethanol increases the activity of large conductance, $Ca^{(2+)}$-activated K^+ channels in isolated neurohypophysial terminals. *Mol. Pharmacol*. 49, 40–48.

Dopico, A. M., and Lovinger, D. M. (2009). Acute alcohol action and desensitization of ligand-gated ion channels. *Pharmacol. Rev*. 61, 98–114. doi: 10.1124/pr.108.000430

Dopico, A. M., Widmer, H., Wang, G., Lemos, J. R., and Treistman, S. N. (1999b). Rat supraoptic magnocellular neurones show distinct large conductance, Ca^{2+}-activated K^+ channel subtypes in cell bodies versus nerve endings. *J. Physiol*. 519, 101–114. doi: 10.1111/j.1469-7793.1999.0101o.x

Dryer, S. E. (2003). Molecular identification of the Na^+-activated K^+ channel. *Neuron* 37, 727–728. doi: 10.1016/S0896-6273(03)00119-3

Dwyer, D. S., and Bradley, R. J. (2000). Chemical properties of alcohols and their protein binding sites. *Cell. Mol. Life Sci*. 57, 265–275. doi: 10.1007/PL00000689

Faber, E. S., and Sah, P. (2002). Physiological role of calcium-activated potassium currents in the rat lateral amygdala. *J. Neurosci*. 22, 1618–1628.

Faber, E. S., and Sah, P. (2003). Ca^{2+}-activated K^+ (BK) channel inactivation contributes to spike broadening during repetitive firing in the rat lateral amygdala. *J. Physiol*. 552, 483–497. doi: 10.1113/jphysiol.2003.050120

Feinberg-Zadek, P. L., and Treistman, S. N. (2007). Beta-subunits are important modulators of the acute response to alcohol in human BK channels. *Alcohol. Clin. Exp. Res.* 31, 737–744. doi: 10.1111/j.1530-0277.2007.00371.x

Ghezzi, A., Al-Hasan, Y. M., Larios, L. E., Bohm, R. A., and Atkinson, N. S. (2004). Slo $K^{(+)}$ channel gene regulation mediates rapid drug tolerance. *Proc. Natl. Acad. Sci. U.S.A*. 101, 17276–17281. doi: 10.1073/pnas.0405584101

Ghezzi, A., and Atkinson, N. S. (2011). Homeostatic control of neural activity: a *Drosophila* model for drug tolerance and dependence. *Int. Rev. Neurobiol*. 99, 23–50. doi: 10.1016/B978-0-12-387003-2.00002-1

Ghezzi, A., Pohl, J. B., Wang, Y., and Atkinson, N. S. (2010). BK channels play a counter-adaptive role in drug tolerance and dependence. *Proc. Natl. Acad. Sci. U.S.A*. 107, 16360–16365. doi: 10.1073/pnas.1005439107

Golding, N. L., Jung, H. Y., Mickus, T., and Spruston, N. (1999). Dendritic calcium spike initiation and repolarization are controlled by distinct potassium channel subtypes in CA1 pyramidal neurons. *J. Neurosci*. 19, 8789–8798.

Gruß, M., Henrich, M., König, P., Hempelmann, G., Vogel, W., and Scholz, A. (2001). Ethanol reduces excitability in a subgroup of primary sensory neurons by activation of $BK_{(Ca)}$ channels. *Eur. J. Neurosci.* 14, 1246–1256. doi: 10.1046/j.0953-816x.2001.01754.x

Handlechner, A. G., Hermann, A., Fuchs, R., and Weiger, T. M. (2013). Acetaldehyde-ethanol interactions on calcium-activated potassium (BK) channels in pituitary tumor (GH3) cells. *Front. Behav. Neurosci*. 7:58. doi: 10.3389/fnbeh.2013.00058

Heath, A. C., Madden, P. A., Bucholz, K. K., Dinwiddie, S. H., Slutske, W. S., Bierut, L. J., et al. (1999). Genetic differences in alcohol sensitivity and the inheritance of alcoholism risk. *Psychol. Med*. 29, 1069–1081. doi: 10.1017/S0033291799008909

Hoshi, T., Pantazis, A., and Olcese, R. (2013). Transduction of voltage and Ca^{2+} signals by Slo1 BK channels. *Physiology (Bethesda)*. 28, 172–189. doi: 10.1152/physiol.00055.2012

Isaacson, J. S., and Murphy, G. J. (2001). Glutamate-mediated extrasynaptic inhibition: direct coupling of NMDA receptors to $Ca^{(2+)}$-activated K^+ channels. *Neuron* 31, 1027–1034. doi: 10.1016/S0896-6273(01)00428-7

Jaggar, J. H., Wellman, G. C., Heppner, T. J., Porter, V. A., Perez, G. J., Gollasch, M., et al. (1998). Ca^{2+} channels, ryanodine receptors and $Ca^{(2+)}$-activated K^+ channels: a functional unit for regulating arterial tone. *Acta Physiol. Scand*. 164, 577–587. doi: 10.1046/j.1365-201X.1998.00462.x

Jakab, M., Schmidt, S., Grundbichler, M., Paulmichl, M., Hermann, A., Weiger, T., et al. (2006). Hypotonicity and ethanol modulate BK channel activity and chloride currents in GH4/C1 pituitary tumour cells. *Acta Physiol. (Oxf)*. 187, 51–59. doi: 10.1111/j.1748-1716.2006.01544.x

Jakab, M., Weiger, T. M., and Hermann, A. (1997). Ethanol activates maxi Ca^{2+}-activated K^+ channels of clonal pituitary (GH3) cells. *J. Membr. Biol*. 157, 237–245. doi: 10.1007/PL00005895

Ji, X., and Martin, G. E. (2012). New rules governing synaptic plasticity in core nucleus accumbens medium spiny neurons. *Eur. J. Neurosci*. 36, 3615–3627. doi: 10.1111/ejn.12002

Jin, W., Sugaya, A., Tsuda, T., Ohguchi, H., and Sugaya, E. (2000). Relationship between large conductance calcium-activated potassium channel and bursting activity. *Brain Res*. 860, 21–28. doi: 10.1016/S0006-8993(00)01943-0

Johnson, B. E., Glauser, D. A., Dan-Glauser, E. S., Halling, D. B., Aldrich, R. W., and Goodman, M. B. (2011). Alternatively spliced domains interact to regulate BK potassium channel gating. *Proc. Natl. Acad. Sci. U.S.A*. 108, 20784–20789. doi: 10.1073/pnas.1116795108

Knott, T. K., Dopico, A. M., Dayanithi, G., Lemos, J., and Treistman, S. N. (2002). Integrated channel plasticity contributes to alcohol tolerance in neurohypophysial terminals. *Mol. Pharmacol.* 62, 135–142. doi: 10.1124/mol.62.1.135

Kreifeldt, M., Le, D., Treistman, S. N., Koob, G. F., and Contet, C. (2013). BK channel β1 and β4 auxiliary subunits exert opposite influences on escalated ethanol drinking in dependent mice. *Front. Integr. Neurosci*. 7:105. doi: 10.3389/fnint.2013.00105

Krnjevic, K. (1972). "Excitable membranes and anesthetics," in *Cellular Biology and Toxicology of Anesthetics*, ed B. R. Fink (Baltimore, MD: Williams & Wilkins), 3–9.

Kuhlmann, C. R., Li, F., Lüdders, D. W., Schaefer, C. A., Most, A. K., Backenköhler, U., et al. (2004). Dose-dependent activation of Ca^{2+}-activated K^+ channels by ethanol contributes to improved endothelial cell functions. *Alcohol. Clin. Exp. Res*. 28, 1005–1011. doi: 10.1097/01.ALC.0000130811.92457.0D

Kyle, B. D., and Braun, A. P. (2014). The regulation of BK channel activity by pre- and post-translational modifications. *Front. Physiol*. 5:316. doi: 10.3389/fphys.2014.00316

Latorre, R., and Brauchi, S. (2006). Large conductance Ca^{2+}-activated K^+ (BK) channel: activation by Ca^{2+} and voltage. *Biol. Res*. 39, 385–401. doi: 10.4067/S0716-97602006000300003

Latorre, R., Oberhauser, A., Labarca, P., and Alvarez, O. (1989). Varieties of calcium-activated potassium channels. *Annu. Rev. Physiol*. 51, 385–399. doi: 10.1146/annurev.ph.51.030189.002125

Lee, U. S., and Cui, J. (2010). BK channel activation: structural and functional insights. *Trends Neurosci*. 33, 415–423. doi: 10.1016/j.tins.2010.06.004

Li, B., Jie, W., Huang, L., Wei, P., Li, S., Luo, Z., et al. (2014). Nuclear BK channels regulate gene expression via the control of nuclear calcium signaling. *Nat. Neurosci*. 17, 1055–1063. doi: 10.1038/nn.3744

Liu, J., Bukiya, A. N., Kuntamallappanavar, G., Singh, A. K., and Dopico, A. M. (2013). Distinct sensitivity of slo1 channel proteins to ethanol. *Mol. Pharmacol*. 83, 235–244. doi: 10.1124/mol.112.081240

Liu, J., Vaithianathan, T., Manivannan, K., Parrill, A., and Dopico, A. M. (2008). Ethanol modulates BK_{Ca} channels by acting as an adjuvant of calcium. *Mol. Pharmacol*. 74, 628–640. doi: 10.1124/mol.108.048694

Liu, P., Liu, J., Huang, W., Li, M. D., and Dopico, A. M. (2003). Distinct regions of the slo subunit determine differential BK_{Ca} channel responses to ethanol. *Alcohol. Clin. Exp. Res*. 27, 1640–1644. doi: 10.1097/01.ALC.0000094756.41638.5D

Liu, P., Xi, Q., Ahmed, A., Jaggar, J. H., and Dopico, A. M. (2004). Essential role for smooth muscle BK channels in alcohol-induced cerebrovascular constriction. *Proc. Natl. Acad. Sci. U.S.A*. 101, 18217–18222. doi: 10.1073/pnas.0406096102

Liu, Y., Savtchouk, I., Acharjee, S., and Liu, S. J. (2011). Inhibition of Ca^{2+}-activated large-conductance K^+ channel activity alters synaptic AMPA receptor phenotype in mouse cerebellar stellate cells. *J. Neurophysiol*. 106, 144–152. doi: 10.1152/jn.01107.2010

MacDermott, A. B., and Weight, F. F. (1982). Action potential repolarization may involve a transient, Ca^{2+}-sensitive outward current in a vertebrate neuron. *Nature* 300, 185–188. doi: 10.1038/300185a0

Madsen, B. W., and Edeson, R. O. (1990). Ethanol enhancement of a calcium-activated potassium current in an identified molluscan neuron. *Brain Res*. 528, 323–326. doi: 10.1016/0006-8993(90)91675-7

Malysz, J., Afeli, S. A., Provence, A., and Petkov, G. V. (2014). Ethanol-mediated relaxation of guinea pig urinary bladder smooth muscle: involvement of BK and L-type Ca^{2+} channels. *Am. J. Physiol. Cell. Physiol*. 306, C45–C58. doi: 10.1152/ajpcell.00047.2013

Marrion, N. V., and Tavalin, S. J. (1998). Selective activation of Ca^{2+}-activated K^+ channels by co-localized Ca^{2+} channels in hippocampal neurons. *Nature* 395, 900–905. doi: 10.1038/27674

Martin, G. E., Hendrickson, L. M., Penta, K. L., Friesen, R. M., Pietrzykowski, A. Z., Tapper, A. R., et al. (2008). Identification of a BK channel auxiliary protein controlling molecular and behavioral tolerance to alcohol. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17543–17548. doi: 10.1073/pnas.0801068105

Martin, G., Puig, S., Pietrzykowski, A., Zadek, P., Emery, P., and Treistman, S. (2004). Somatic localization of a specific large-conductance calcium-activated potassium channel subtype controls compartmentalized ethanol sensitivity in the nucleus accumbens. *J. Neurosci*. 24, 6563–6572. doi: 10.1523/JNEUROSCI.0684-04.2004

McIntire, S. L. (2010). Ethanol. *Worm Book* 29, 1–6. doi: 10.1895/wormbook.1.40.1

Meredith, A. L., Wiler, S. W., Miller, B. H., Takahashi, J. S., Fodor, A. A., Ruby, N. F., et al. (2006). BK calcium-activated potassium channels regulate circadian behavioral rhythms and pacemaker output. *Nat. Neurosci*. 9, 1041–1049. doi: 10.1038/nn1740

Mulholland, P. J., Hopf, F. W., Bukiya, A. N., Martin, G. E., Liu, J., Dopico, A. M., et al. (2009). Sizing up ethanol-induced plasticity: the role of small and large

conductance calcium-activated potassium channels. *Alcohol. Clin. Exp. Res.* 33, 1125–1135. doi: 10.1111/j.1530-0277.2009.00936.x

Narayanan, D., Adebiyi, A., and Jaggar, J. H. (2012). Inositol trisphosphate receptors in smooth muscle cells. *Am. J. Physiol. Heart Circ. Physiol.* 302, H2190–H2210. doi: 10.1152/ajpheart.01146.2011

Nicoll, R. A., and Madison, D. V. (1982). General anesthetics hyperpolarize neurons in the vertebrate central nervous system. *Science* 217, 1055–1057. doi: 10.1126/science.7112112

Niesen, C. E., Baskys, A., and Carlen, P. L. (1988). Reversed ethanol effects on potassium conductances in aged hippocampal dentate granule neurons. *Brain Res.* 445, 137–141. doi: 10.1016/0006-8993(88)91082-7

Niu, X., Qian, X., and Magleby, K. L. (2004). Linker-gating ring complex as passive spring and $Ca^{(2+)}$-dependent machine for a voltage- and $Ca^{(2+)}$-activated potassium channel. *Neuron* 42, 745–756. doi: 10.1016/j.neuron.2004.05.001

Orio, P., Rojas, P., Ferreira, G., and Latorre, R. (2002). New disguises for an old channel: maxiK channel beta-subunits. *News Physiol. Sci.* 17, 156–161.

Petrik, D., and Brenner, R. (2007). Regulation of STREX exon large conductance, calcium-activated potassium channels by the beta4 accessory subunit. *Neuroscience* 149, 789–803. doi: 10.1016/j.neuroscience.2007.07.066

Pietrzykowski, A. Z., Friesen, R. M., Martin, G. E., Puig, S. I., Nowak, C. L., Wynne, P. M., et al. (2008). Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron* 59, 274–287. doi: 10.1016/j.neuron.2008.05.032

Pietrzykowski, A. Z., Martin, G. E., Puig, S. I., Knott, T. K., Lemos, J. R., and Treistman, S. N. (2004). Alcohol tolerance in large-conductance, calcium-activated potassium channels of CNS terminals is intrinsic and includes two components: decreased ethanol potentiation and decreased channel density. *J. Neurosci.* 24, 8322–8332. doi: 10.1523/JNEUROSCI.1536-04.2004

Raffaelli, G., Saviane, C., Mohajerani, M. H., Pedarzani, P., and Cherubini, E. (2004). BK potassium channels control transmitter release at CA3-CA3 synapses in the rat hippocampus. *J. Physiol.* 557, 147–157. doi: 10.1113/jphysiol.2004.062661

Rancz, E. A., and Häusser, M. (2006). Dendritic calcium spikes are tunable triggers of cannabinoid release and short-term synaptic plasticity in cerebellar Purkinje neurons. *J. Neurosci.* 26, 5428–5437. doi: 10.1523/JNEUROSCI.5284-05.2006

Sah, P. (1996). $Ca^{(2+)}$-activated K^+ currents in neurones: types, physiological roles and modulation. *Trends Neurosci.* 19, 150–154. doi: 10.1016/S0166-2236(96)80026-9

Salkoff, L., Butler, A., Ferreira, G., Santi, C., and Wei, A. (2006). High-conductance potassium channels of the SLO family. *Nat. Rev. Neurosci.* 7, 921–931. doi: 10.1038/nrn1992

Schreiber, M., Wei, A., Yuan, A., Gaut, J., Saito, M., and Salkoff, L. (1998). Slo3, a novel pH-sensitive K^+ channel from mammalian spermatocytes. *J. Biol. Chem.* 273, 3509–3516. doi: 10.1074/jbc.273.6.3509

Schuckit, M. A. (1985a). Studies of populations at high risk for alcoholism. *Psychiatr. Dev.* 3, 31–63.

Schuckit, M. A. (1985b). Genetics and the risk for alcoholism. *JAMA* 254, 2614–2617. doi: 10.1001/jama.1985.03360180118037

Schuckit, M. A. (1994). Low level of response to alcohol as a predictor of future alcoholism. *Am. J. Psychiatry* 151, 184–189.

Singh, A. K., McMillan, J., Bukiya, A. N., Burton, B., Parrill, A. L., and Dopico, A. M. (2012). Multiple cholesterol recognition/interaction amino acid consensus (CRAC) motifs in cytosolic C tail of Slo1 subunit determine cholesterol sensitivity of Ca^{2+}- and voltage-gated K^+ (BK) channels. *J. Biol. Chem.* 287, 20509–20521. doi: 10.1074/jbc.M112.356261

Stocker, M. (2004). $Ca^{(2+)}$-activated K^+ channels: molecular determinants and function of the SK family. *Nat. Rev. Neurosci.* 5, 758–770. doi: 10.1038/nrn1516

Stojilkovic, S. S., Zemkova, H., and Van Goor, F. (2005). Biophysical basis of pituitary cell type-specific Ca^{2+} signaling-secretion coupling. *Trends Endocrinol. Metab.* 16, 152–159. doi: 10.1016/j.tem.2005.03.003

Strbak, V. (2006). Cell volume and peptide hormone secretion. *Contrib. Nephrol.* 152, 210–220. doi: 10.1159/000096325

Thombs, D. L., Olds, R. S., and Snyder, B. M. (2003). Field assessment of BAC data to study late-night college drinking. *J. Stud. Alcohol.* 64, 322–330.

Toro, L., Wallner, M., Meera, P., and Tanaka, Y. (1998). Maxi-$K_{(Ca)}$, a Unique Member of the Voltage-Gated K Channel Superfamily. *News Physiol. Sci.* 13, 112–117.

Treistman, S. N., and Martin, G. E. (2009). BK Channels: mediators and models for alcohol tolerance. *Trends Neurosci.* 32, 629–637. doi: 10.1016/j.tins.2009.08.001

Vaithianathan, T., Narayanan, D., Asuncion-Chin, M. T., Jeyakumar, L. H., Liu, J., Fleischer, S., et al. (2010). Subtype identification and functional characterization of ryanodine receptors in rat cerebral artery myocytes. *Am. J. Physiol. Cell. Physiol.* 299, C264–C278. doi: 10.1152/ajpcell.00318.2009

Van Goor, F., Li, Y. X., and Stojilkovic, S. S. (2001). Paradoxical role of large-conductance calcium-activated K+ (BK) channels in controlling action potential-driven Ca^{2+} entry in anterior pituitary cells. *J. Neurosci.* 21, 5902–5915.

Velàzquez-Marrero, C., Seale, G. E., Treistman, S. N., and Martin, G. E. (2014). Large conductance voltage− and Ca^{2+}-gated potassium (BK) channel ß4 subunit influences sensitivity and tolerance to alcohol by altering Its response to kinases. *J. Biol. Chem.* 289, 29261–29272. doi: 10.1074/jbc.M114.604306

Velázquez-Marrero, C., Wynne, P., Bernardo, A., Palacio, S., Martin, G., and Treistman, S. N. (2011). The relationship between duration of initial alcohol exposure and persistence of molecular tolerance is markedly nonlinear. *J. Neurosci.* 31, 2436–2446. doi: 10.1523/JNEUROSCI.5429-10.2011

Walters, F. S., Covarrubias, M., and Ellingson, J. S. (2000). Potent inhibition of the aortic smooth muscle maxi-K channel by clinical doses of ethanol. *Am. J. Physiol. Cell. Physiol.* 279, C1107–C1115.

Wang, L., and Sigworth, F. J. (2009). Structure of the BK potassium channel in a lipid membrane from electron cryomicroscopy. *Nature* 461, 292–295. doi: 10.1038/nature08291

Wang, Q., Kalogeris, T. J., Wang, M., Jones, A. W., and Korthuis, R. J. (2010). Antecedent ethanol attenuates cerebral ischemia/reperfusion-induced leukocyte-endothelial adhesive interactions and delayed neuronal death: role of large conductance, Ca^{2+}-activated K^+ channels. *Microcirculation* 17, 427–438.

Wang, X., Wang, G., Lemos, J. R., and Treistman, S. N. (1994). Ethanol directly modulates gating of a dihydropyridine-sensitive Ca^{2+} channel in neurohypophysial terminals. *J. Neurosci.* 14, 5453–5460.

Wang, Z. W. (2008). Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Mol. Neurobiol.* 38, 153–166. doi: 10.1007/s12035-008-8039-7

Warbington, L., Hillman, T., Adams, C., and Stern, M. (1996). Reduced transmitter release conferred by mutations in the slowpoke-encoded $Ca^{2(+)}$-activated K^+ channel gene of *Drosophila*. *Invert. Neurosci.* 2, 51–60. doi: 10.1007/BF02336660

Weaver, A. K., Olsen, M. L., McFerrin, M. B., and Sontheimer, H. (2007). BK channels are linked to inositol 1,4,5-triphosphate receptors via lipid rafts: a novel mechanism for coupling $[Ca^{(2+)}]_{(i)}$ to ion channel activation. *J. Biol. Chem.* 282, 31558–31568. doi: 10.1074/jbc.M702866200

Weiger, T. M., Hermann, A., and Levitan, I. B. (2002). Modulation of calcium-activated potassium channels. *J. Comp. Physiol. A. Neuroethol. Sens. Neural. Behav. Physiol.* 188, 79–87. doi: 10.1007/s00359-002-0281-2

Wessel, R., Kristan, W. B. Jr., and Kleinfeld, D. (1999). Dendritic $Ca^{(2+)}$-activated $K^{(+)}$ conductances regulate electrical signal propagation in an invertebrate neuron. *J. Neurosci.* 19, 8319–8326.

Wong, R. K., and Prince, D. A. (1981). Afterpotential generation in hippocampal pyramidal cells. *J. Neurophysiol.* 45, 86–97.

Wu, Y., Yang, Y., Ye, S., and Jiang, Y. (2010). Structure of the gating ring from the human large-conductance $Ca^{(2+)}$-gated $K^{(+)}$ channel. *Nature* 466, 393–397. doi: 10.1038/nature09252

Wynne, P. M., Puig, S. I., Martin, G. E., and Treistman, S. N. (2009). Compartmentalized beta subunit distribution determines characteristics and ethanol sensitivity of somatic, dendritic, and terminal large-conductance calcium-activated potassium channels in the rat central nervous system. *J. Pharmacol. Exp. Ther.* 329, 978–986. doi: 10.1124/jpet.108.146175

Xia, X. M., Zhang, X., and Lingle, C. J. (2004). Ligand-dependent activation of Slo family channels is defined by interchangeable cytosolic domains. *J. Neurosci.* 24, 5585–5591. doi: 10.1523/JNEUROSCI.1296-04.2004

Xie, J., and McCobb, D. P. (1998). Control of alternative splicing of potassium channels by stress hormones. *Science* 280, 443–446. doi: 10.1126/science.280.5362.443

Ye, Y., Jian, K., Jaggar, J. H., Bukiya, A. N., and Dopico, A. M. (2014). Type 2 ryanodine receptors are highly sensitive to alcohol. *FEBS Lett.* 588, 1659–1665. doi: 10.1016/j.febslet.2014.03.005

Yuan, C., Chen, M., Covey, D. F., Johnston, L. J., and Treistman, S. N. (2011). Cholesterol tuning of BK ethanol response is enantioselective, and is a function of accompanying lipids. *PLoS ONE* 6:e27572. doi: 10.1371/journal.pone.0027572

Yuan, C., O'Connell, R. J., Jacob, R. F., Mason, R. P., and Treistman, S. N. (2007). Regulation of the gating of BK_{Ca} channel by lipid bilayer thickness. *J. Biol. Chem.* 282, 7276–7286. doi: 10.1074/jbc.M607593200

Yuan, C., O'Connell, R. J., Wilson, A., Pietrzykowski, A. Z., and Treistman, S. N. (2008). Acute alcohol tolerance is intrinsic to the BK_{Ca} protein, but is modulated by the lipid environment. *J. Biol. Chem.* 283, 5090–5098. doi: 10.1074/jbc.M708214200

Yuan, P., Leonetti, M. D., Pico, A. R., Hsiung, Y., and MacKinnon, R. (2010). Structure of the human BK channel Ca^{2+}-activation apparatus at 3.0 A resolution. *Science* 329, 182–186. doi: 10.1126/science.1190414

Zhang, X. F., Gopalakrishnan, M., and Shieh, C. C. (2003). Modulation of action potential firing by iberiotoxin and NS1619 in rat dorsal root ganglion neurons. *Neuroscience* 122, 1003–1011. doi: 10.1016/j.neuroscience.2003.08.035

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 September 2014; accepted: 13 November 2014; published online: 02 December 2014.

Citation: Dopico AM, Bukiya AN and Martin GE (2014) Ethanol modulation of mammalian BK channels in excitable tissues: molecular targets and their possible contribution to alcohol-induced altered behavior. Front. Physiol. ***5****:466. doi: 10.3389/fphys.2014.00466*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 09 September 2014
doi: 10.3389/fphys.2014.00346

The role of the BK channel in ethanol response behaviors: evidence from model organism and human studies

Jill C. Bettinger* and Andrew G. Davies

Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA, USA

Edited by:
Alex M. Dopico, The University of Tennessee Health Science Center, USA

Reviewed by:
Teresa Giraldez, La Laguna University, Spain
Habibeh Khoshbouei, Univeristy of Florida, USA

****Correspondence:***
Jill C. Bettinger, Department of Pharmacology and Toxicology, Virginia Commonwealth University, PO Box 980613, Richmond, VA 23298, USA
e-mail: jcbettinger@vcu.edu

Alcohol abuse is a significant public health problem. Understanding the molecular effects of ethanol is important for the identification of at risk individuals, as well as the development of novel pharmacotherapies. The large conductance calcium sensitive potassium (BK) channel has emerged as an important player in the behavioral response to ethanol in genetic studies in several model organisms and in humans. The BK channel, *slo-1*, was identified in a forward genetics screen as a major ethanol target in *C. elegans* for the effects of ethanol on locomotion and egg-laying behaviors. Regulation of the expression of the BK channel, *slo*, in *Drosophila* underlies the development of rapid tolerance to ethanol and benzyl alcohol sedation. Rodent expression studies of the BK-encoding *KCNMA1* gene have identified regulation of mRNA levels in response to ethanol exposure, and knock out studies in mice have demonstrated that the β subunits of the BK channel, β1 and β4, can modulate ethanol sensitivity of the channel in electrophysiological preparations, and can influence drinking behavior. In human genetics studies, both *KCNMA1* and the genes encoding β subunits of the BK channel have been associated with alcohol dependence. This review describes the genetic data for a role for BK channels in mediating behavioral responses to ethanol across these species.

Keywords: BK, *slo*, KCNMA1, *slo-1*, potassium channel, ethanol, genetics

INTRODUCTION

Alcohol abuse is a significant worldwide socioeconomic problem, for which there are very few treatments. Despite the severity and prevalence of the disease, the molecular mechanisms by which alcohol exerts the effects that are relevant to drinking behavior remain incompletely understood. One major difficulty has been identifying behaviorally relevant ethanol targets. Ethanol interacts with many molecules *in vitro*, but translating information into an understanding of the behavioral relevance of the interactions requires different experimental approaches.

It is clear that there are physiological differences between individuals that influence their susceptibility to developing an alcohol use disorder, and that these differences are genetically influenced (Rodriguez et al., 1993; Schuckit and Smith, 1996; Prescott and Kendler, 1999). This indicates that there are differences in the human population in the neurobiological response to alcohol, and that these differences underlie at least some of the risk to abuse the drug.

One approach to identifying the mechanisms by which ethanol mediates its effects on behavior is to use genetic analysis, which allows for *in vivo* examination of the effect of ethanol on the function of a gene in mediating a behavioral phenotype. There are several types of genetic analysis, which can be divided into two major categories, forward and reverse genetics. In forward genetic analysis, the functions of all genes in a genome are examined using large-scale mutant screens. One important aspect of this approach is that there are no assumptions made about the relevance of any particular gene, and genes emerge from this analysis only if they have detectable effects on the phenotype being studied. Reverse genetics involves examining the function of particular targets *in vivo* by manipulating the expression or function of the gene. Reverse genetics allows for exquisite dissection of the function of particular genes in a phenotype of interest.

Model organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, provide powerful genetic tools, short generation times and relatively simple nervous systems with which to approach the problem of identifying the targets of a neuroactive drug and the physiological responses associated with the development of tolerance to such a drug (Schafer, 2004; Giacomotto and Ségalat, 2010; Devineni and Heberlein, 2013). Genetic analysis in rodent models is more laborious, but rodents allow for the genetic analysis of much more elaborate nervous systems, as well as very complex behaviors, such as voluntary ethanol drinking.

Using forward and reverse genetics approaches, many different laboratories across several different model organisms and humans have identified the large conductance voltage and calcium-sensitive potassium (BK) channel as being important in modulating the behavioral effects of ethanol. The purpose of this review is to describe genetic evidence supporting a major role for BK as a central mediator of ethanol's effects on behavior.

C. ELEGANS: IDENTIFICATION OF BK AS THE MAJOR MEDIATOR OF THE DEPRESSIVE BEHAVIORAL EFFECTS OF ETHANOL

One of the most powerful approaches for the identification of novel genes that are important for a phenotype is a forward genetic screen (Jorgensen and Mango, 2002). Wild-type animals are treated with a mutagen to induce random mutations in the germline and subsequent progeny bearing the induced mutations are screened for a phenotype of interest, such as reduced sensitivity to the effects of a drug. Such an approach, in theory, can assess the *in vivo* impact of a mutation in every gene on the phenotype of interest and it does so with minimal bias and no *a priori* assumptions about which gene is important. We used this approach with *C. elegans* to identify potential direct targets of ethanol using a behavioral response as the means of selection.

In a screen for mutant animals that were less sensitive to the depressive effects of ethanol on the speed of locomotion, we identified multiple independent mutations in the *slo-1* gene (Davies et al., 2003), the *C. elegans* homolog of the mammalian *KCNMA1* gene (Wang et al., 2001), which encodes the α subunit of the BK channel. The *slo-1* mutants had the largest level of ethanol resistance for any mutant found, suggesting that with loss of *slo-1,* a significant effect of ethanol had been blocked. In this genetic screen of randomly induced mutations, the mutations in *slo-1* were identified without bias from the findings that the BK channel was already known to be a target of ethanol based on numerous *in vitro* assays (Dopico et al., 1996, 1998, 1999; Jakab et al., 1997; Chu et al., 1998; Walters et al., 2000; Gruss et al., 2001). In *C. elegans*, the BK channel is expressed in neurons and in muscle (Wang et al., 2001). We confirmed a requirement for BK channels in the nervous system for ethanol to produce its effects by expressing wild-type SLO-1 only in neurons in an otherwise *slo-1* null mutant background and restoring wild-type sensitivity to the effects of ethanol on locomotion speed (Davies et al., 2003). Electrophysiology was performed on intact *C. elegans* neurons, which showed that ethanol increased BK channel currents but failed to do so in *slo-1* null mutant neurons (Davies et al., 2003). Two gain-of-function *slo-1* mutants displayed phenotypes that were characteristic of ethanol intoxication, providing further confirmation that hyperactivation of BK channels can produce intoxicated behaviors (Davies et al., 2003).

From the same genetic screen, mutations that affected members of the dystrophin-associated protein complex (DAPC) were also identified as being resistant to the effects of ethanol (Davies et al., 2003). These mutants did not have the same level of ethanol resistance as *slo-1* mutants, but they did share certain basal (observed in the absence of ethanol) mutant phenotypes with *slo-1* mutants. These phenotypic similarities led to the identification of an interaction between the DAPC and BK channels in muscle, and the observation that BK channels are localized by the DAPC (Kim et al., 2009).

More recently, we have shown that the BK channel plays a role in the development of acute functional (within session) tolerance (AFT) to ethanol, but, importantly, that loss of *slo-1* does not eliminate the ability to develop AFT (Bettinger et al., 2012). This latter observation implicates other mechanisms of action for both ethanol's effects and for the development of tolerance to those effects. In this study, we showed that a triacylglycerol (TAG) lipase plays a significant role in regulating the rate of development of acute functional tolerance. By manipulating the levels of TAGs through a mutation in the TAG lipase, *lips-7*, we could alter the basal behavioral phenotypes associated with the hyperactivated gain-of-function mutations in the *slo-1* gene (Bettinger et al., 2012). This result provides genetic evidence for a link between lipid environment and BK channel function. Such an interaction between BK and the lipid environment has been well documented *in vitro* as having significant effects on the ethanol sensitivity of a BK channel (Crowley et al., 2003, 2005; Yuan et al., 2008, 2011a,b).

Dillon et al. (2013) made use of the *C. elegans* pharynx and associated neurons, a relatively closed neuromuscular circuit, to assess the effect of a concentration range of ethanol on the rhythmic pumping of the pharynx. In this system, *slo-1* is expressed only in the neurons. At low doses, with the *slo-1* null mutant, they did not see the typical stimulatory effects of ethanol, whereas at high concentrations, where ethanol inhibits pharyngeal activity, loss of *slo-1* had no effect. This may point to other targets of ethanol in the pharyngeal neurons or a direct action of ethanol on the pharyngeal muscle itself.

C. ELEGANS: USE OF GENETICS TO IDENTIFY AN ETHANOL INTERACTING DOMAIN IN THE SLO-1 BK CHANNEL

Recently, Davis et al. (2014) used a combination of forward and reverse genetics strategies to identify a domain on the BK channel that is ethanol responsive. This group took advantage of a large collection of 32 mutant strains carrying amino acid substitution mutations in the *C. elegans slo-1* gene, which they assayed *in vivo* for changes in ethanol sensitivity. The goal was to identify a mutation that altered ethanol sensitivity without altering BK channel function, with the idea that the mutation may specifically alter the ability of ethanol to directly interact with the BK channel. One mutation, resulting in a T381I substitution, produced significant resistance to the depressing effects of ethanol on the neuromuscular-controlled behaviors of egg laying and locomotion (Davis et al., 2014). The threonine affected by the mutation is conserved in mammals and flies and is located in the N-terminal portion of the RCK1 calcium-sensing domain. The substitution had very mild effects on normal SLO-1 functions; the only departure from wild-type phenotypes was a partial effect on sensitivity to an acetylcholinesterase inhibitor that is used as a measure of acetylcholine release. Therefore, the T381I mutation has a strong impact on ethanol sensitivity of the BK channel with little effect on normal channel function. The relevance of this amino acid residue across phyla was tested by creating an identical mutation in the human BK channel (T352I) and expressing that version in *C. elegans*. Transformation with the wild-type human BK channel could rescue *slo-1* null mutant animals, whereas the mutant human BK channel acted like the worm T381I mutant, it failed to restore ethanol sensitivity but did restore the other *slo-1* null mutant phenotypes (Davis et al., 2014). Single-channel patch clamp recordings of the human BK channel expressed in *C. elegans* neurons showed that in both the non-mutated and the mutated forms, currents could be detected that displayed all of the expected characteristics of the human channel, suggesting that the mutation did not alter normal BK channel properties.

When these channels were expressed in HEK293 cells, only a minor difference in open probability across different voltages was detected between the non-mutated and mutant forms, suggesting the possibility of a subtle impact of the mutation on normal BK channel function, and furthermore, ethanol-induced potentiation was eliminated in the mutated human channel (Davis et al., 2014). The structural impact of the T381I mutation is not yet known but the equivalent amino acid in mammals (T352) is only 9 amino acids from the hydrogen-bonding lysine residue identified by Bukiya et al. (2014) as part of an ethanol interacting domain in the mammalian channel. The T381I mutation, which replaces a smaller polar residue with a larger nonpolar residue, may impact the same pocket either through hindrance of ethanol or through electrical charge dynamics.

DROSOPHILA: THE ROLE OF THE BK CHANNEL IN RAPID TOLERANCE TO ETHANOL

The fruit fly, *Drosophila melanogaster*, has proved to be an excellent model in which to carefully dissect the contribution of BK channels to the *in vivo* behavioral effects of ethanol. A single gene, *slowpoke*, encodes the BK channel, *slo*, in *Drosophila*, and its role in the response to alcohol sedation has been extensively characterized.

slo function in the fly response to drugs has been characterized primarily in an adaptive drug response phenotype, the development of rapid tolerance. These studies have used two drugs, ethanol and benzyl alcohol (BA). In these assays, flies are exposed to an ethanol or BA vapor in a closed chamber. Flies accumulate ethanol from the air and, after a brief initial startle activation response, become sedated (Rothenfluh and Heberlein, 2002), and similar kinetics are observed when flies are treated with BA (Ghezzi et al., 2004). When the flies are all sedated, the vapor is removed from the air flow, and the time that it takes the flies to recover the ability to stand is recorded. If the flies are then sedated a second time, they recover their postural control significantly more quickly than after the first sedation, a phenotype that is referred to as rapid functional tolerance (Ghezzi et al., 2004; Cowmeadow et al., 2005). Rapid tolerance is observable for at least 7 but fewer than 14 days after the initial sedation, and represents a long-lasting modification of nervous system function in response to the drug exposure. Sedation with benzyl alcohol or ethanol causes cross-tolerance with the other (Cowmeadow et al., 2006), strongly suggesting that the mechanism of rapid tolerance is the same for the two drugs.

slo function is required for the development of rapid tolerance, as *slo* null mutant animals do not become tolerant to either drug after an initial sedation (Ghezzi et al., 2004; Cowmeadow et al., 2005). This group has also been able to take advantage of an excellent genetic tool, the $ash2^{18}$ deletion mutant, which removes only the promoter region driving expression of *slo* in neurons, but leaves the expression of *slo* in muscles intact, to map the requirement for *slo* function in rapid tolerance to the nervous system (Ghezzi et al., 2004; Cowmeadow et al., 2005).

Sedation with ethanol, BA, chloroform, CO_2, cold temperatures, or sub-lethal doses of tetrodotoxin all cause a transient increase in *slo* mRNA levels in neurons that can be observed at 6 h post sedation (Ghezzi et al., 2004; Cowmeadow et al., 2006). This suggested that the drug-induced increase in *slo* expression may be responsible for the development of rapid tolerance, and indeed, when *slo* expression was induced with a heat-shock inducible transgene, the recovery kinetics from the first sedation resembled those of animals that had developed rapid tolerance (Cowmeadow et al., 2006).

Epigenetic modifications control this drug-induced increase in *slo* expression; these act on specific promoter sequences that are conserved across *Drosophila* species (Wang et al., 2007). There is a complex pattern of histone acetylation and deacetylation in the *slo* regulatory region that occurs over the course of 48 h after sedation (Wang et al., 2007), and these histone acetylation changes require binding of CREB to the *slo* promoter; loss of function of *Drosophila* dCREB2 eliminates drug-induced upregulation of *slo*, and eliminates rapid tolerance (Wang et al., 2009).

It is interesting that an increase in *slo* expression should cause a tolerance phenotype, which suggests that in this context, BK channels act to increase the excitability of cells. Ghezzi et al. (2010) explored the mechanism of this role of BK in tolerance to alcohol sedation in *Drosophila*. In these experiments, the firing capacity of the giant fiber neuron pathway was measured; the ability of the pathway to fire repeatedly is a measure of neuronal excitability (Tanouye and Wyman, 1980). An electrical stimulation was delivered into the eyes of the fly, and the firing of the giant fiber neuron pathway was measured in the dorsal-longitudinal flight muscles. Twenty-four hours after sedation with BA or ethanol, giant fiber neurons demonstrated an increase in excitability that was dependent on *slo* expression in neurons (Ghezzi et al., 2010, 2012). The interpretation of these results is that the drug-induced increase in *slo* expression causes a decrease in the refractory period in neurons, thereby increasing neuronal excitability. This increase in excitability acts to counter the depressing effects of BA and ethanol, and describes a potential mechanism for the development of rapid tolerance.

Additional evidence for this hypothesis comes from the observation that sedation with BA or ethanol also leads to an increase in the seizure susceptibility of the giant fiber pathway neurons that is due to the drug-induced increase in *slo* expression (Ghezzi et al., 2010, 2012), further indicating that these neurons have become hyper-excitable. This phenotype has characteristics in common with the seizure susceptibility that is sometimes observed in alcohol-dependent humans during alcohol withdrawal (Rogawski, 2005).

RODENT EXPRESSION STUDIES: THE EFFECT OF ETHANOL EXPOSURE ON BK CHANNEL MESSAGE LEVELS

The two commonly used inbred mouse strains, C57BL/6J (C57) and DBA/2J (D2), show significant differences in their behavioral responses to ethanol and in their ethanol drinking preferences (Crawley et al., 1997). DBA/2J mice show significant ethanol-induced locomotor stimulation and reduced alcohol consumption relative to C57BL/6J. Kerns et al. (2005) took advantage of these behavioral differences, and examined global gene expression using microarrays in these two strains in the nucleus accumbens, prefrontal cortex, and ventral tegmental area 4 h following acute ethanol (2 g/kg) or saline injection. The BK channel encoding gene, *KCNMA1*, was one of only 307 genes found to be regulated

in response to ethanol in either strain in any of the brain regions. *KCNMA1* expression was upregulated in the nucleus accumbens in D2 mice but was relatively unchanged in C57 mice. Wolen et al. (2012) further explored the differences between these two mouse strains, and increased the power of this approach by using the recombinant inbred BXD strains that were derived from an initial cross between C57 and D2 and then inbred for homozygosity to produce over 100 strains in which the original C57 and D2 genomes are recombined into unique combinations (Peirce et al., 2004). Wolen et al. (2012) used microarrays to examine global gene expression in the parent strains and in at least 27 of the BXD strains (depending on the brain region studied). They assessed gene expression in the nucleus accumbens, the prefrontal cortex, and the ventral midbrain 4 h following intraperitoneal injection of ethanol (1.8 g/kg) or saline, and found that *KCNMA1* gene expression was ethanol-responsive in all brain regions examined. In addition, expression of the *KCNMB4* gene, which encodes the β4 subunit of the BK channel in mammals, was found to be ethanol-responsive in the ventral midbrain. Focusing on the prefrontal cortex data, they identified networks of genes whose expression was highly correlated either basally, following ethanol exposure, or both. *KCNMA1* was identified as a hub gene based on the bioinformatic measures of connectivity and betweenness centrality in one of these large gene networks (Wolen et al., 2012). Hub genes are considered to be likely regulators of the transcriptional response to ethanol treatment so it is notable that *KCNMA1* falls into this category. The difference in *KCNMA1* regulation in response to ethanol in these different genetic backgrounds suggests that there is important genetic variation in the mechanisms of regulation in these strains, and points to important avenues for future study.

The increased expression of *KCNMA1* in ethanol-treated D2 mice is consistent with the *Drosophila* rapid tolerance studies, which showed increased BK channel expression following ethanol treatment (Cowmeadow et al., 2006; Ghezzi et al., 2010). In contrast, in a study of the effects of ethanol on *KCNMA1* gene expression, Pietrzykowski et al. (2008) showed that ethanol exposure of rat supraoptic nucleus (SON) neurons results in a down regulation of total *KCNMA1* mRNA. In primary cultured striatal neurons and in SON neurons in organotypic explants there is a rapid decrease in total *KCNMA1* mRNA levels within 15 min of ethanol (20 mM) exposure.

Pietrzykowski et al. (2008) also made the intriguing observation that ethanol exposure alters which splice forms of *KCNMA1* mRNA are present in these neurons. The authors examined the variety of alternatively spliced *KCNMA1* mRNAs and found that in untreated SON neurons, eight mRNA variants were detected, however, in 24 h-ethanol treated SON neurons, only two mRNA variants were found. These data suggest that ethanol is causing regulation of specific *KCNMA1* mRNAs. The rapid action (within 15 min of exposure) of ethanol on the level of *KCNMA1* message suggests that the regulation acts on preexisting mRNAs rather than on transcription levels. Particular targets of this down-regulation were mRNAs containing exon 29, which was dubbed ALCOREX (Pietrzykowski et al., 2008). Loss of mRNAs containing the ALCOREX exon is likely to impact the ethanol sensitivity of BK channels because when expressed transiently in HEK293 cells, BK channel isoforms that contain ALCOREX are more sensitive to the potentiating effects of ethanol and that potentiation is longer lasting than that in other isoforms lacking ALCOREX. To identify the mediators of the ethanol effect in down regulating specific *KCNMA1* mRNAs, the authors looked to regulation by specific microRNAs. Expression of miR-9 increases on a timescale equivalent to the down-regulation effects on *KCNMA1* mRNA. miR-9 is predicted to target ALCOREX-containing *KCNMA1* mRNAs based on the presence of and complementarity with an alternative 3′UTR (called 3′UTR-2.1) that is associated with ALCOREX-containing mRNAs (Pietrzykowski et al., 2008). These data identify miR-9 as a key regulator of BK channel ethanol tolerance although the *in vivo* consequences of this regulation remains to be tested, particularly in combination with beta subunit effects on sensitivity and tolerance.

MOUSE KNOCKOUT STUDIES: THE ROLE OF THE β SUBUNITS OF THE BK CHANNEL IN THE ELECTROPHYSIOLOGICAL AND BEHAVIORAL RESPONSES TO ETHANOL

The use of gene knockout techniques in mice allows for an *in vivo* examination of the function of a gene, including its role in behavioral responses. The knockout mutations used to examine the role of BK channel beta subunits have been particularly informative, although, as with any complete knockout in any organism, the consequences of loss of the gene must be interpreted with the possibility of compensation in the form of up or down regulation of other functionally interacting genes and/or the possibility that loss of the gene may result in developmental alterations.

There are four β subunits (β1–β4) that can associate with the BK alpha subunit to alter its physiological and pharmacological properties (reviewed by Torres et al., 2007; Pongs and Schwarz, 2010). These subunits show tissue specific expression, with some overlap. The subunits of particular importance to the effects of ethanol on BK channels are the β1 and β4 subunits, which are encoded in mammals by the *KCNMB1* and *KCNMB4* genes, respectively.

The β4 subunit is abundant in the CNS (Brenner et al., 2000; Weiger et al., 2000). Martin et al. (2008) examined the effect of β4 subunits on the development of tolerance to the effects of ethanol on BK channels. Expressed alone in HEK293 cells, the BK α subunit showed potentiation of activity by ethanol that diminishes within 10 min despite continuous ethanol exposure. In contrast, when the α subunit is co-expressed with the β4 subunit, tolerance to the potentiating effects of ethanol does not occur, suggesting that the β4 subunit is interfering with the mechanism of tolerance without altering the potentiating effect of ethanol. This outcome was replicated with medium spiny neurons from the mouse ventral striatum in wild-type and *KCMNB4* (β4) knockout animals, in which activation of the BK channel depresses excitability of the neurons, decreasing the number of action potentials (APs) evoked by current injection in striatal slice preparations. Loss of β4 allowed tolerance to develop to potentiating effects of ethanol on the BK channel (Martin et al., 2008). In slices from wild-type mice, ethanol decreased the number of APs for at least 8 min of recording, whereas in slices prepared from β4 knockout mice, the degree of ethanol-induced reduction in APs diminished over the course of the 8 min of recording, showing that tolerance could

develop to the effect of ethanol in the absence of β4 subunits (Martin et al., 2008). Two behavioral assays were then performed using the β4 knockout mice. First, the sensitivity to ethanol intoxication was measured. C57BL/6 mice show significantly decreased rates of acute (5–15 min) locomotor activity in the presence of 2 mg/kg ethanol. In wild-type mice, tolerance to this effect is observable at the 15 min post-injection time point, but only after 4 days of identical treatment (Martin et al., 2008). This effect somewhat resembles the rapid tolerance displayed by *Drosophila* that have seen a previous ethanol exposure (Cowmeadow et al., 2006). In contrast, β4 knockout mice showed significant within-session acute functional tolerance (AFT) to the decreased speed of locomotion effects of ethanol at the 10 and 15 min time points, even on the first day of ethanol administration (Martin et al., 2008), an effect that resembles the development of AFT seen in *C. elegans* for the effect of ethanol on locomotion (Davies et al., 2004; Bettinger et al., 2012). The simplest explanation for these outcomes is that the β4 subunit has a negative effect, *in vivo*, on the development of tolerance to the potentiating effects of ethanol on the BK channel, and that tolerance can only develop slowly in the presence of β4.

To examine the impact of the lack of tolerance associated with β4 knockout on drinking behavior, Martin et al. (2008) went on to assess voluntary ethanol drinking using a "drinking in the dark" paradigm, which restricts access to ethanol. The β4 knockout mice consumed greater quantities of ethanol during the first three access periods and achieved a higher blood alcohol level than the wild-type mice, without changing their water intake. This increased level of ethanol drinking might reflect a decreased sensitivity to one or more effects of ethanol as tolerance to the drug would be predicted to be greater in the β4 knockout animals.

A separate genetic study has confirmed that the presence of particular β subunits can alter the direction of ethanol's effect on BK channels, such that inhibition of the BK channel by ethanol is also possible. The β1 subunit is highly expressed in smooth muscle (Behrens et al., 2000). Focusing on ethanol's effects on smooth muscle, Bukiya et al. (2009) examined the requirement for the β1 subunit for ethanol-induced inhibition of BK channels in myocytes that results in cerebrovascular constriction. Depending on the intracellular Ca^{2+} concentration, ethanol can potentiate (lower Ca^{2+}) or inhibit (higher Ca^{2+}) BK potassium currents. When associated with a β1 subunit, the BK channel is inhibited at significantly lower intracellular calcium concentrations (Bukiya et al., 2009). A comparison of the effects of ethanol on cerebral artery myocytes derived from wild-type and *KCNMB1* (β1) knockout mice showed that the β1 subunit was able to completely change the effect of ethanol on the BK channel. In wild-type myocytes, ethanol inhibited BK channel activity, whereas in the absence of the β1 subunit, ethanol potentiated BK channel activity. An effect on the contraction of cerebral arteries that is consistent with the β1 subunit promoting BK channel inhibition was also observed; arteries from β1 knockout mice fail to contract with ethanol exposure while arteries from wild-type animals (β1-containing) showed a 10-15% reduction in diameter (Bukiya et al., 2009).

While the β1 subunit is highly expressed in smooth muscle (Behrens et al., 2000) the β1 subunit also has limited expression elsewhere, including the brain (Martin et al., 2004). Kreifeldt et al. (2013) examined the impact of β1 or β4 knockouts in a C57BL/6J genetic background on voluntary ethanol consumption in mice. In a paradigm of voluntary ethanol consumption, two-bottle choice continuous access followed by intermittent access, loss of β1 and β4 had no effect on drinking levels. Note that this is seemingly in contrast to the data presented above, where β4 knockout mice consumed more alcohol during a restricted access (drinking in the dark) paradigm (Martin et al., 2008). It is possible that the drinking paradigms used in the two studies were sufficiently different to expose a mutant phenotype in one paradigm but not the other. An alternative possibility that is proposed, is that genetic background of the control strains used in the Martin et al. (2008) study may differ from the β4 knockout animals that were tested (Kreifeldt et al., 2013). The most interesting outcome of the comparison of β1 and β4 knockout mice was found when the mice were first made ethanol-dependent before their ethanol consumption was assessed. Chronic intermittent exposure (CIE) to ethanol vapor with repeated withdrawals produces symptoms of physiological ethanol dependence, and when these treated mice are then given limited access to ethanol they have been shown to escalate their ethanol consumption during the limited access periods over time (Becker and Lopez, 2004). When β1 and β4 knockout mice were made ethanol dependent by CIE treatment, the β1 knockout mice were found to escalate their ethanol consumption to a greater extent than the wild-type mice, while the β4 knockout mice had a lower rate of escalation of consumption than the wild-type animals (Kreifeldt et al., 2013). This surprising combination of phenotypes highlights both the complexity of the ethanol drinking phenotypes and the complex roles of BK channels in those behaviors. Simplistically, one might have predicted that loss of β1 should increase ethanol sensitivity because the presence of β1 prevents potentiation of BK by ethanol. In contrast, loss of β4 should decrease ethanol sensitivity via the rapid development of tolerance; β4 prevents the development of tolerance and, in its absence, tolerance to ethanol's effects can occur more readily. As appears to be the case in human populations (Schuckit and Smith, 1996), an increase in sensitivity to ethanol may be predicted to decrease voluntary drinking, whereas a decrease in sensitivity may increase drinking behavior. However, this model is clearly too simple. The combination of the adaptive changes that must be occurring with the generation of ethanol dependence and the as yet not fully understood roles for the beta subunits in modifying BK channel function, as well as the potential for different tissue specific subunit localization, makes a complete interpretation of this data difficult. It is clear, however, that beta subunit availability is an important factor in specific ethanol effects and in drinking behaviors.

HUMAN POPULATION STUDIES: ASSOCIATION OF BK CHANNEL GENES WITH ALCOHOL RESPONSE PHENOTYPES AND ALCOHOL DEPENDENCE

Ultimately, the goal of the work in the model organisms is to develop an understanding of the relevant mediators of the ethanol response that can be applied to the human alcohol response. Alcohol dependence (AD) is a complex phenotype, and its development is influenced by many factors. Genetics plays a

significant role in the risk for AD (Prescott and Kendler, 1999) and much effort has been focused on identifying "liability loci," alleles that predispose individuals to developing AD. Despite several large-scale genome wide analyses for genes associated with AD, few good candidates have been identified, and almost none, with the exception of metabolic enzymes, have been replicated in more than one study. This is likely to be due, at least in part, to the complex genetic architecture of risk for AD (Kendler et al., 2012), which suggests that there will not be individual genes that have a very large effect on liability, but rather that there will be many genes that have small, but real, effects. A second complexity in these analyses is that for phenotypes such as an increased liability to develop alcohol dependence, the severity of effects of the alleles on gene function are likely to be somewhat subtle. That is, in the model organism studies, we are able to strongly decrease or strongly potentiate function of a gene, whereas in human populations, allelic variation is likely to cause more minor changes in gene function; the allelic variation in human populations will include change of function mutations, and completely null mutations will be more rare. Taken together, this suggests that any signals that are detected are likely to be weak. Therefore, weaker signals, in particular those that appear in more than one study, may identify biologically relevant genes for AD. It is particularly intriguing, therefore, that signals have been reported in the BK channel-encoding gene, *KCNMA1*, in several different human population studies.

In 2005, Schuckit et al. reported a study of a population of sibling pairs in which they used linkage analysis to identify association of particular chromosomal regions with alcohol response phenotypes. They studied the level of response to alcohol, which is the degree of effect of a given dose of alcohol on objective and subjective measurements. The most consistent results were found for the Subjective High Assessment Scale (SHAS) measure with linkage on chromosome 10. The investigators looked specifically SNPs in the *KCNMA1* gene locus, which is within the chromosome 10 interval of interest. Six of 44 SNPs produced statistically significant association with the SHAS measure, but were not significant after correction for multiple testing, possibly due to a small sample size.

A second signal in *KCNMA1* was detected by Kendler et al. (2011) in a genome wide association study (GWAS) for alcohol dependence symptoms. In this study, the most significant intragenic single nucleotide polymorphism (SNP) in a European-American population was in the *KCNMA1* gene, which was accompanied by multiple additional SNPs with low p values. The finding of several nominally associated SNPs within the same gene lends support to the observation of association with *KCNMA1*. However, as with the Schuckit et al. (2005) study, none of the SNPs identified in this study (for any gene) approached genome-wide significance.

Edenberg et al. (2010) found in a GWAS of alcohol dependence using the COGA (Collaborative Study on the Genetics of Alcoholism) that among the top 985 SNPs that were highly ranked for association with alcohol dependence in European Americans were six SNPs that showed nominal ($p < 0.05$) significance in a smaller African American case-control study. One of these SNPs was in the *KCNMA1* gene. In this study, again, no SNP in any gene met the criteria for genome-wide significance. Edenberg et al. (2010) also found a SNP in *KCNMA1* was nominally significant ($p < 0.05$) for early-onset alcohol dependence in a family-based association analysis.

Recently, Han et al. (2013) used an approach that combined GWAS data sets (COGA and the Study of Addiction: Genetics and Environment (SAGE)) with human protein interaction networks to identify modules of networked genes that were enriched for highly ranked genes associated with alcohol dependence. When they tested their final 39-gene network, which included both *KCNMA1* and *KCNMB1*, for association with alcohol dependence, they found significant associations in both European Americans and African Americans in the merged COGA and SAGE GWAS data sets. The association of the gene network with AD was replicated in two European American samples and one African American sample.

CONCLUSIONS

Genetic studies in *C. elegans*, *Drosophila melanogaster*, and mice have all demonstrated that BK channels are central to the behavioral effects of ethanol across these diverse phyla. It is interesting to note that while an important role for BK in ethanol response behaviors is apparently conserved, the actual behavioral outcomes of altering the function of BK channels are different across these models, which points to the complexity of the effects of ethanol on BK channels, and to the complexity of the roles of BK channels in ethanol response behaviors.

In *C. elegans* and in *Drosophila,* the roles for BK channels in ethanol response behaviors appear to be both strong and reasonably straightforward, although these effects are quite different in the two models. In worms, the activation of the *slo-1* BK channel by ethanol is a primary mechanism of the initial sensitivity to the intoxicating effects of ethanol, and loss of *slo-1* causes resistance to ethanol. Worm BK channels are also likely to be negatively modulated to develop acute functional tolerance, and this modulation may involve modification of the lipid bilayer. In sharp contrast, in flies, ethanol induces expression of BK, and this increase in BK activity leads to a decrease in sensitivity to ethanol, this can be observed as resistance to ethanol when *slo* is induced experimentally, or as rapid tolerance when ethanol induces the response.

In mammals, the story is substantially more complex than it is in either of the invertebrate models. BK channels can be activated or inactivated by ethanol, depending on calcium levels and the presence or absence of specific β subunits, and there is dynamic regulation of the channels during the development of tolerance. The differential expression of *KCNMA1*, either basally or in response to ethanol treatment, in two inbred mouse strains and their progeny that display different behavioral responses to ethanol, points to the importance of genetic background and genetic factors that could regulate levels and localization of the BK channel and its β subunits. Very acutely, *KCNMA1* expression in rats is decreased by miR-9 in response to ethanol treatment, but induction of *KCNMA1* has been observed in mice in separate studies in specific brain regions 4 h after an injection of ethanol. Loss of different β subunits can alter drinking behavior in opposite directions. All of these observations highlight the

complex relationship between the alpha and beta subunits, the tissues where they are expressed, and the dynamic changes that can occur with ethanol treatment.

Together, these observations across different organisms and experimental paradigms all highlight the importance of BK channels in the behavioral response to ethanol. It may be that the invertebrate models may each feature a different aspect of the rich mammalian involvement of BK in ethanol behavioral responses.

One goal of studies of the molecular effects of ethanol on neuronal function is to ultimately be able to identify and intervene with individuals who are at high genetic risk of developing an alcohol use disorder before they begin to abuse the drug. BK channels, therefore, represent an excellent candidate for examination in studies of genes that impact the genetic liability to develop an abuse disorder. Several human studies have found suggestive evidence for allelic variation in BK channels being associated with alcohol dependence. Each of these signals individually failed to achieve genome wide significance, but this is perhaps not surprising, given the difficulties that have been encountered in identifying AD liability loci across studies. Indeed, the repeated finding of nominally significant signals in *KNCMA1* in many different studies lends support to each individual result. Together, these data strongly support a model in which allelic variation in the human BK channel-encoding gene is a risk factor for developing AD.

The genetic and molecular analyses of BK channels are a rich resource for suggesting additional candidate genes for association with AD in human population studies. If variation in BK channels themselves can modify liability, as is suggested by the repeated identification of *KCNMA1* in human studies, then it seems likely that variation in modifiers of BK function would also be potential liability loci. The well-established roles for the microRNA miR-9 and the BK β subunits in altering BK function, and, specifically, in modifying the ethanol responsiveness of BK, make them excellent targets for study. In addition, we are particularly intrigued by the genes that regulate the lipid milieu in which the BK channels reside.

An additional goal of these studies is to provide information for the rational development of pharmaceutical interventions for alcohol dependence. The recent identification of specific ethanol interaction domains in the BK channel in both worms and mammals (Bukiya et al., 2014; Davis et al., 2014), provides promising specific targets for the development of molecules that prevent the BK mediated effects of ethanol.

Finally, there remains much that we do not know about the roles of BK channels in responses to ethanol. Very recently, there has been an exciting report describing a newly identified function of BK channels in the nucleus of neuronal cells. Li et al. (2014) report that functional BK channels reside on the nuclear envelope of mammalian hippocampal neurons. Their finding that blockade of BK function can affect nuclear Ca^{2+} levels and modify activity dependent gene transcription opens an entirely new field of study of the role of BK channels in the effects of ethanol exposure on brain function.

ACKNOWLEDGMENT

Support for this work was provided by R01AA016837 (Jill C. Bettinger).

REFERENCES

Becker, H. C., and Lopez, M. F. (2004). Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice. *Alcohol. Clin. Exp. Res.* 28, 1829–1838. doi: 10.1097/01.ALC.0000149977.95306.3A

Behrens, R., Nolting, A., Reimann, F., Schwarz, M., Waldschutz, R., and Pongs, O. (2000). hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family. *FEBS Lett.* 474, 99–106. doi: 10.1016/S0014-5793(00)01584-2

Bettinger, J. C., Leung, K., Bolling, M. H., Goldsmith, A. D., and Davies, A. G. (2012). Lipid environment modulates the development of acute tolerance to ethanol in *Caenorhabditis elegans*. *PLoS ONE* 7:e35129. doi: 10.1371/journal.pone.0035192

Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y., and Aldrich, R. W. (2000). Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. *J. Biol. Chem.* 275, 6453–6461. doi: 10.1074/jbc.275.9.6453

Bukiya, A. N., Kuntamallappanavar, G., Edwards, J., Singh, A. K., Shivakumar, B., and Dopico, A. M. (2014). An alcohol-sensing site in the calcium- and voltage-gated, large conductance potassium (BK) channel. *Proc. Natl. Acad. Sci. U.S.A.* 111, 9313–9318. doi: 10.1073/pnas.1317363111

Bukiya, A. N., Liu, J., and Dopico, A. M. (2009). The BK channel accessory beta1 subunit determines alcohol-induced cerebrovascular constriction. *FEBS Lett.* 583, 2779–2784. doi: 10.1016/j.febslet.2009.07.019

Chu, B., Dopico, A. M., Lemos, J. R., and Treistman, S. N. (1998). Ethanol potentiation of calcium-activated potassium channels reconstituted into planar lipid bilayers. *Mol. Pharmacol.* 54, 397–406.

Cowmeadow, R. B., Krishnan, H. R., and Atkinson, N. S. (2005). The slowpoke gene is necessary for rapid ethanol tolerance in *Drosophila*. *Alcohol. Clin. Exp. Res.* 29, 1777–1786. doi: 10.1097/01.alc.0000183232.56788.62

Cowmeadow, R. B., Krishnan, H. R., Ghezzi, A., Al'Hasan, Y. M., Wang, Y. Z., and Atkinson, N. S. (2006). Ethanol tolerance caused by slowpoke induction in *Drosophila*. *Alcohol. Clin. Exp. Res.* 30, 745–753. doi: 10.1111/j.1530-0277.2006.00087.x

Crawley, J. N., Belknap, J. K., Collins, A., Crabbe, J. C., Frankel, W., Henderson, N., et al. (1997). Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. *Psychopharmacology* 132, 107–124. doi: 10.1007/s002130050327

Crowley, J. J., Treistman, S. N., and Dopico, A. M. (2003). Cholesterol antagonizes ethanol potentiation of human brain BK_{Ca} channels reconstituted into phospholipid bilayers. *Mol. Pharmacol.* 64, 365–372. doi: 10.1124/mol.64.2.365

Crowley, J. J., Treistman, S. N., and Dopico, A. M. (2005). Distinct structural features of phospholipids differentially determine ethanol sensitivity and basal function of BK channels. *Mol. Pharmacol.* 68, 4–10. doi: 10.1124/mol.105.012971

Davies, A. G., Bettinger, J. C., Thiele, T. R., Judy, M. E., and McIntire, S. L. (2004). Natural variation in the *npr-1* gene modifies ethanol responses of wild strains of *C.* elegans. *Neuron* 42, 731–743. doi: 10.1016/j.neuron.2004.05.004

Davies, A. G., Pierce-Shimomura, J. T., Kim, H., VanHoven, M. K., Thiele, T. R., Bonci, A., et al. (2003). A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. *Cell* 115, 655–666. doi: 10.1016/S0092-8674(03)00979-6

Davis, S. J., Scott, L. L., Hu, K., and Pierce-Shimomura, J. T. (2014). Conserved single residue in the BK potassium channel required for activation by alcohol and intoxication in *C.* elegans. *J. Neurosci.* 34, 9562–9573. doi: 10.1523/JNEUROSCI.0838-14.2014

Devineni, A. V., and Heberlein, U. (2013). The evolution of *Drosophila melanogaster* as a model for alcohol research. *Annu. Rev. Neurosci.* 36, 121–138. doi: 10.1146/annurev-neuro-062012-170256

Dillon, J., Andrianakis, I., Mould, R., Ient, B., Liu, W., James, C., et al. (2013). Distinct molecular targets including SLO-1 and gap junctions are engaged across a continuum of ethanol concentrations in *Caenorhabditis elegans*. *FASEB J.* 27, 4266–4278. doi: 10.1096/fj.11-189340

Dopico, A. M., Anantharam, V., and Treistman, S. N. (1998). Ethanol increases the activity of Ca^{++}-dependent K^{+} (mslo) channels: functional interaction with cytosolic Ca^{++}. *J. Pharmacol. Exp. Ther.* 284, 258–268.

Dopico, A. M., Lemos, J. R., and Treistman, S. N. (1996). Ethanol increases the activity of large conductance, Ca^{2+}-activated K^+ channels in isolated neurohypophysial terminals. *Mol. Pharmacol.* 49, 40–48.

Dopico, A. M., Widmer, H., Wang, G., Lemos, J. R., and Treistman, S. N. (1999). Rat supraoptic magnocellular neurones show distinct large conductance, Ca^{2+}-activated K^+ channel subtypes in cell bodies versus nerve endings. *J. Physiol.* 519(Pt. 1), 101–114. doi: 10.1111/j.1469-7793.1999.0101o.x

Edenberg, H. J., Koller, D. L., Xuei, X., Wetherill, L., McClintick, J. N., Almasy, L., et al. (2010). Genome-wide association study of alcohol dependence implicates a region on chromosome 11. *Alcohol. Clin. Exp. Res.* 34, 840–852. doi: 10.1111/j.1530-0277.2010.01156.x

Ghezzi, A., Al'Hasan, Y. M., Larios, L. E., Bohm, R. A., and Atkinson, N. S. (2004). slo K^+ channel gene regulation mediates rapid drug tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 101, 17276–17281. doi: 10.1073/pnas.0405584101

Ghezzi, A., Krishnan, H. R., and Atkinson, N. S. (2012). Susceptibility to ethanol withdrawal seizures is produced by BK channel gene expression. *Addict. Biol.* 19, 332–337. doi: 10.1111/j.1369-1600.2012.00465.x

Ghezzi, A., Pohl, J. B., Wang, Y., and Atkinson, N. S. (2010). BK channels play a counter-adaptive role in drug tolerance and dependence. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16360–16365. doi: 10.1073/pnas.1005439107

Giacomotto, J., and Ségalat, L. (2010). High-throughput screening and small animal models, where are we? *Br. J. Pharmacol.* 160, 204–216. doi: 10.1111/j.1476-5381.2010.00725.x

Gruss, M., Henrich, M., König, P., Hempelmann, G., Vogel, W., and Scholz, A. (2001). Ethanol reduces excitability in a subgroup of primary sensory neurons by activation of BK_{Ca} channels. *Eur. J. Neurosci.* 14, 1246–1256. doi: 10.1046/j.0953-816x.2001.01754.x

Han, S., Yang, B. Z., Kranzler, H. R., Liu, X., Zhao, H., Farrer, L. A., et al. (2013). Integrating GWASs and human protein interaction networks identifies a gene subnetwork underlying alcohol dependence. *Am. J. Hum. Genet.* 93, 1027–1034. doi: 10.1016/j.ajhg.2013.10.021

Jakab, M., Weiger, T. M., and Hermann, A. (1997). Ethanol activates maxi Ca^{2+}-activated K^+ channels of clonal pituitary (GH3) cells. *J. Membr. Biol.* 157, 237–245. doi: 10.1007/PL00005895

Jorgensen, E. M., and Mango, S. E. (2002). The art and design of genetic screens: *Caenorhabditis elegans*. *Nat. Rev. Genet.* 3, 356–369. doi: 10.1038/nrg794

Kendler, K. S., Chen, X., Dick, D., Maes, H., Gillespie, N., Neale, M. C., et al. (2012). Recent advances in the genetic epidemiology and molecular genetics of substance use disorders. *Nat. Neurosci.* 15, 181–189. doi: 10.1038/nn.3018

Kendler, K. S., Kalsi, G., Holmans, P. A., Sanders, A. R., Aggen, S. H., Dick, D. M., et al. (2011). Genomewide association analysis of symptoms of alcohol dependence in the molecular genetics of schizophrenia (MGS2) control sample. *Alcohol. Clin. Exp. Res.* 35, 963–975. doi: 10.1111/j.1530-0277.2010.01427.x

Kerns, R. T., Ravindranathan, A., Hassan, S., Cage, M. P., York, T., Sikela, J. M., et al. (2005). Ethanol-responsive brain region expression networks: implications for behavioral responses to acute ethanol in DBA/2J versus C57BL/6J mice. *J. Neurosci.* 25, 2255–2266. doi: 10.1523/JNEUROSCI.4372-04.2005

Kim, H., Pierce-Shimomura, J. T., Oh, H. J., Johnson, B. E., Goodman, M. B., and McIntire, S. L. (2009). The dystrophin complex controls BK channel localization and muscle activity in *Caenorhabditis elegans*. *PLoS Genet.* 5:e1000780. doi: 10.1371/journal.pgen.1000780

Kreifeldt, M., Le, D., Treistman, S. N., Koob, G. F., and Contet, C. (2013). BK channel beta1 and beta4 auxiliary subunits exert opposite influences on escalated ethanol drinking in dependent mice. *Front. Integr. Neurosci.* 7:105. doi: 10.3389/fnint.2013.00105

Li, B., Jie, W., Huang, L., Wei, P., Li, S., Luo, Z., et al. (2014). Nuclear BK channels regulate gene expression via the control of nuclear calcium signaling. *Nat. Neurosci.* 17, 1055–1063. doi: 10.1038/nn.3744

Martin, G. E., Hendrickson, L. M., Penta, K. L., Friesen, R. M., Pietrzykowski, A. Z., Tapper, A. R., et al. (2008). Identification of a BK channel auxiliary protein controlling molecular and behavioral tolerance to alcohol. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17543–17548. doi: 10.1073/pnas.0801068105

Martin, G., Puig, S. I., Pietrzykowski, A. Z., Zadek, P., Emery, P., and Treistman, S. N. (2004). Somatic localization of a specific large-conductance calcium-activated potassium channel subtype controls compartmentalized ethanol sensitivity in the nucleus accumbens. *J. Neurosci.* 24, 6563–6572. doi: 10.1523/JNEUROSCI.0684-04.2004

Peirce, J. L., Lu, L., Gu, J., Silver, L. M., and Williams, R. W. (2004). A new set of BXD recombinant inbred lines from advanced intercross populations in mice. *BMC Genet.* 5:7. doi: 10.1186/1471-2156-5-7

Pietrzykowski, A. Z., Friesen, R. M., Martin, G. E., Puig, S. I., Nowak, C. L., Wynne, P. M., et al. (2008). Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron* 59, 274–287. doi: 10.1016/j.neuron.2008.05.032

Pongs, O., and Schwarz, J. R. (2010). Ancillary subunits associated with voltage-dependent K^+ channels. *Physiol. Rev.* 90, 755–796. doi: 10.1152/physrev.00020.2009

Prescott, C. A., and Kendler, K. S. (1999). Genetic and environmental contributions to alcohol abuse and dependence in a population-based sample of male twins. *Am. J. Psychiatry* 156, 34–40.

Rodriguez, L. A., Wilson, J. R., and Nagoshi, C. T. (1993). Does psychomotor sensitivity to alcohol predict subsequent alcohol use? *Alcohol. Clin. Exp. Res.* 17, 155–161. doi: 10.1111/j.1530-0277.1993.tb00741.x

Rogawski, M. A. (2005). Update on the neurobiology of alcohol withdrawal seizures. *Epilepsy Curr.* 5, 225–230. doi: 10.1111/j.1535-7511.2005.00071.x

Rothenfluh, A., and Heberlein, U. (2002). Drugs, flies, and videotape: the effects of ethanol and cocaine on *Drosophila* locomotion. *Curr. Opin. Neurobiol.* 12, 639–645. doi: 10.1016/S0959-4388(02)00380-X

Schafer, W. R. (2004). Addiction research in a simple animal model: the nematode *Caenorhabditis elegans*. *Neuropharmacology* 47(Suppl. 1), 123–131. doi: 10.1016/j.neuropharm.2004.06.026

Schuckit, M. A., and Smith, T. L. (1996). An 8-year follow-up of 450 sons of alcoholic and control subjects. *Arch. Gen. Psychiatry* 53, 202–210. doi: 10.1001/archpsyc.1996.01830030020005

Schuckit, M. A., Wilhelmsen, K., Smith, T. L., Feiler, H. S., Lind, P., Lange, L. A., et al. (2005). Autosomal linkage analysis for the level of response to alcohol. *Alcohol. Clin. Exp. Res.* 29, 1976–1982. doi: 10.1097/01.alc.0000187598.82921.27

Tanouye, M. A., and Wyman, R. J. (1980). Motor outputs of giant nerve fiber in *Drosophila*. *J. Neurophysiol.* 44, 405–421.

Torres, Y. P., Morera, F. J., Carvacho, I., and Latorre, R. (2007). A marriage of convenience: beta-subunits and voltage-dependent K^+ channels. *J. Biol. Chem.* 282, 24485–24489. doi: 10.1074/jbc.R700022200

Walters, F. S., Covarrubias, M., and Ellingson, J. S. (2000). Potent inhibition of the aortic smooth muscle maxi-K channel by clinical doses of ethanol. *Am. J. Physiol. Heart Circ. Physiol.* 279, C1107–C1115.

Wang, Y., Ghezzi, A., Yin, J. C., and Atkinson, N. S. (2009). CREB regulation of BK channel gene expression underlies rapid drug tolerance. *Genes Brain Behav.* 8, 369–376. doi: 10.1111/j.1601-183X.2009.00479.x

Wang, Y., Krishnan, H. R., Ghezzi, A., Yin, J. C., and Atkinson, N. S. (2007). Drug-induced epigenetic changes produce drug tolerance. *PLoS Biol.* 5:e265. doi: 10.1371/journal.pbio.0050265

Wang, Z. W., Saifee, O., Nonet, M. L., and Salkoff, L. (2001). SLO-1 potassium channels control quantal content of neurotransmitter release at the *C. elegans* neuromuscular junction. *Neuron* 32, 867–881. doi: 10.1016/S0896-6273(01)00522-0

Weiger, T. M., Holmqvist, M. H., Levitan, I. B., Clark, F. T., Sprague, S., Huang, W. J., et al. (2000). A novel nervous system beta subunit that downregulates human large conductance calcium-dependent potassium channels. *J. Neurosci.* 20, 3563–3570.

Wolen, A. R., Phillips, C. A., Langston, M. A., Putman, A. H., Vorster, P. J., Bruce, N. A., et al. (2012). Genetic dissection of acute ethanol responsive gene networks in prefrontal cortex: functional and mechanistic implications. *PLoS ONE* 7:e33575. doi: 10.1371/journal.pone.0033575

Yuan, C., Chen, M., Covey, D. F., Johnston, L. J., and Treistman, S. N. (2011a). Cholesterol tuning of BK ethanol response is enantioselective, and is a function of accompanying lipids. *PLoS ONE* 6:e27572. doi: 10.1371/journal.pone.0027572

Yuan, C., O'Connell, R. J., Wilson, A., Pietrzykowski, A. Z., and Treistman, S. N. (2008). Acute alcohol tolerance is intrinsic to the BK_{Ca} protein, but is modulated by the lipid environment. *J. Biol. Chem.* 283, 5090–5098. doi: 10.1074/jbc.M708214200

Yuan, C., Treistman, S. N., Covey, D. F., Chen, M., and Johnston, L. J. (2011b). Cholesterol regulates the basal functions and ethanol sensitivity of large conductance, Ca^{2+}-sensitive K^+ channel through specific cholesterol-protein interaction. *Biophys. J.* 100:583. doi: 10.1016/j.bpj.2010.12.3369

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 July 2014; accepted: 22 August 2014; published online: 09 September 2014.
Citation: Bettinger JC and Davies AG (2014) The role of the BK channel in ethanol response behaviors: evidence from model organism and human studies. Front. Physiol. ***5****:346. doi: 10.3389/fphys.2014.00346*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 12 November 2014
doi: 10.3389/fphys.2014.00431

Phosphorylation of BK channels modulates the sensitivity to hydrogen sulfide (H_2S)

Guzel F. Sitdikova[1], Roman Fuchs[2], Verena Kainz[3], Thomas M. Weiger[3] and Anton Hermann[3]*

[1] Department of Physiology of Man and Animals, Kazan Federal University, Kazan, Russia
[2] Neurosignaling Unit, Department of Organismic Biology, University of Salzburg, Salzburg, Austria
[3] Division of Cellular and Molecular Neurobiology, Department of Cell Biology, University of Salzburg, Salzburg, Austria

Edited by:
Ali Mobasheri, University of Surrey, UK

Reviewed by:
Richard Barrett-Jolley, University of Liverpool, UK
Hideo Kimura, National Institute of Neuroscience, Japan
Atsufumi Kawabara, Kinki University, Japan

***Correspondence:**
Anton Hermann, Division of Cellular and Molecular Neurobiology, Department of Cell Biology, University of Salzburg, Hellbrunnerstr. 34, A-5020 Salzburg, Austria
e-mail: anton.hermann@sbg.ac.at

Introduction: Gases, such as nitric oxide (NO), carbon monoxide (CO), or hydrogen sulfide (H_2S), termed gasotransmitters, play an increasingly important role in understanding of how electrical signaling of cells is modulated. H_2S is well-known to act on various ion channels and receptors. In a previous study we reported that H_2S increased calcium-activated potassium (BK) channel activity.

Aims: The goal of the present study is to investigate the modulatory effect of BK channel phosphorylation on the action of H_2S on the channel as well as to recalculate and determine the H_2S concentrations in aqueous sodium hydrogen sulfide (NaHS) solutions.

Methods: Single channel recordings of GH3, GH4, and GH4 STREX cells were used to analyze channel open probability, amplitude, and open dwell times. H_2S was measured with an anion selective electrode.

Results: The concentration of H_2S produced from NaHS was recalculated taking pH, temperature salinity of the perfusate, and evaporation of H_2S into account. The results indicate that from a concentration of 300 μM NaHS, only 11–13%, i.e., 34–41 μM is effective as H_2S in solution. GH3, GH4, and GH4 STREX cells respond differently to phosphorylation. BK channel open probability (Po) of all cells lines used was increased by H_2S in ATP-containing solutions. PKA prevented the action of H_2S on channel Po in GH4 and GH4 STREX, but not in GH3 cells. H_2S, high significantly increased Po of all PKG pretreated cells. In the presence of PKC, which lowers channel activity, H_2S increased channel Po of GH4 and GH4 STREX, but not those of GH3 cells. H_2S increased open dwell times of GH3 cells in the absence of ATP significantly. A significant increase of dwell times with H_2S was also observed in the presence of okadaic acid.

Conclusions: Our results suggest that phosphorylation by PKG primes the channels for H_2S activation and indicate that channel phosphorylation plays an important role in the response to H_2S.

Keywords: gasotransmitters, hydrogen sulfide (H_2S), maxi calcium-activated potassium (BK) channels, patch clamp, phosphorylation, GH cells

INTRODUCTION

Hydrogen sulfide (H_2S), although it is extremely toxic in higher concentrations, similar to the other established gasotransmitters, nitric oxide (NO) and carbon monoxide (CO), serves as a cellular signaling molecule in low concentrations being involved in a vast variety of physiological actions (Goodwin et al., 1989; Abe and Kimura, 1996; Nagai et al., 2004; Kimura et al., 2010; Olson and Whitfield, 2010; Tang et al., 2013) reviewed in (Mustafa et al., 2009; Gadalla and Snyder, 2010; Wang, 2010, 2012, 2014; Hu et al., 2011; Hermann et al., 2012b; Kimura et al., 2012; Kabil et al., 2014) and pathophysiological incidences (i.e., Kimura and Kimura, 2004; Kimura et al., 2012; Mani et al., 2014). H_2S is endogenously produced in living cells from the amino acid cysteine through various enzymatic pathways (Łowicka and Bełtowski, 2007; Ishigami et al., 2009; Shibuya et al., 2009, 2013; Kimura, 2011; Renga, 2011). Although H_2S is well-known to modulate receptors and ion channels, such as NMDA receptors (Abe and Kimura, 1996), ATP (adenosine triphosphate)-dependent potassium (K^+) channels (Zhao and Wang, 2002; Jiang et al., 2010; Tang et al., 2010; Liang et al., 2011; Liu et al., 2011) or calcium (Ca^{2+}) channels (Kawabata et al., 2007; García-Bereguiaín et al., 2008; Sun et al., 2008; Maeda et al., 2009; Skovgaard et al., 2011; Matsunami et al., 2012), it was only recently shown to modulate maxi Ca^{2+}-activated K^+ (BK) channels (Telezhkin et al., 2009, 2010; Sitdikova et al., 2010; Jackson-Weaver et al., 2011; Zhao et al., 2013).

The physiological concentrations of H_2S concentrations are presently under discussion. Previous *in vivo* concentrations reported in the range of 40–160 μM H_2S (Goodwin et al., 1989; Savage and Gould, 1990) were possibly overestimated since H_2S

derived from various sources were included in the measurements (discussed in Kimura et al., 2012). Recent studies indicate tissue/plasma or blood H_2S levels of nanomolar (~15 nM in mouse brain/liver homogenates (Furne et al., 2008) to low micromolar (0.4–0.9 μM in rat blood (Wintner et al., 2010); cerebrospinal fluid, pig, ~600 nM (Leffler et al., 2011); ~32 μM in mouse blood (Peng et al., 2011); 34 μM in mouse plasma, (Li et al., 2005) which could rise to low micromolar quantities during pathophysiological conditions (i.e., during hypercapnia in cerebrospinal fluid, pig, 4–5 μM (Leffler et al., 2011). The actual H_2S concentrations at the target sites are unknown, however, since exogenous H_2S is highly volatile, rapidly removed, bound or metabolized (cf. Szabó, 2007; Whitfield et al., 2008; Wintner et al., 2010). These H_2S levels therefore may be of limited relevance to the effective H_2S concentrations at the target site(s). Nevertheless, action appeared required to carefully estimate the H_2S concentrations derived from the H_2S donor (sodium hydrogen sulfide, NaHS) used in our experiments.

BK channels are present in a great variety of non-excitable and excitable cells. Recent detailed studies of BK channels created a vast amount of knowledge regarding their biophysical, structural and functional, physiological, pathophysiological, and pharmacological properties (reviewed in: Ghatta et al., 2006; Salkoff et al., 2006; Cui et al., 2009; Berkefeld et al., 2010; Cui, 2010; Grimm and Sansom, 2010; Hill et al., 2010; Lee and Cui, 2010; Stojilkovic et al., 2010; Wu et al., 2010; Hermann et al., 2012a). BK channels are essential in controlling electrical activity of cells, hormone secretion, vasoregulation, auditory tuning of the hair cells or circadian rhythm generation and participate in mediating drug actions such as ethanol or acetaldehyde. Mutations of the BK channel protein are involved in disorders such as epilepsy, paroxysmal movements, or in erectile dysfunctions. BK channels are synergistically gated by both Ca^{2+} as well as by membrane voltage and are modulated by a wide variety of intra- and extracellular factors, including protein kinases, phosphatases causing phosphorylation/dephosphorylation or changes in their redox environment in particular at the C-terminal region (Reinhart et al., 1991; Levitan, 1994; Tian et al., 2001; Zhou et al., 2001, 2010; Weiger et al., 2002; Lu et al., 2006; Hou et al., 2009; for reviews see Wang, 2008; Dai et al., 2009; Hermann et al., 2012a). Protein kinase G (PKG) in many tissues acts as BK channel activator (Alioua et al., 1998; Kyle et al., 2013), protein kinase A (PKA) can act both ways—activating as well as inhibitory depending on the splice variant of the channel (Hall and Armstrong, 2000; Tian et al., 2001, 2004; Zhou et al., 2001), while protein kinase C (PKC) in many cases acts inhibitory (Shipston and Armstrong, 1996; Schubert and Nelson, 2001; Tian et al., 2004; Kizub et al., 2010; Zhou et al., 2010, 2012; van Welie and du Lac, 2011). PCK-dependent conditional phosphorylation of the channels may be important for PKA-dependent activation (Widmer et al., 2003) or specific isoforms of PKC (PKCδ) may up-regulate BK channel activity (Barman et al., 2004; Kim and Park, 2008). BK channels are also a target of gasotransmitters such as NO and CO which act via intracellular signaling or directly at the channels (reviewed in Hermann et al., 2012c). However, the interaction of BK channel phosphorylation and hydrogen sulfide has not been studied.

In previous experiments we found that H_2S caused a concentration dependent and reversible increase of membrane outward currents in whole cell experiments (Sitdikova et al., 2010). In single channel recordings H_2S reversibly increased BK channel open probability in a voltage-dependent, but Ca^{2+} independent manner. Redox modulation of the channels further indicated that the augmenting effect of H_2S on BK channel activity may be linked to its reducing action on sulfhydryl groups of the channel protein. The aim of the present study was to evaluate the effects of BK channel phosphorylation in the context of H_2S application employing patch clamp recordings at different types of rat GH pituitary tumor cells (GH3, GH4, and GH4 STREX). We used these three different but related cell lines, because they all express BK channels who differ in their sequences and respond differently to phosphorylation or in the responsiveness to arachidonic acid. The sequences of BK α-subunits in GH3 and GH4 splice variants differ most prominently by the presence or absence of 27 amino acids in the COOH terminus of the channel (Li et al., 2010). In contrast GH4 STREX cells which express a cysteine rich 59 amino-acid insert in the channel tail, contain an additional PKA phosphorylation site (Xie and McCobb, 1998; Tian et al., 2001).

Since BK channels variants of these cell lines respond differently to phosphorylation it appeared interesting to study the impact of H_2S on these channels types. From our results we hypothesize that the state of BK channel phosphorylation plays an important role in the response to H_2S.

MATERIALS AND METHODS

CELLS

We used GH3, GH4C1, and GH4 STREX, rat pituitary tumor cells, for investigation of BK channels. GH cells secrete growth hormone (somatotropin) and prolactin and express various receptors. Stressors applied to GH cells lead to the expression of a 59 amino acid cysteine rich insert at the pore forming α-subunit C-terminus of BK channels—termed GH STREX cells (Xie and McCobb, 1998; Erxleben et al., 2002). In our case the BK-STREX variant was induced by growing GH4/C1 cells in culture medium supplemented with phenol red (10 mg/ml) for at least 10 days. Once the cell line has been established cells can be kept permanently in this medium (Hall and Armstrong, 2000; Erxleben et al., 2002).

Techniques for cell culturing, electrophysiology, and standard solutions used have been described in detail previously (Sitdikova et al., 2010). In brief: cells were cultured at 37°C and 90% humidity in MEM (Minimal Essential Medium), supplemented with 7% fetal calf serum (FCS) and 3% horse serum (HS) for GH3 cells, and HAM F10 plus L-glutamine supplemented with 2.5% FCS and 15% HS for GH4 cells. For experiments cells were grown on poly-D-lysine coated coverslips and used for recordings 3 to 4 days after seeding. Culture media were from Sigma (Vienna, Austria), and sera from Invitrogen (Lofer, Austria), all other chemicals were from Sigma.

ELECTROPHYSIOLOGY

In brief: pipettes for single channel patch clamp recordings were drawn from borosilicate glass (Science Products/FRG) with

resistances of 3–6 MegaOhm (cf. Sitdikova et al., 2010). As reference electrode an agar bridge containing a silver/silver chloride (Ag/AgCl) pellet was used to avoid H_2S reaction with Ag to silver sulfide (Ag_2S) and hence destabilization of the reference electrode. Recordings from excised outside-out patches were made with an Axopatch-200B amplifier connected to a Digidata 1322A interface using pClamp10 software for data acquisition and analysis. Data were filtered at 1 kHz offline and analyzed with Clampfit software (Axon Instruments/Molecular Devices, USA). Raw recordings were idealized with the built in feature of pClamp's Clampfit module using the half-amplitude threshold method with automatic baseline and level tracking. Dwell time analysis was done by fitting open dwell time distributions to standard exponentials with the appropriate number of terms to get an optimal fit. Since BK channels are localized in clusters in cell membranes, all of our patches contained more than one channel. For this reason we analyzed only open dwell times but not closed channel kinetics. Single channel current amplitudes were calculated by fitting amplitude histograms to a Gaussian distribution. Channel open probability was expressed as $P_{open} = NPo/n$, where $NPo = [(to)/(to + tc)]$, Po = open probability for one channel, to = sum of open times, tc = sum of closed times, N = actual number of channels in the patch, and n = maximum number of individual channels observed in the patch. All equations used were standard built in equations from Clampfit. Experiments were repeated at least three times and the mean, as well as the s.e.m. (standard error of the mean) were calculated.

Experimental solutions were applied via a gravity-driven, electronically switched perfusion system (ALA Scientific Instruments, USA). For rapid solution exchange (about 300–500 ms) membrane patches were held in a stream of the experimental solution from a second pipette.

SOLUTIONS AND CHEMICALS

The standard experimental bath solution contained in mM: 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.2. The regular pipette solution contained in mM: 145 KCl, 1 $MgCl_2$, 10 HEPES, pH 7.2, and 5 EGTA, 3.63 $CaCl_2$ – resulting in 0.5 μM free Ca^{2+} as calculated using the Webmaxc extended calculator (http://www.stanford.edu/~cpatton/webmaxcE.htm). Experiments were carried out at room temperature between 20 and 22°C. Sodium hydrosulfide (NaHS, Sigma, Vienna) was used as a source of H_2S. NaHS at concentrations usually used in the present study did not change the pH of the buffered solution. NaHS solutions prepared shortly before experiments were clear and were usually used 3–5 min but no longer than 20 min. For details of making and working with H_2S, see Hughes et al. (2009).

Drugs added to the pipette solution: ATP – 1 mM; PKA catalytic subunit 50 units/ml; PKC subunit (PKCsu) 0.1 units/ml; PKC inhibitor fragment 19–31 (PKCin) – 500 nM; PKG – 400 units/ml plus 50 μM cGMP; Staurosporine (STS) – 1 μM; Okadaic Acid (OA) – 100 nM.

DETERMINATION OF H_2S CONCENTRATIONS

NaHS salt dissociates in watery solution to Na^+ and HS^-, and HS^- associates with H^+ to produce H_2S. Previously as a rule of thumb we calculated for neutral solutions that one-third of NaHS exists as H_2S and the remaining two-thirds are present as HS^- (Beauchamp et al., 1984). This provides a solution of H_2S at a concentration that is about 66% less compared to the original concentration of NaHS.

In order to obtain more precise measurements for our experimental situation we have determined H_2S concentrations using an anion selective electrode (ISO-H2S-2) together with an Apollo 1000 free radical Analyser (WPI, Germany). The response time of the sensor is less than 5 s, with a sensitivity of 2 pA/nM and a detection range from 5 nM to 100 μM H_2S. For calibration of the sensor instructions according to the WPI manual were used. After we had established a calibration curve we determined the rate of loss of H_2S versus time for the NaHS donor. As depicted from **Figure 1B** the loss of H_2S obtained after 20 min is 42%. A loss of about 33% after 15 min and 90% after 30 min was reported previously by Kimura et al. (2006).

The H_2S sensor measures the dissolved H_2S gas which is only one component of the total sulfide equilibrium system. The total sulfide concentration $[S_{tot}]$ in solution can be calculated from: $[H_2S] = [S_{tot}]/\{1+K_1/[H^+] + K_1K_2/[H^+]\}$, with $pK_1 = 6.89$, where $K_1 = 10^{-6.89}$ and $pK_2 = 19$, where $K_2 = 10^{-19}$ at pH = 7.21, where $[H^+] = 10^{-7.21}$ M. As K_2 is very small and not significant at pH < 9 we can use a simplified equation: $[H_2S] = [S_{tot}]/(1+K_1/[H^+])$. For NaHS = 300 μM and $pK_1 = 6.89$, the H_2S concentration calculates to 97 μM, which is quite similar to the rule of thumb of 1/3 H_2S in solution (see above). Calculated from $pK_1 = 7.04$ for standard conditions (20°C, which is close to our experimental conditions) $K_1 = 10^{-7.04}$ (Lide, 1998).Using the pK1 value of 7.04 and 300 μM of NaHS we calculate 121 μM of H_2S. However, pK_1 is also dependent on salinity. The equation derived by Millero (Millero et al., 1988) gives for: $pK_1 = -98.08 + 5765.4/T + 15.04555 \times LN(T) + (-0.157 \times (S^{0.5})) + 0.0135 \times S$ (with T = temperature in Kelvin, LN(T) = is the natural logarithm of T, and S = salinity). Taken salinity as zero for water and temperature 20°C equal to 293.15 Kelvin we calculate $pK_1 = 7.056$, which is very close to literature value (Lide, 1998). The salinity of our extracellular solution is 11.5 gram of salts per liter.

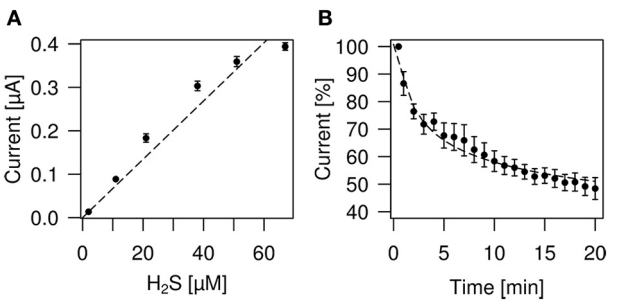


FIGURE 1 | Determination of H_2S concentrations and loss of H_2S by evaporation. (A) Plot of the H_2S concentrations derived from NaHS donor vs. H_2S sensor current (nA). The relationship is linear up to about 50 μM H_2S, respectively 226 μM NaHS (stippled line fitted to data points). **(B)** Measurement of H_2S evaporation from physiological bath solution. The plot shows sensor current readings vs. time. One hundred percent indicates the beginning of the measurement which started immediately after preparation of the solution.

Taken this salinity value $pK_1 = 6.679$. For 300 μM of NaHS the H_2S concentration calculates to 68 μM, which is 22.3% of the total sulfide concentration. Hence it is important in addition to pH and temperature to take salinity into account to calculate H_2S concentrations.

CALIBRATION

H_2S liberated from NaHS evaporates quickly from the solution (DeLeon et al., 2012; cf. Olson, 2012). We therefore took measures to avoid loss of H_2S during calibration or perfusion. A stock solution of 2 mM NaHS was prepared in distilled water or in experimental bath solution in an Eppendorf vial and thoroughly covered using parafilm. 100, 500, 1000, 2000, or 3000 μl of stock solution was added to 20 ml bath solution to obtain the desired NaHS concentration. **Figure 1A** shows a plot of H_2S concentrations derived from NaHS donor vs. H_2S sensor current (nA). The relationship is linear up to about 50 μM H_2S, respectively 226 μM NaHS. **Table 1** shows values of NaHS solutions, the sensor current and the calculated H_2S concentrations. During dilution from the stock to the final solution which took about 30–60 s we may have lost about 5–10% H_2S by evaporation (see **Figure 1B**). The container, holding the NaHS solution had a volume of 20 ml and was covered with parafilm. The fluid was continuously stirred as required for the correct function of the H_2S sensor using the lowest stirring speed to avoid mechanical perturbation of the sensor.

From our measurements of H_2S concentrations we know that during the first 5 min about 30–40% of H_2S evaporates from the solution (**Figure 1**). Over the following 5 min, which was the actual application period, about 6–8% H_2S evaporated. This gives a total of approximately 40–50% loss of H_2S, i.e., an effective H_2S concentration during our experimental settings of 11–13%, i.e., 34–41 μM H_2S from a 300 μM NaHS solution.

PERFUSION SETTINGS DURING RECORDINGS

We used a closed perfusion system comprising 5 ml syringes (Henke-Sass, Wolf, HSW, FRG) containing the experimental fluid. The syringe opening was covered with parafilm with a small whole inserted to allow for pressure exchange. The tip of the perfusion pipette was located in the recording chamber at about 1 mm distance to the measured cell or patch. The constant perfusion rate of 1 ml/80 s ensured that the channels recorded were always submerged by the desired H_2S concentration with little further loss due to evaporation.

Table 1 | List of NaHS solutions prepared in standard bath solution (first row), the measured sensor current ± standard error of the mean (s.e.m.) (middle row) and the calculated amount of H_2S (last row).

NaHS, μM	Sensor current, nA (±s.e.m.)	H_2S, μM
10	13.67 ± 0.33	2
48.7	88.67 ± 2.4	11
95.23	183.33 ± 9.61	21
170.2	303.33 ± 11.02	38
226	359.33 ± 11.66	51
300	394.00 ± 8.62	67

STATISTICAL METHODS

There are some problematic features of proportional data, like open probabilities (Po), which often hinder the use of parametric statistical procedures, such as ANOVAs and regression analysis. Basic assumptions of these methods are often violated, because all values coming from single channel recordings are set within the interval $0 < Po < 1$. To be more precise, proportional data tend to be non-normally distributed and heteroscedastic in nature, which actually means that the size of sample variances is not evenly distributed along the interval of $0 < Po < 1$.

Besides using less powerful non-parametric methods, there are other strategies to overcome these limitations, e.g., data transformation (Sokal and Rohlf, 1995) and/or logistic regression. Here, Po-values were transformed with the "logit-function" $p = \ln(p/(1 - p))$, which "normalized" this kind of data and substantially reduced heteroscedasticity. After this transformation, "usual" parametric methods could be used. Due to similar reasons, dwell-time measurements were also log-transformed prior to statistical analysis.

Since our experiments also include repeated measurements on similar membrane patches, all statistical procedures had to be adapted accordingly. The number of repetitions of each experiment "n" refers to the number of patches from different cells measured. In electrophysiology, this problem is usually addressed by calculating "fold-changes" of variables, i.e., dividing all measurements of open probabilities with their matched "control values." Nevertheless, there are also more powerful statistical procedures to handle the problem of self-correlated or repeated measurements, one of them is the use of "mixed models" (Venables and Ripley, 2002; Bolker et al., 2009; Zuur et al., 2009). With this method, not only "fixed factors," such as experimental conditions, are used but also so called "random factors," which usually comprise "uncontrolled" variables (Venables and Ripley, 2002; Zuur et al., 2009), such as test subjects, or in this particular case, membrane spots. Since more familiar "*post-hoc* analysis," such as a "TukeyHSD" or "Scheffe"-test are only feasible in simple univariate ANOVA designs (Sokal and Rohlf, 1995), specific hypothesis tests by linear contrasts had to be used for multiple comparisons (Hothorn et al., 2008). All statistical procedures were performed with R 3.0.1 (R Development Core Team, 2011) and its additional packages "nlme" (Pinheiro et al., 2013) and "multcomp" (Hothorn et al., 2008).

Evaluation of statistic analysis is given as: significant (one star *, $0.05 > p > 0.01$), high significant (two stars **, $0.01 > p > 0.001$), and most significant (three stars ***, $p < 0.001$). Data described in the text as percentage were calculated by setting control values to 100% and expressing experimental values as percentual change of controls.

RESULTS

Original recording of single channel activities are depicted in **Figure 2**. Patches were held at +30 mV in all recordings.

PHOSPHORYLATION OF BK CHANNELS

GH3 Cells

Experimental values of BK single channel open probability (Po) for GH3, GH4, and GH4 STREX cells are listed in **Table 2**.

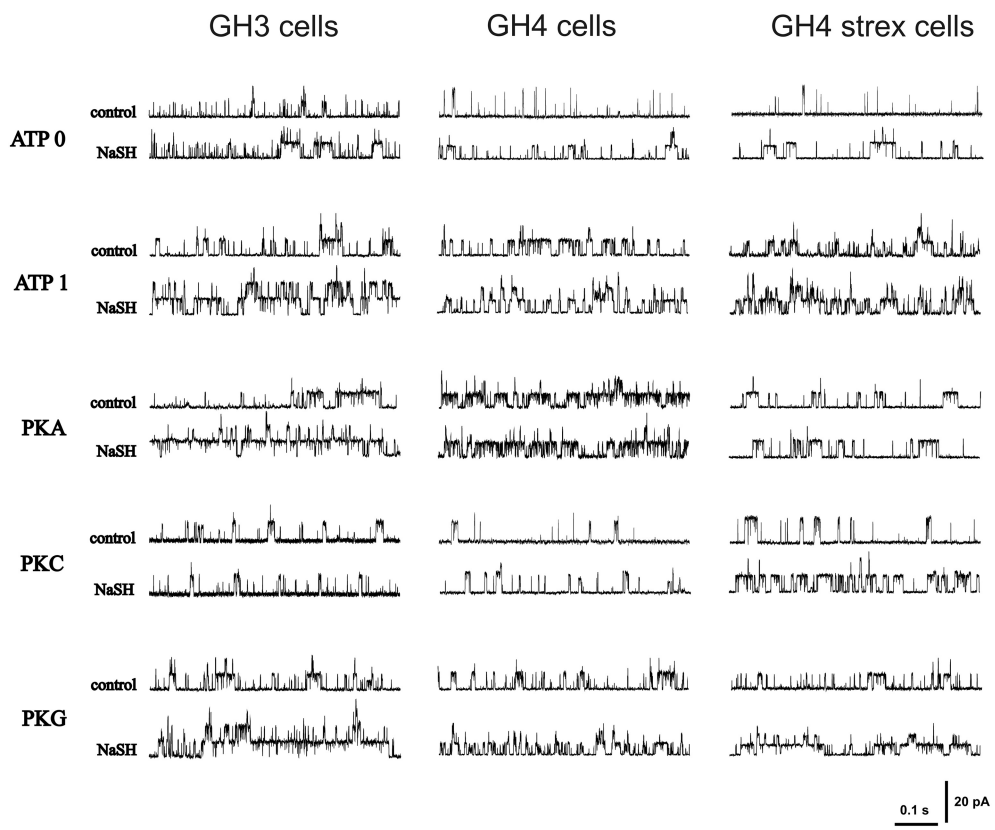


FIGURE 2 | Original recordings of H_2S effects on BK single channel open probability (P_{open}) during different phosphorylation conditions. The figure shows recordings from various GH cell types (GH3, GH4, and GH4 STREX) during various phosphorylation conditions (control) and after NaHS (300 μM NaHS, equivalent to effective 34–41 μM H_2S) application. Holding potential +30 mV.

Table 2 | Phosphorylation of BK channels.

	P_{open}		
	GH3	**GH4**	**GH4 STREX**
ATP 0 $n = 12$	0.0152 ± 0.0030	0.0096 ± 0.0028	0.0065 ± 0.0022
ATP 1 $n = 12$	0.0887 ± 0.0135	0.1164 ± 0.0196	0.0342 ± 0.0093
PKA subunit $n = 11$	0.1087 ± 0.0166	0.1256 ± 0.0222	0.0822 ± 0.0321
PKG subunit $n = 8$	0.0724 ± 0.0118	0.0522 ± 0.0173	0.0504 ± 0.0133
PKC subunit $n = 10$	0.0443 ± 0.0075	0.0277 ± 0.0064	0.0253 ± 0.0060
PKC inhib $n = 6$	0.0740 ± 0.0180	0.0208 ± 0.0055	0.0400 ± 0.0087
Staurosporine $n = 6$	0.0468 ± 0.0149	0.0505 ± 0.0147	0.0375 ± 0.0097
Okadaic acid $n = 7$	0.3075 ± 0.0581	0.4422 ± 0.0403	0.3992 ± 0.0962

Experimental values of BK single channel open probability (P_{open}) for GH3, GH4, and GH4 STREX cells. ATP0 (no ATP added to the patch pipette); ATP (1 mM added to the patch pipette; all further solutions contained 1 mM ATP during application of PKA, protein kinase A; PKG, protein kinase G; PKC, protein kinase C; STS, staurosporine; OA, okadaic acid; Po-values are presented as mean ± standard error of mean, and n is the number of patches/cells.

Po-values are presented as mean ± standard error of mean. ATP was added to the pipette solution (intracellular membrane face). In a physiological pipette solution containing no ATP (assigned ATP0), BK single channel open probability (Po) was generally low. Addition of 1 mM ATP (ATP1) to the pipette solution most significantly increased Po (***) (**Table 2** and **Figure 3A**). Similar findings have been reported previously from GH3 cells (Denson et al., 2001; Zhou et al., 2012), and reviewed for other cell types (Schubert and Nelson, 2001). All further solutions contained 1 mM ATP. PKA catalytic subunit (50 units/ml) altered Po, but both values were non-significant compared to ATP1 (**Figure 3A**), however, they were both significantly (***) different

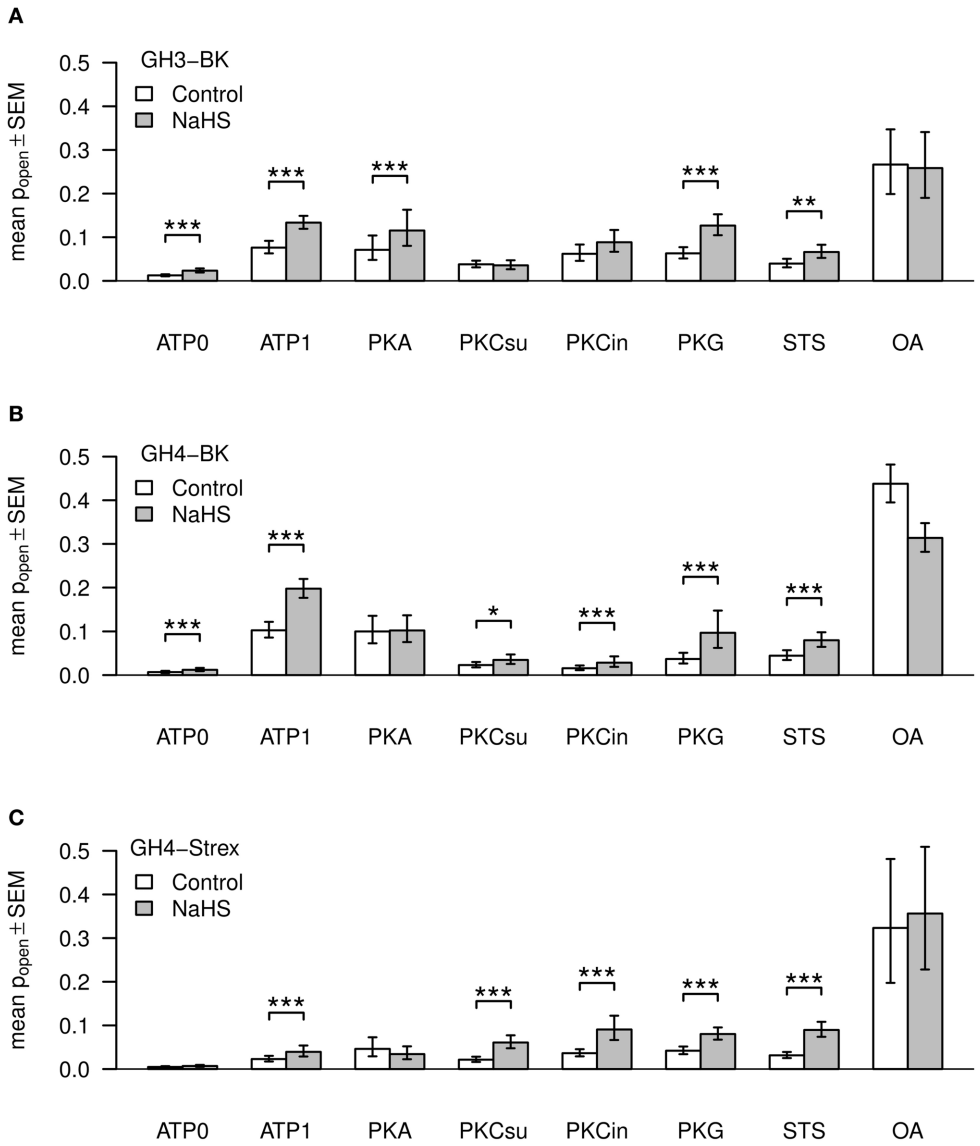


FIGURE 3 | Mean values of BK channel open time probabilities (P_{open}). Three types of channels, GH3-BK **(A)**, GH4-BK **(B)**, and GH4-STREX **(C)** cells, were investigated. Bar graphs show Po-values under control conditions (left bars, white) and after extracellular application of NaHS (right bars, gray). ATP, kinase, kinase inhibitor, kinase inhibitor staurosporine (STS), or phosphatase blocker okadaic acid (OA) were added to the pipette solution; ATP0 – no ATP added; ATP1 – 1 mM ATP added; PKA (50 units); PKCsu (0.1 units/ml); PKCin – 500 nM; STS – 1 μM; OA – 100 nM. Figures are based on back transformed means and s.e.m. values (error bars), respectively. Asteriks indicate significance of H_2S (300 μM NaHS equivalent to effective 34–41 μM H_2S) effects according to the logit transformed data.

to ATP0. PKC (PKC catalytic subunit, PKCsu, 0.1 units/ml) in the pipette solution decreased BK Po non-significantly compared to ATP1, but Po was significantly different from ATP0 (*). PKC is known to cause inhibition of BK channel activity from GH cells (Shipston and Armstrong, 1996; Hall and Armstrong, 2000; Wu et al., 2007; Zhou et al., 2012, 2010). With protein kinase inhibitor pseudo-substrate (PKCin, fragment 19–31, 500 nM) Po increased non-significantly if compared to PKCsu or ATP1, but was significantly higher than during ATP0 conditions (**) if compared to ATP1. The experiments support previous results of an inhibitory role of PKC at BK channels from GH3 cells (Shipston and Armstrong, 1996). Staurosporin (1 μM), an ATP-competitive kinase inhibitor, non-significantly reduced Po, whereas okadaic acid (100 nM), an inhibitor of serine/threonine phosphatase, significantly increased Po compared to 1 mM ATP.

GH4/C1 cells

All drug concentrations used for these cells were similar to those used with GH3 cells. With ATP1 Po increased high significantly compared to ATP0 (***) (**Table 2** and **Figure 3B**). Using PKA subunit or PKG showed no significant difference to ATP1 containing solution. PKC-subunit reduced Po about 4-fold (**) with high

significance compared to ATP1, as previously reported by Zhou et al. (2012). Use of PKC inhibitor pseudo-substrate had a similar effect. With the kinase inhibitor staurosporin Po was statistically not significantly different to ATP1 (**Table 2**, **Figure 3B**). With the phosphatase inhibitor okadaic acid, however, Po significantly increased if compared to ATP1, PKA, PKCin, PKCsu, staurosporine, or ATP0.

GH4/C1 STREX cells

These cells contain a 59 amino-acid insert, so called STREX (stress axis regulated) exon at the BK channel α-subunit C terminus, located between the two regulatory domains of K^+ conductance (RCK1 and RCK2). This insert bears various cysteine residues which can be phosphorylated. The STREX insert speeds activation and slows deactivation kinetics and their half-activation voltage is shifted to the left (Xie and McCobb, 1998). Again, as in GH3 or GH4/C1 cells Po-values in ATP0 were low (**Table 2**, **Figure 3C**), in fact they were the lowest of all three cell types. Po increased with ATP1 with low significance (*). The increase caused by ATP was, however, less compared to BK channels from GH3 or GH4 cells (**Table 2**, **Figure 3C**). PKA is known to phosphorylate the channels at the STREX insert and changes channel activity from stimulatory to inhibitory whereas channels that lack the STREX insert are activated by PKA (Tian et al., 2001). PKA increased Po significantly when compared with ATP0, but this increase was not significant when compared to ATP1. With PKG Po was not significant different to ATP1. Using PKC-subunit, PKC inhibitor, or staurosporine, again, no significant alteration of Po compared to ATP1 was observed. However, okadaic acid significantly increased Po (***) when compared to all other conditions, implying that inhibition of the BK channel attached phosphatase allows for unrestricted phosphorylation of the channel protein.

H_2S MODULATES BK CHANNEL ACTIVITY DEPENDENT ON PHOSPHORYLATION

We again present data separately for all three cell types used in our study. Original recordings of H_2S effects are shown in **Figure 2**.

GH3 cells

As reported previously (Sitdikova et al., 2010) and shown in **Figure 3A**, NaHS (300 μM – effective H_2S concentration 34–41 μM, see Materials and Methods), applied to the extracellular bath solution most significantly increased Po of BK channels from 0.01522 ± 0.00302 to 0.0295 ± 0.00722 ($n = 12$, ***) (internal ATP free pipette solution (ATP0), $V = +30$ mV), equal to 190.58 ± 13.81% of control (=100%) during the first minute of H_2S perfusion. Po decayed after 1 min H_2S application during the following 3 min. A similar transient response was observed previously using ethanol (Jakab et al., 1997) and H_2S (Sitdikova et al., 2010). In ATP1 solution Po further increased from 0.08872 ± 0.01352 control most significantly to 0.14082 ± 0.01439, $n = 12$ (***) with NaHS, i.e., to 191.65 ± 23.52% of control (=100%) (**Figure 3A**). All further experimental solutions contained 1 mM ATP. Using PKA subunit (50 units/ml), NaHS increased Po with high significance from 0.10873 ± 0.01664 (control) to 0.15475 ± 0.02218, $n = 11$ (***), i.e., to 169.42 ± 19.99% of control. PKG (50 units/ml) also increased Po from 0.0724 ± 0.0118 (control) to 0.1395 ± 0.0236($n = 8$) in NaHS by 205.56 ± 19.08% (***). PKC catalytic subunit (PKCsu, 0.1 units/ml) decreased BK channel activity to 0.04434 ± 0.00752 and NaHS had no significant effect (Po = 0.04609 ± 0.00869, $n = 10$, equal to 104.92 ± 16.09%). After addition of PKC inhibitor pseudo-substrate (PKCin, fragment 19–31, 500 nM), Po was 0.07408 ± 0.01808 (control), but NaHS again had no significant effect (Po 0.10292 ± 0.02299, $n = 6$, i.e., to 145.45 ± 14.082%). During application of the kinase inhibitor staurosporine (STS, 1 μM) Po was decreased to 0.04683 ± 0.01493, and in this case NaHS significantly increased Po to 0.07483 ± 0.01892 ($n = 6$, **), i.e., to 168.56 ± 11.87% of control. The most significant increase of Po compared to either ATP0 or ATP1 was obtained with the phosphatase inhibitor okadaic acid (100 nM) which increased Po to 0.3075 ± 0.0581, however, Po was not further significantly altered by NaHS (0.3039 ± 0.05523, $n = 8$, 102.10 ± 13.86%).

GH4/C1 cells

All basic conditions and concentrations of drugs were similar to the experiments using GH3 cells. In ATP0, Po increased highly significant from 0.00968 ± 0.00285 (control) to 0.01632 ± 0.00452, $n = 8$ (***), i.e., to 183.48 ± 22.12% (***) after application of NaHS. With ATP1 solution, Po increased significant from 0.11647 ± 0.01969 (control) to 0.20591 ± 0.02146, in NaHS, $n = 11$ (***), i.e., to 201.36 ± 19.12%. After application of PKA, Po was 0.12569 ± 0.02224 (control), but with NaHS no significant alteration of Po 0.12693 ± 0.02414, $n = 11$, i.e., to 107.33 ± 11.86% was observed. With PKG Po increased highly significant from 0.05229 ± 0.01732 (control) to 0.1430 ± 0.04916 ($n = 7$) in NaHS, i.e., by 258.95 ± 33.78% (***). Application of PKC subunit (PKCsu, 0.1 units/ml) decreased Po to 0.02771 ± 0.00647 and NaHS increased Po significantly to 0.0465 ± 0.01669, i.e., to 161 ± 28%, $n = 7$ (*). A similar Po of 0.02089 ± 0.00553 was obtained with PKC inhibitor (PKCin, 500 nM), and Po was high significantly increased with NaHS to 0.04093 ± 0.0108, $n = 7$ (***), i.e., to 186.78 ± 19.85%. With staurosporine (STS, 1 μM) Po was 0.0505 ± 0.01474 and increased with NaHS highly significant to 0.087 ± 0.02195, $n = 5$ (***), i.e., to 181.66 ± 15.35%. Okadaic acid (100 nM) substantially increased Po to 0.44223 ± 0.0403 compared to either ATP0 or ATP1 controls, whereas NaHS decreased Po under this condition to 0.31953 ± 0.03142, $n = 7$ (***), i.e., to 74.841 ± 7.8%.

GH4/C1 STREX cells

In ATP0 BK channel Po of 0.00652 ± 0.00224 (control) was low compared to Po of GH3 and GH4 cells. NaHS non-significantly increased Po to 0.00921 ± 0.00286, $n = 7$, i.e., to 148 ± 11.99%. With ATP1 Po increased to 0.03428 ± 0.00931, $n = 13$ compared to ATP0, but this increase was much less compared to Po-values obtained in GH3 and GH4 cells (**Figure 3**). NaHS in ATP1 increased Po at high significance to 0.06233 ± 0.01687, $n = 13$ (***), i.e., to 177.74 ± 15.91%. PKA subunit (50 units/ml) increased Po to 0.08221 ± 0.03214, $n = 8$ under control conditions, however, NaHS application had no significant effect on Po, 0.05739 ± 0.02129, $n = 8$, i.e., 78.65 ± 9.38% of control. PKG high-significantly increased Po from 0.05043 ± 0.01330

(control) to 0.08829 ± 0.01635 in NaHS, by 201.94 ± 40.37%, $n = 8$ (***).

As with previous results from GH3 and GH4 cells, using PKC subunit (PKCsu, 0.1 units/ml) BK channel Po was low, 0.02533 ± 0.00604, $n = 6$ (control), however, NaHS increased Po to 0.06833 ± 0.01267, i.e., to 322.44 ± 74.48%, although with low significance (*). With PKC inhibitor (PKCin, 500 nM) Po of 0.0400 ± 0.00879, $n = 5$ was similar to ATP1, and increased with NaHS to 0.10552 ± 0.02854, $n = 5$, i.e., to 253.07 ± 28.82%, however not significantly. Also staurosporine (STS, 1 μM) non-significantly increased Po from 0.0375 ± 0.00975 (control) to 0.10114 ± 0.02397 in NaHS, $n = 8$, i.e., to 306.14 ± 49.76%. In okadaic acid (100 nM) which significantly increased Po to 0.39927 ± 0.09621 compared to ATP1, NaHS had no additional significant effect on Po, 0.42796 ± 0.08584, $n = 7$, i.e., to 114.93 ± 9.22%.

In summary, our result show (**Table 3**) that Po of BK channels from GH3 and GH4 containing no ATP (ATP0) were high significantly increased in the presence of H_2S, whereas BK channels from GH4-STREX cell did not respond to H_2S. Po of BK channels of all cell types phosphorylated by ATP was highly significantly increased and H_2S further increased Po at high significance. PKA and PKG were not able to increase Po beyond the ATP1 effect. H_2S was able to increase Po of BK channels from GH3, but not those of GH4 and GH4 STREX cells. H_2S high significantly increased Po of all cell types pretreated with PKG. PKC-subunit (PKCsu) either had no effect (GH4-STREX) or decreased Po (GH3, GH4) but H_2S increased Po of BK channels pretreated with PKCsu in GH4 and GH4 STREX but not those of GH3 cells. Staurosporin had no effect on BK channel Po activity but did not prevent the activating effect of H_2S. Okadaic acid most significantly increased BK channel Po of all cell types and H_2S had no further effect on channel Po, it even decreased BK channel Po of GH4 cells.

Table 3 | Summary, of open probability (Po) of BK channels in the absence (ATP0, first line) and presence of ATP1 (1 mM, all other lines), during application of PKA (protein kinase A), PKG (protein kinase G), PKC (protein kinase C), STS (staurosporine), OA (okadaic acid), and after application of H_2S.

GH3	Po	GH4	Po	GH4 Strex	Po
ATP0		ATP0		ATP0	
+H_2S	+	+H_2S	+	+H_2S	0
ATP1	+	ATP 1	+	ATP1	+
+H_2S	+	+H_2S	+	+H_2S	+
PKA	0	PKA	0	P KA	0
+H_2S	+	+H_2S	0	+H_2S	0
PKG	0	PKG	0	PKG	0
+H_2S	+	+H_2S	+	+H_2S	+
PKCsu	−	PKCsu	−	PKCsu	0
+H_2S	0	+H_2S	+	+H_2S	+
STS	0	STS	0	STS	0
+H_2S	+	+H_2S	+	+H_2S	+
OA	+	OA	+	OA	+
+H_2S	0	+H_2S	−	+H_2S	0

All experimental values were compared to ATP1, except ATP0.

+, Po increase; 0, no effect; −, decrease of Po.

CHANNEL OPEN DWELL TIMES AND AMPLITUDES

Values of BK channel Po, amplitude and dwell time are shown in **Figures 4**, **5**. Channel open dwell-time indicates the time a channel spends at the open current level. Analysis of BK channels from GH3 cells revealed that ATP most significantly (***) increased mean open dwell times from 0.74 ± 0.087 ms (ATP0) to 2.022 ± 0.18 ms (ATP1). Open dwell times were also significantly increased with PKA to 1.78 ± 0.22 ms (***), with PKG to 1.56 ± 0.15 ms (**) and with PKC subunit to 1.78 ± 0.6 ms (*), compared to an ATP0 solution. A significant increase of mean dwell times was also observed with BK channels from GH4 cells from 0.77 ± 0.10 ms (ATP0) to 1.49 ± 0.11 ms (**) after application of ATP1. With PKA dwell times increased to 3.00 ± 0.44 ms (***) and with PKG to 1.54 ± 0.12 ms (**) if compared to ATP1 (**Figure 4B**). Significant decreases of dwell times were seen with paired comparison for PKCsu vs. PKA (***), PKCin vs. PKA (***), staurosporine vs. PKA (***) and okadaic acid vs. PKA (***) and PKG vs. PKA (*). NaHS had a significant increasing effect on open dwell times only in okadaic acid (***). In GH4 STREX cells open dwell times were only significantly increased when PKG is compared with ATP0 (*).

Addition of NaHS to GH3 increased BK channel dwell times significantly only in ATP0 from 0.74 ± 0.087 ms to 1.02 ± 0.11 ms (**), with GH4 cells in okadaic acid from 0.10 ± 26 ms to 1.73 ± 0.46 ms (***), and in ATP0 in GH4 STREX cells from 0.69 ± 0.10 ms to 0.99 ± 0.16 ms (*). NaHS had no effect at dwell times with all other experimental settings (**Figure 4C**).

Mean amplitudes of single BK channel currents from GH3 cells were not altered under ATP0 or any of the phosphorylation procedures. Only with PKG and H_2S channel amplitudes were significantly increased (**Figure 5A**). In GH4 cells channel amplitudes significantly increased in ATP1 vs. ATP0. In all other settings the values could not be differentiated to ATP1 (which was always present in these experiments). H_2S significantly increased amplitudes only with ATP1 (**Figure 5B**). Also in GH4 STREX cells BK channels amplitudes were increased at low significance with okadaic acid vs. ATP0 and staurosporin. H_2S increased channel amplitudes with ATP1 and PKCsu (**Figure 5C**).

Channel amplitudes and open dwell time histograms are listed as Supplementary Material.

DISCUSSION

RECALCULATION OF H_2S CONCENTRATIONS

Experimentally used H_2S concentrations are of major concern in the context of physiological quantities. In the present study we recalculated the concentration of H_2S liberated from the donor NaHS. In our calculations and estimations we were taking in addition to temperature and pH the salinity of the solution and evaporation of H_2S into account. The results indicate that a concentration of 300 μM NaHS, which was usually used in our experiments (Sitdikova et al., 2010) contains approximately

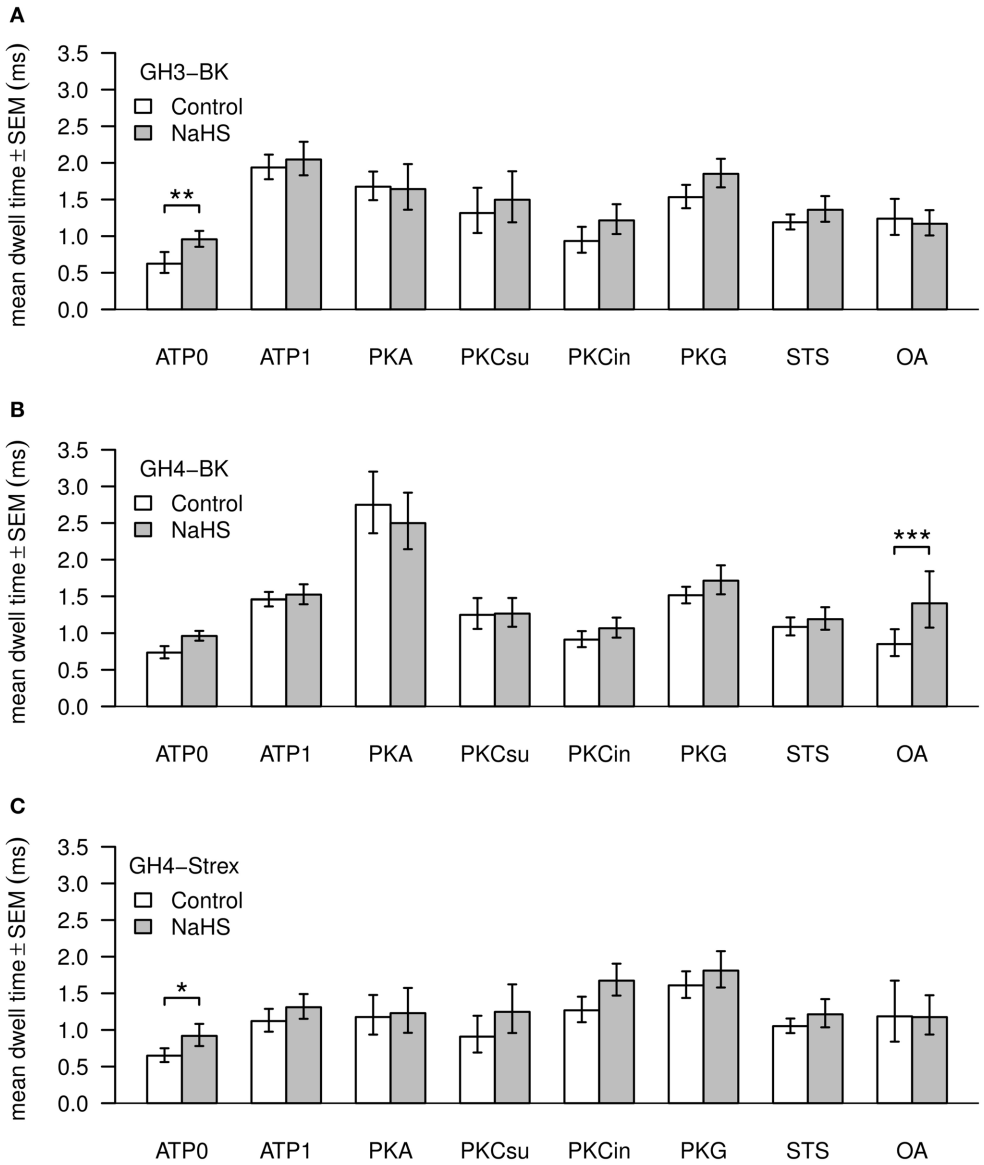


FIGURE 4 | Mean values of BK channel open dwell times. Three types of channels—GH3-BK **(A)**, GH4-BK **(B)**, and GH4-STREX **(C)** cells were investigated. Bar graphs show open dwell values in ms under control conditions (left bars, white) and after extracellular application of 300 μM NaHS (right bars, gray). All drug concentrations were the same as shown in **Figure 3**. Significant $*0.05 > p > 0.01$; high significant $**0.01 > p > 0.001$; most significant $***p < 0.001$.

68 μM H_2S, i.e., 22.3% of the NaHS concentration is as H_2S in solution. Furthermore, taking evaporation of H_2S into account we estimated a loss of approximately 40–50% of H_2S which gives an *effective* H_2S concentration during our experimental settings of 11–13%, i.e., 34–41 μM H_2S from a 300 μM NaHS solution.

Our previous estimations of the effective NaHS concentrations (EC50) at BK channel Po (Sitdikova et al., 2010) gave a low range value of 169 μM and a high range value of 2000 μM. With the newly calculated values we obtain for the low range NaHS value an EC50 of 18.8–22.6μM, for the high range EC50 223–267.6 μM of actual H_2S in solution. Early estimations of the amount of H_2S concentrations in the rat, bovine, and human brain gave values of 50–160 μM (Goodwin et al., 1989; Warenycia et al., 1989; Savage and Gould, 1990). These values appear overestimated since with the method used in these studies H_2S from acid-labile sulfur was measured in addition to free H_2S (Kimura et al., 2012). Some recent experimental H_2S estimations suggest extremely low values of basal submicromolar concentrations increasing to single-digit micromolar levels during application of cysteine or hypercapnia (Leffler et al., 2011). In our studies we usually used a standard concentration of 300 μM NaHS (34–41 μM effective H_2S) to (1) obtain reliable, well visible effects at the channels, and (2) being able to compare our results with previous investigations. Previous studies (Sitdikova et al., 2010) indicated that the lowest H_2S concentration that significantly ($p < 0.05$) increased Po was about 30 μM NaHS, which effectively amounts to 3.4–4.1 μM

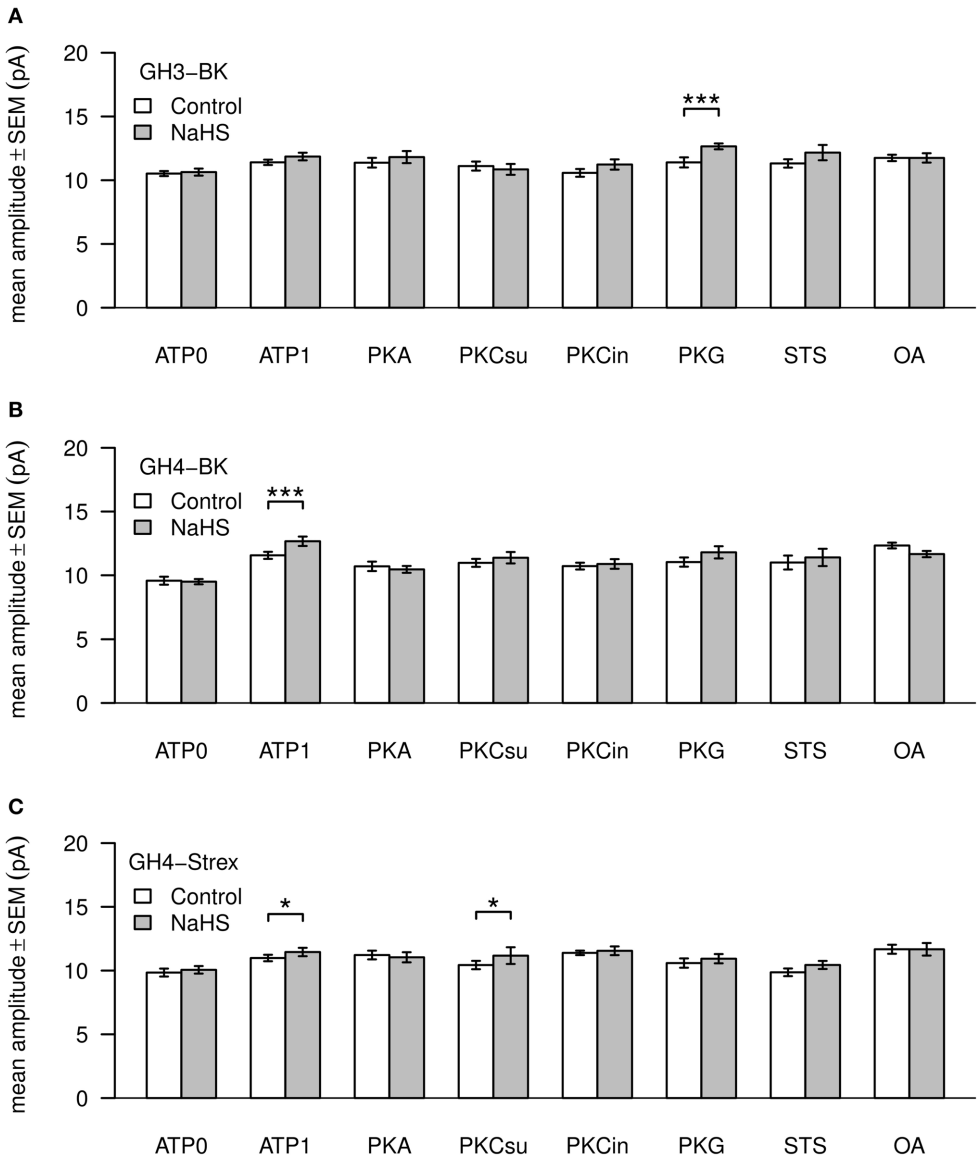


FIGURE 5 | Mean values of BK channel amplitudes. Three types of channels—GH3-BK **(A)**, GH4-BK **(B)**, and GH4-STREX **(C)** cells were investigated. Bar graphs show amplitude values in pA under control conditions (left bars, white) and after extracellular application of 300 μM NaHS (right bars, gray). All drug concentrations were the same as depicted in **Figure 3**. Significant *$0.05 > p > 0.01$; most significant ***$p < 0.001$.

free H_2S. Hence, H_2S appears to induce BK channel activation at a physiological relevant concentration range. The effective H_2S level at its target site(s) is unknown, however. H_2S may be stored as bound sulfane sulfur and/or neuronal excitation can trigger its release from surrounding astrocytes (Ishigami et al., 2009). It may be possible that subcellular local high concentrations of H_2S may be effective at ion channels for a short period of time which is supported by the fact that even very high concentrations at BK channels (400–500 μM) applied for a few minutes were completely reversible (Sitdikova et al., 2010). Therefore, not only the amount of H_2S appears important but the concentration per application time.

Our results indicate that the effect of NaHS on single channel currents was maximal within 1 min of application but then decreased within the following minutes. The decline of Po may be caused by the evaporation of H_2S from the perfusate. This appears unlikely, however, since the decline of H_2S within the 5 minute actual experimental time (see Materials and method) was only about 6–8%. As indicated from a previous dose-response plot (Sitdikova et al., 2010, Figure 4), this will result in a Po variation within the experimental error and therefore is unable to explain the decrease of Po. And, most importantly—we continuously perfuse the patches which gives a constant concentration of H_2S at the channels. We therefore interpret this transient response of the BK Po as development of tolerance to the gas. A similar transient effect has been reported for the action of ethanol on BK channels from GH3 cells (Jakab et al., 1997; Treistman and Martin, 2009). The transient activation of ion channels may be physiologically

relevant since this mechanism would allow cells to readjust to pre-stimulation electrical signaling conditions even in the presence of high levels of the activating drug or it may play a role in the rapid adaptation of other excitable cells, such as receptor cells.

In physiological solutions at pH 7.4, H_2S (∼20%), HS^- (∼80%), and traces of S^{2-}, are always simultaneously present in the solution or in the cytosol. This mixture is usually referred to in the literature as H_2S for simplicity (Liu et al., 2012; Greiner et al., 2013). HS^- should not be able to cross the membrane since it is charged and negative surface charges at the membrane make a passage unlikely. H_2S, as a neutral molecule appears more likely to readily cross the membrane. An internal action of H_2S at BK channels appears indicated since the effect of NaHS was prevented when the reducing agent dithiothreitol was applied inside through the pipette of excised outside-out patches, whereas the oxidizing agent thimerosal increased Po. Added to the external solution these agents had no effect (Sitdikova et al., 2010). The experiment suggests that H_2S is able to penetrate the membrane and acts on an internal site at the channels. Since H_2S once inside the cell is able to dissociate HS^- may be present again. Hence, it is still not clear which, H_2S or HS^-, or both may be considered as acting agents. However, an inner ring of negative charges produced by the RCK1,2 C-terminal part of the channel protein (Carvacho et al., 2008) may prevent access of HS^- to the channel favoring H_2S as the acting molecule.

To further complicate the H_2S issue, recent studies indicate that whatever form of H_2S donor or even H_2S itself is used in solutions results in the formation of polysulfides (Greiner et al., 2013) and polysulfides activating TRP receptors are orders of magnitude more effective compared to H_2S donors (Kimura et al., 2013). Since NaHS contains polysulfides it is possible that at least parts of the results are mediated by polysulfides. Further experimentation is needed to investigate these questions.

Considering the physiological relevance of our findings we speculate that the activation of BK channels causes a decrease of excitability or shorten the duration of action potentials which may lead to an increase of excitability which is modulated by the status of channels phosphorylation. Again, further experimentation is required.

PHOSPHORYLATION AND H_2S EFFECTS

ATP with high significance increased Po of all three types of cells investigated, GH3, GH4/C1, and GH4/C1 STREX by 583, 1203, and 525%, respectively, compared to ATP0. Kinases and phosphatases which are constitutively attached to BK channels, arranged in so called nano-domains, appear to provide an equilibrium of phosphorylation/dephosphorylation dependent on the presence of ATP. The most significant effect of ATP observed with GH4 cells suggests that they are particularly amenable to phosphorylation. As to the impact of ATP on BK channels it has been reported that ATP per see can inhibit BK channel activity (Clark et al., 1999; Hirano et al., 2001). This, however, appears not to apply to our situation since ATP in all BK channels from the different cell types investigated increased channel activity. It may be also possible that channel phosphatases are blocked by an ATP-dependent mechanism which could explain the substantial stimulatory effect of ATP. In GH4/C1 cells BK channels were found to be activated by PKG and inhibited by PKA and PKC (White et al., 1991; Shipston and Armstrong, 1996), but this can be altered by PKC-dependent pre-phosphorylation (Zhou et al., 2001). Stimulation of BK channels by ATP, therefore, may depend on the preexisting status of phosphorylation before ATP is supplied.

With pipette solutions containing no ATP H_2S significantly increased Po of GH3 and GH4 cells but had no significant effect on GH4 STREX cells. This may be explained by the particular arrangement at BK STREX channels where the 59 amino acid insert may prevent access of H_2S to its activation site. H_2S application to the bath in ATP-containing pipette solutions significantly increased Po in all cell types. The experiment indicates that phosphorylation (or an ATP/kinase activated blockade of a phosphatase) which in turn may prime the channels for H_2S activation.

In all three cell lines PKA effect on Po is not significant different compared to ATP1. The result indicates that the channels are already sufficiently phosphorylated in the presence of ATP. PKA has been reported to activate, inhibit or have no effect on BK channels as reviewed by Schubert and Nelson (2001). These different actions appear to depend on alternative BK splicing or could reflect variants in α and β subunit composition of the channel complexes (Hall and Armstrong, 2000; Tian et al., 2001, 2004). At PKA primed channels H_2S only had a high significant effect on BK channel Po from GH3 cells but had no effect on GH4 and GH4 STREX cells. Phosphorylation of BK channels by PKA therefore appears to prevent further H_2S activation in these cells. PKG had no significant effect on Po compared to ATP1 of all cell types (GH3, GH4, and GH4 STREX cells), whereas NaHS significantly increased Po of BK channels phosphorylated by PKG of all cell types. In all of our experiments the initial phosphorylation state of BK channels at the start of experiments was not determined and remains unknown. Also the amount of phosphorylation by ATP or the addition of kinases was not measured. This should be kept in mind when considering the interpretation of the results.

PKC subunit and PKC inhibitor decrease channel Po but this effect was only significant with GH4 cells. Our data are in congruence with a decrease of channel activity by PKC reported previously (Shipston and Armstrong, 1996; Schubert and Nelson, 2001; Tian et al., 2004; Kizub et al., 2010; Zhou et al., 2010, 2012; van Welie and du Lac, 2011). H_2S in this context did not alter BK channel Po from GH3 cells, but increased Po of GH4 and even more of GH4 STREX cells. With PKC inhibitor, H_2S had no effect at GH3 cells but high significantly increased Po of BK channels from GH4 and GH4 STREX cells. Although PKC phosphorylation suppresses Po of BK channels H_2S appears able to overcome this effect and to stimulate the channels. The kinase inhibitor, staurosporine, had no significant effect on BK channel Po of all three cell lines. H_2S under these conditions was able, however, to significantly increase Po. Hence, in all three cell types Po of non-phosphorylated (ATP0) and phosphorylated channels by ATP1 and by PKG can be elevated by H_2S. PKC phosphorylation prevented the Po increasing H_2S effect in GH3 cells but increased Po in GH4 and GH4 STREX cells. PKA also differently affected cell types with increasing Po of GH3 cells but

not of GH4 and GH4 STREX cells (which appear even decreased by H_2S).

The protein phosphatase inhibitor, okadaic acid, had the most significant effect in increasing Po under normal conditions (pipette solution containing 1 mM ATP). This peculiar action of okadaic acid may be due to the fact that a very active protein phosphatase is associated with the BK channels (Reinhart and Levitan, 1995; Zhou et al., 2010) and on site acts by dephosphorylating the channels. Inhibition of dephosphorylation by okadaic acid promotes phosphorylation and therefore appears able to convey a major impact on Po activation. Although phosphorylated channels are activated by H_2S okadaic acid had no further increasing effect on BK channels from GH3 and GH4 STREX cells. We interpret this result by assuming that channel phosphorylation may be at its maximum and therefore H_2S had no further effect.

The PKC subunit tends to decrease Po but this was only significant with channels from GH4 cells. In the presence of PKC H_2S increased channel Po of GH4 and GH4 STREX significantly but not those of GH3 cells. The PKC inhibitor had no significant effect on Po compared to PKC subunit, whereas H_2S had a similar increasing effect in GH4 and GH4 STREX cells. No significant difference was observed between ATP1 and staurosporine, but in all cells channel Po was increased during H_2S application. Our results indicate that H_2S modulates diverse BK splice variants in a different way.

A significant increase in open dwell times was observed with ATP1 vs. ATP0 in GH3 cells, in all other settings there was no significant effect beyond the ATP1 increase. In GH3 cells H_2S only increased open dwell times in ATP0 significantly. With GH4 cells open dwell times increased in ATP1 vs. ATP0 and with ATP1 vs. PKA, but decreased with PKC, PKG, staurosporin, and okadaic acid vs. PKA. A significant increase of dwell times was seen with H_2S in the presence of okadaic acid. With GH4 STREX cells a low significant increase of dwell times was only observed with PKG vs. ATP0 and with H_2S in ATP0. Closed dwell times were not analyzed since most of our single channels recording contained more than one channel.

BK channel amplitudes were little altered by phosphorylation or by H_2S. Exceptions appear to be BK channels from GH3 and GH4 cells which increase in amplitude significantly with PKG or with ATP1, respectively. The mechanisms underlying these alteration in Po, dwell times or amplitude variations caused by phosphorylation of channels and the impact by H_2S are unknown.

In summary, Po of BK channels of GH3, GH4 cell types, either in their non-phosphorylated state (with no ATP or with staurosporine in the pipette solution) or if phosphorylated by ATP can be activated by H_2S. GH4 STREX BK channels do not respond to H_2S in ATP0 whereas in the phosphorylated mode (ATP1) channel Po is increased. PKA prevented the action of H_2S on channel Po in GH4 and GH4 STREX but increased BK Po of GH3 cells at high significance. This suggests that PKA phosphorylates the channels of GH4 and GH4 STREX cells at some other site which rearranges the protein in a way that H_2S is no longer able to act at the channels. Zhou et al. (2012) showed that only BK channels which are dephosphorylated as well as depalmitoylated can be stimulated by PKA whereas if phosphorylated by PKC and palmitoylated the channels are insensitive to PKA. PKG vs. ATP0 increased Po of BK channels of all cell types but had no further effect at Po if ATP was present. H_2S, high significantly increased Po of PKG pretreated cells. This suggests that the phosphorylation by PKG primes the channels for H_2S activation. PKC decreased Po under control conditions of all BK channels types but BK channel Po of GH4 and GH4 STREX cells was increased with H_2S at high significance. In experiments using the phosphatase inhibitor okadaic acid, the effect of H_2S on Po was prevented or even reduced indicating that either phosphorylation at the BK channel protein mediates this effect or the channel activity is already at a maximum preventing any further increase (**Table 3**).

Our experimental results indicate that phosphorylation may prime or prevent the action of H_2S on BK channels Po which may be dependent on the channels conformation which exposes or impedes phosphorylation sites and this way may govern the access of H_2S to reach its effective location.

ACKNOWLEDGMENTS

The study was funded in part by travel grants to Guzel F. Sitdikova, by the Stiftungs- und Förderungsgesellschaft der Universität Salzburg (Austria) and by the Russian Scientific Fund № 14-15-00618.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphys.2014.00431/abstract

REFERENCES

Abe, K., and Kimura, H. (1996). The possible role of hydrogen sulfide as an endogenous neuromodulator. *J. Neurosci* 16, 1066–1071.

Alioua, A., Tanaka, Y., Wallner, M., Hofmann, F., Ruth, P., Meera, P., et al. (1998). The large conductance, voltage-dependent, and calcium-sensitive K+ channel, Hslo, is a target of cGMP-dependent protein kinase phosphorylation *in vivo*. *J. Biol. Chem.* 273, 32950–32956. doi: 10.1074/jbc.273.49.32950

Barman, S. A., Zhu, S., and White, R. E. (2004). PKC activates BKCa channels in rat pulmonary arterial smooth muscle via cGMP-dependent protein kinase. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 286, L1275–L1281.

Beauchamp, R. O. Jr., Bus, J. S., Popp, J. A., Boreiko, C. J., and Andjelkovich, D. A. (1984). A critical review of the literature on hydrogen sulfide toxicity. *Crit. Rev. Toxicol.* 13, 25–97. doi: 10.3109/10408448409029321

Berkefeld, H., Fakler, B., and Schulte, U. (2010). Ca2+-activated K+ channels: from protein complexes to function. *Physiol. Rev.* 90, 1437–1459. doi: 10.1152/physrev.00049.2009

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H., et al. (2009). Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol. Evol. (Amst.)* 24, 127–135. doi: 10.1016/j.tree.2008.10.008

Carvacho, I., Gonzalez, W., Torres, Y. P., Brauchi, S., Alvarez, O., Gonzalez-Nilo, F. D., et al. (2008). Intrinsic electrostatic potential in the BK channel pore: role in determining single channel conductance and block. *J. Gen. Physiol.* 131, 147–161. doi: 10.1085/jgp.200709862

Clark, A. G., Hall, S. K., and Shipston, M. J. (1999). ATP inhibition of a mouse brain large-conductance K+ (mslo) channel variant by a mechanism independent of protein phosphorylation. *J. Physiol. (Lond.)* 516(Pt 1), 45–53. doi: 10.1111/j.1469-7793.1999.045aa.x

Cui, J. (2010). BK-type calcium-activated potassium channels: coupling of metal ions and voltage sensing. *J. Physiol. (Lond.)* 588, 4651–4658. doi: 10.1113/jphysiol.2010.194514

Cui, J., Yang, H., and Lee, U. S. (2009). Molecular mechanisms of BK channel activation. *Cell. Mol. Life Sci.* 66, 852–875. doi: 10.1007/s00018-008-8609-x

Dai, S., Hall, D. D., and Hell, J. W. (2009). Supramolecular assemblies and localized regulation of voltage-gated ion channels. *Physiol. Rev.* 89, 411–452. doi: 10.1152/physrev.00029.2007

DeLeon, E. R., Stoy, G. F., and Olson, K. R. (2012). Passive loss of hydrogen sulfide in biological experiments. *Anal. Biochem.* 421, 203–207. doi: 10.1016/j.ab.2011.10.016

Denson, D. D., Wang, X., Worrell, R. T., AlKhalili, O., and Eaton, D. C. (2001). Cytosolic phospholipase A2 is required for optimal ATP activation of BK channels in GH(3) cells. *J. Biol. Chem.* 276, 7136–7142. doi: 10.1074/jbc.M009566200

Erxleben, C., Everhart, A. L., Romeo, C., Florance, H., Bauer, M. B., Alcorta, D. A., et al. (2002). Interacting effects of N-terminal variation and strex exon splicing on slo potassium channel regulation by calcium, phosphorylation, and oxidation. *J. Biol. Chem.* 277, 27045–27052. doi: 10.1074/jbc.M203087200

Furne, J., Saeed, A., and Levitt, M. D. (2008). Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 295, R1479–R1485. doi: 10.1152/ajpregu.90566.2008

Gadalla, M. M., and Snyder, S. H. (2010). Hydrogen sulfide as a gasotransmitter. *J. Neurochem.* 113, 14–26. doi: 10.1111/j.1471-4159.2010.06580.x

García-Bereguiaín, M. A., Samhan-Arias, A. K., Martín-Romero, F. J., and Gutiérrez-Merino, C. (2008). Hydrogen sulfide raises cytosolic calcium in neurons through activation of L-type Ca2+ channels. *Antioxid. Redox Signal.* 10, 31–42. doi: 10.1089/ars.2007.1656

Ghatta, S., Nimmagadda, D., Xu, X., and O'Rourke, S. T. (2006). Large-conductance, calcium-activated potassium channels: structural and functional implications. *Pharmacol. Ther.* 110, 103–116. doi: 10.1016/j.pharmthera.2005.10.007

Goodwin, L. R., Francom, D., Dieken, F. P., Taylor, J. D., Warenycia, M. W., Reiffenstein, R. J., et al. (1989). Determination of sulfide in brain tissue by gas dialysis/ion chromatography: postmortem studies and two case reports. *J. Anal. Toxicol.* 13, 105–109. doi: 10.1093/jat/13.2.105

Greiner, R., Pálinkás, Z., Bäsell, K., Becher, D., Antelmann, H., Nagy, P., et al. (2013). Polysulfides link H2S to protein thiol oxidation. *Antioxid. Redox Signal.* 19, 1749–1765. doi: 10.1089/ars.2012.5041

Grimm, P. R., and Sansom, S. C. (2010). BK channels and a new form of hypertension. *Kidney Int.* 78, 956–962. doi: 10.1038/ki.2010.272

Hall, S. K., and Armstrong, D. L. (2000). Conditional and unconditional inhibition of calcium-activated potassium channels by reversible protein phosphorylation. *J. Biol. Chem.* 275, 3749–3754. doi: 10.1074/jbc.275.6.3749

Hermann, A., Sitdikova, G. F., and Weiger, T. M. (2012a). "BK channels – focus on polyamines, ethanol/acetaldehyde and hydrogen sulfide (H2S)," in *Patch Clamp Technique*, ed F. Shad Kaneez (InTech). Available online at: http://www.intechopen.com/statistics/33631 (Accessed September 20, 2013).

Hermann, A., Sitdikova, G. F., and Weiger, T. M. (2012b). *Gasotransmitters: Physiology and Pathophysiology*. Berlin; Heidelberg: Springer. Available online at: http://www.springer.com/biomed/human+physiology/book/978-3-642-30337-1 (Accessed September 27, 2013).

Hermann, A., Sitdikova, G. F., and Weiger, T. M. (2012c). "Modulated by gasotransmitters: BK channels," in *Gasotransmitters: Physiology and Pathophysiology*, eds A. Hermann, G. F. Sitdikova, and T. M. Weiger (Berlin; Heidelberg: Springer), 163–201. Available online at: http://link.springer.com/chapter/10.1007/978-3-642-30338-8_6 (Accessed September 20, 2013).

Hill, M. A., Yang, Y., Ella, S. R., Davis, M. J., and Braun, A. P. (2010). Large conductance, Ca2+-activated K+ channels (BKCa) and arteriolar myogenic signaling. *FEBS Lett.* 584, 2033–2042. doi: 10.1016/j.febslet.2010.02.045

Hirano, J., Nakamura, K., and Kubokawa, M. (2001). Properties of a Ca(2+)-activated large conductance K(+) channel with ATP sensitivity in human renal proximal tubule cells. *Jpn. J. Physiol.* 51, 481–489. doi: 10.2170/jjphysiol.51.481

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biom. J.* 50, 346–363. doi: 10.1002/bimj.200810425

Hou, S., Heinemann, S. H., and Hoshi, T. (2009). Modulation of BKCa channel gating by endogenous signaling molecules. *Physiology (Bethesda)* 24, 26–35. doi: 10.1152/physiol.00032.2008

Hu, L.-F., Lu, M., Hon Wong, P. T., and Bian, J.-S. (2011). Hydrogen sulfide: neurophysiology and neuropathology. *Antioxid. Redox Signal.* 15, 405–419. doi: 10.1089/ars.2010.3517

Hughes, M. N., Centelles, M. N., and Moore, K. P. (2009). Making and working with hydrogen sulfide: the chemistry and generation of hydrogen sulfide *in vitro* and its measurement *in vivo*: a review. *Free Radic. Biol. Med.* 47, 1346–1353. doi: 10.1016/j.freeradbiomed.2009.09.018

Ishigami, M., Hiraki, K., Umemura, K., Ogasawara, Y., Ishii, K., and Kimura, H. (2009). A source of hydrogen sulfide and a mechanism of its release in the brain. *Antioxid. Redox Signal.* 11, 205–214. doi: 10.1089/ars.2008.2132

Jackson-Weaver, O., Paredes, D. A., Gonzalez Bosc, L. V., Walker, B. R., and Kanagy, N. L. (2011). Intermittent hypoxia in rats increases myogenic tone through loss of hydrogen sulfide activation of large-conductance Ca(2+)-activated potassium channels. *Circ. Res.* 108, 1439–1447. doi: 10.1161/CIRCRESAHA.110.228999

Jakab, M., Weiger, T. M., and Hermann, A. (1997). Ethanol activates maxi Ca2+-activated K+ channels of clonal pituitary (GH3) cells. *J. Membr. Biol.* 157, 237–245. doi: 10.1007/PL00005895

Jiang, B., Tang, G., Cao, K., Wu, L., and Wang, R. (2010). Molecular mechanism for H(2)S-induced activation of K(ATP) channels. *Antioxid. Redox Signal.* 12, 1167–1178. doi: 10.1089/ars.2009.2894

Kabil, O., Motl, N., and Banerjee, R. (2014). H2S and its role in redox signaling. *Biochim. Biophys. Acta* 1844, 1355–1366. doi: 10.1016/j.bbapap.2014.01.002

Kawabata, A., Ishiki, T., Nagasawa, K., Yoshida, S., Maeda, Y., Takahashi, T., et al. (2007). Hydrogen sulfide as a novel nociceptive messenger. *Pain* 132, 74–81. doi: 10.1016/j.pain.2007.01.026

Kim, J.-Y., and Park, C.-S. (2008). Potentiation of large-conductance calcium-activated potassium (BK(Ca)) channels by a specific isoform of protein kinase C. *Biochem. Biophys. Res. Commun.* 365, 459–465. doi: 10.1016/j.bbrc.2007.10.179

Kimura, H. (2011). Hydrogen sulfide: its production and functions. *Exp. Physiol.* 96, 833–835.

Kimura, Y., Dargusch, R., Schubert, D., and Kimura, H. (2006). Hydrogen sulfide protects HT22 neuronal cells from oxidative stress. *Antioxid. Redox Signal.* 8, 661–670. doi: 10.1089/ars.2006.8.661

Kimura, Y., and Kimura, H. (2004). Hydrogen sulfide protects neurons from oxidative stress. *FASEB J.* 18, 1165–1167. doi: 10.1096/fj.04-1815fje

Kimura, H., Shibuya, N., and Kimura, Y. (2012). Hydrogen sulfide is a signaling molecule and a cytoprotectant. *Antioxid. Redox Signal.* 17, 45–57. doi: 10.1089/ars.2011.4345

Kimura, Y., Goto, Y.-I., and Kimura, H. (2010). Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. *Antioxid. Redox Signal.* 12, 1–13. doi: 10.1089/ars.2008.2282

Kimura, Y., Mikami, Y., Osumi, K., Tsugane, M., Oka, J., and Kimura, H. (2013). Polysulfides are possible H2S-derived signaling molecules in rat brain. *FASEB J.* 27, 2451–2457. doi: 10.1096/fj.12-226415

Kizub, I. V., Pavlova, O. O., Ivanova, I. V., and Soloviev, A. I. (2010). Protein kinase C-dependent inhibition of BK(Ca) current in rat aorta smooth muscle cells following gamma-irradiation. *Int. J. Radiat. Biol.* 86, 291–299. doi: 10.3109/09553000903564042

Kyle, B. D., Hurst, S., Swayze, R. D., Sheng, J., and Braun, A. P. (2013). Specific phosphorylation sites underlie the stimulation of a large conductance, Ca(2+)-activated K(+) channel by cGMP-dependent protein kinase. *FASEB J.* 27, 2027–2038. doi: 10.1096/fj.12-223669

Lee, U. S., and Cui, J. (2010). BK channel activation: structural and functional insights. *Trends Neurosci.* 33, 415–423. doi: 10.1016/j.tins.2010.06.004

Leffler, C. W., Parfenova, H., Basuroy, S., Jaggar, J. H., Umstot, E. S., and Fedinec, A. L. (2011). Hydrogen sulfide and cerebral microvascular tone in newborn pigs. *Am. J. Physiol. Heart Circ. Physiol.* 300, H440–H447. doi: 10.1152/ajpheart.00722.2010

Levitan, I. B. (1994). Modulation of ion channels by protein phosphorylation and dephosphorylation. *Annu. Rev. Physiol.* 56, 193–212. doi: 10.1146/annurev.ph.56.030194.001205

Li, J., Al-Khalili, O., Ramosevac, S., Eaton, D. C., and Denson, D. D. (2010). Protein-protein interaction between cPLA2 and splice variants of α-subunit of BK channels. *Am. J. Physiol. Cell Physiol.* 298, C251–C262. doi: 10.1152/ajpcell.00221.2009

Li, L., Bhatia, M., Zhu, Y. Z., Zhu, Y. C., Ramnath, R. D., Wang, Z. J., et al. (2005). Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *FASEB J.* 19, 1196–1198. doi: 10.1096/fj.04-3583fje

Liang, G. H., Adebiyi, A., Leo, M. D., McNally, E. M., Leffler, C. W., and Jaggar, J. H. (2011). Hydrogen sulfide dilates cerebral arterioles by activating smooth muscle cell plasma membrane KATP channels. *Am. J. Physiol. Heart Circ. Physiol.* 300, H2088–H2095. doi: 10.1152/ajpheart.01290.2010

Lide, D. R. (1998). *CRC Handbook of Chemistry and Physics, 79th Edn*. Boca Raton, FL: Taylor & Francis.

Liu, W.-Q., Chai, C., Li, X.-Y., Yuan, W.-J., Wang, W.-Z., and Lu, Y. (2011). The cardiovascular effects of central hydrogen sulfide are related to K(ATP) channels activation. *Physiol. Res.* 60, 729–738.

Liu, Y.-H., Lu, M., Hu, L.-F., Wong, P. T.-H., Webb, G. D., and Bian, J.-S. (2012). Hydrogen sulfide in the mammalian cardiovascular system. *Antioxid. Redox Signal.* 17, 141–185. doi: 10.1089/ars.2011.4005

Łowicka, E., and Bełtowski, J. (2007). Hydrogen sulfide (H_2S) - the third gas of interest for pharmacologists. *Pharmacol. Rep.* 59, 4–24.

Lu, R., Alioua, A., Kumar, Y., Eghbali, M., Stefani, E., and Toro, L. (2006). MaxiK channel partners: physiological impact. *J. Physiol. (Lond.)* 570, 65–72. doi: 10.1113/jphysiol.2005.098913

Maeda, Y., Aoki, Y., Sekiguchi, F., Matsunami, M., Takahashi, T., Nishikawa, H., et al. (2009). Hyperalgesia induced by spinal and peripheral hydrogen sulfide: evidence for involvement of Cav3.2 T-type calcium channels. *Pain* 142, 127–132. doi: 10.1016/j.pain.2008.12.021

Mani, S., Untereiner, A., Wu, L., and Wang, R. (2014). Hydrogen sulfide and the pathogenesis of atherosclerosis. *Antioxid. Redox Signal.* 20, 805–817. doi: 10.1089/ars.2013.5324

Matsunami, M., Kirishi, S., Okui, T., and Kawabata, A. (2012). Hydrogen sulfide-induced colonic mucosal cytoprotection involves T-type calcium channel-dependent neuronal excitation in rats. *J. Physiol. Pharmacol.* 63, 61–68.

Millero, F. J., Plese, T., and Fernandez, M. (1988). The dissociation of hydrogen sulfide in seawater. *Limnol. Oceanogr.* 33, 269–274. doi: 10.4319/lo.1988.33.2.0269

Mustafa, A. K., Gadalla, M. M., and Snyder, S. H. (2009). Signaling by gasotransmitters. *Sci Signal* 2:re2. doi: 10.1126/scisignal.268re2

Nagai, Y., Tsugane, M., Oka, J.-I., and Kimura, H. (2004). Hydrogen sulfide induces calcium waves in astrocytes. *FASEB J.* 18, 557–559. doi: 10.1096/fj.03-1052fje

Olson, K. R. (2012). A practical look at the chemistry and biology of hydrogen sulfide. *Antioxid. Redox Signal.* 17, 32–44. doi: 10.1089/ars.2011.4401

Olson, K. R., and Whitfield, N. L. (2010). Hydrogen sulfide and oxygen sensing in the cardiovascular system. *Antioxid. Redox Signal.* 12, 1219–1234. doi: 10.1089/ars.2009.2921

Peng, H., Cheng, Y., Dai, C., King, A. L., Predmore, B. L., Lefer, D. J., et al. (2011). A fluorescent probe for fast and quantitative detection of hydrogen sulfide in blood. *Angew. Chem. Int. Ed. Engl.* 50, 9672–9675. doi: 10.1002/anie.201104236

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and R Core Team. (2013). *nlme: Linear and Nonlinear Mixed Effects Models*. Available online at: http://CRAN.R-project.org/package=nlme

R Development Core Team. (2011). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: http://www.r-project.org

Reinhart, P. H., Chung, S., Martin, B. L., Brautigan, D. L., and Levitan, I. B. (1991). Modulation of calcium-activated potassium channels from rat brain by protein kinase A and phosphatase 2A. *J. Neurosci.* 11, 1627–1635.

Reinhart, P. H., and Levitan, I. B. (1995). Kinase and phosphatase activities intimately associated with a reconstituted calcium-dependent potassium channel. *J. Neurosci.* 15, 4572–4579.

Renga, B. (2011). Hydrogen sulfide generation in mammals: the molecular biology of cystathionine-β- synthase (CBS) and cystathionine-γ-lyase (CSE). *Inflamm. Allergy Drug Targets* 10, 85–91. doi: 10.2174/187152811794776286

Salkoff, L., Butler, A., Ferreira, G., Santi, C., and Wei, A. (2006). High-conductance potassium channels of the SLO family. *Nat. Rev. Neurosci.* 7, 921–931. doi: 10.1038/nrn1992

Savage, J. C., and Gould, D. H. (1990). Determination of sulfide in brain tissue and rumen fluid by ion-interaction reversed-phase high-performance liquid chromatography. *J. Chromatogr.* 526, 540–545. doi: 10.1016/S0378-4347(00)82537-2

Schubert, R., and Nelson, M. T. (2001). Protein kinases: tuners of the BKCa channel in smooth muscle. *Trends Pharmacol. Sci.* 22, 505–512. doi: 10.1016/S0165-6147(00)01775-2

Shibuya, N., Koike, S., Tanaka, M., Ishigami-Yuasa, M., Kimura, Y., Ogasawara, Y., et al. (2013). A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells. *Nat Commun* 4, 1366. doi: 10.1038/ncomms2371

Shibuya, N., Mikami, Y., Kimura, Y., Nagahara, N., and Kimura, H. (2009). Vascular endothelium expresses 3-mercaptopyruvate sulfurtransferase and produces hydrogen sulfide. *J. Biochem.* 146, 623–626. doi: 10.1093/jb/mvp111

Shipston, M. J., and Armstrong, D. L. (1996). Activation of protein kinase C inhibits calcium-activated potassium channels in rat pituitary tumour cells. *J. Physiol. (Lond.)* 493(Pt 3), 665–672.

Sitdikova, G. F., Weiger, T. M., and Hermann, A. (2010). Hydrogen sulfide increases calcium-activated potassium (BK) channel activity of rat pituitary tumor cells. *Pflugers Arch.* 459, 389–397. doi: 10.1007/s00424-009-0737-0

Skovgaard, N., Gouliaev, A., Aalling, M., and Simonsen, U. (2011). The role of endogenous H2S in cardiovascular physiology. *Curr. Pharm. Biotechnol.* 12, 1385–1393. doi: 10.2174/138920111798280956

Sokal, R. R., and Rohlf, F. J. (1995). *Biometry: the Principles and Practice of Statistics in Biological Research*. New York, NY: W. H. Freeman.

Stojilkovic, S. S., Tabak, J., and Bertram, R. (2010). Ion channels and signaling in the pituitary gland. *Endocr. Rev.* 31, 845–915. doi: 10.1210/er.2010-0005

Sun, Y.-G., Cao, Y.-X., Wang, W.-W., Ma, S.-F., Yao, T., and Zhu, Y.-C. (2008). Hydrogen sulphide is an inhibitor of L-type calcium channels and mechanical contraction in rat cardiomyocytes. *Cardiovasc. Res.* 79, 632–641. doi: 10.1093/cvr/cvn140

Szabó, C. (2007). Hydrogen sulphide and its therapeutic potential. *Nat. Rev. Drug Discov.* 6, 917–935. doi: 10.1038/nrd2425

Tang, G., Wu, L., and Wang, R. (2010). Interaction of hydrogen sulfide with ion channels. *Clin. Exp. Pharmacol. Physiol.* 37, 753–763. doi: 10.1111/j.1440-1681.2010.05351.x

Tang, G., Yang, G., Jiang, B., Ju, Y., Wu, L., and Wang, R. (2013). H2S is an endothelium-derived hyperpolarizing factor. *Antioxid. Redox Signal.* 19, 1634–1646. doi: 10.1089/ars.2012.4805

Telezhkin, V., Brazier, S. P., Cayzac, S. H., Wilkinson, W. J., Riccardi, D., and Kemp, P. J. (2010). Mechanism of inhibition by hydrogen sulfide of native and recombinant BKCa channels. *Respir. Physiol. Neurobiol.* 172, 169–178. doi: 10.1016/j.resp.2010.05.016

Telezhkin, V., Brazier, S. P., Cayzac, S., Müller, C. T., Riccardi, D., and Kemp, P. J. (2009). Hydrogen sulfide inhibits human BK(Ca) channels. *Adv. Exp. Med. Biol.* 648, 65–72. doi: 10.1007/978-90-481-2259-2_7

Tian, L., Coghill, L. S., McClafferty, H., MacDonald, S. H.-F., Antoni, F. A., Ruth, P., et al. (2004). Distinct stoichiometry of BKCa channel tetramer phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11897–11902. doi: 10.1073/pnas.0402590101

Tian, L., Duncan, R. R., Hammond, M. S., Coghill, L. S., Wen, H., Rusinova, R., et al. (2001). Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J. Biol. Chem.* 276, 7717–7720. doi: 10.1074/jbc.C000741200

Treistman, S. N., and Martin, G. E. (2009). BK Channels: mediators and models for alcohol tolerance. *Trends Neurosci.* 32, 629–637. doi: 10.1016/j.tins.2009.08.001

van Welie, I., and du Lac, S. (2011). Bidirectional control of BK channel open probability by CAMKII and PKC in medial vestibular nucleus neurons. *J. Neurophysiol.* 105, 1651–1659. doi: 10.1152/jn.00058.2011

Venables, W. N., and Ripley, B. D. (2002). *Modern Applied Statistics with S*. New York, NY: Springer.

Wang, R. (2010). Hydrogen sulfide: the third gasotransmitter in biology and medicine. *Antioxid. Redox Signal.* 12, 1061–1064. doi: 10.1089/ars.2009.2938

Wang, R. (2012). Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Physiol. Rev.* 92, 791–896. doi: 10.1152/physrev.00017.2011

Wang, R. (2014). Gasotransmitters: growing pains and joys. *Trends Biochem. Sci.* 39, 227–232. doi: 10.1016/j.tibs.2014.03.003

Wang, Z.-W. (2008). Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Mol. Neurobiol.* 38, 153–166. doi: 10.1007/s12035-008-8039-7

Warenycia, M. W., Goodwin, L. R., Benishin, C. G., Reiffenstein, R. J., Francom, D. M., Taylor, J. D., et al. (1989). Acute hydrogen sulfide poisoning. Demonstration of selective uptake of sulfide by the brainstem by measurement of brain sulfide levels. *Biochem. Pharmacol.* 38, 973–981. doi: 10.1016/0006-2952(89)90288-8

Weiger, T. M., Hermann, A., and Levitan, I. B. (2002). Modulation of calcium-activated potassium channels. *J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* 188, 79–87. doi: 10.1007/s00359-002-0281-2

White, R. E., Schonbrunn, A., and Armstrong, D. L. (1991). Somatostatin stimulates Ca(2+)-activated K+ channels through protein dephosphorylation. *Nature* 351, 570–573. doi: 10.1038/351570a0

Whitfield, N. L., Kreimier, E. L., Verdial, F. C., Skovgaard, N., and Olson, K. R. (2008). Reappraisal of H2S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 294, R1930–R1937. doi: 10.1152/ajpregu.00025.2008

Widmer, H. A., Rowe, I. C. M., and Shipston, M. J. (2003). Conditional protein phosphorylation regulates BK channel activity in rat cerebellar

Purkinje neurons. *J. Physiol. (Lond.)* 552, 379–391. doi: 10.1113/jphysiol.2003.046441

Wintner, E. A., Deckwerth, T. L., Langston, W., Bengtsson, A., Leviten, D., Hill, P., et al. (2010). A monobromobimane-based assay to measure the pharmacokinetic profile of reactive sulphide species in blood. *Br. J. Pharmacol.* 160, 941–957. doi: 10.1111/j.1476-5381.2010.00704.x

Wu, S.-N., Wang, Y.-J., and Lin, M.-W. (2007). Potent stimulation of large-conductance Ca2+-activated K+ channels by rottlerin, an inhibitor of protein kinase C-delta, in pituitary tumor (GH3) cells and in cortical neuronal (HCN-1A) cells. *J. Cell. Physiol.* 210, 655–666. doi: 10.1002/jcp.20866

Wu, Y., Yang, Y., Ye, S., and Jiang, Y. (2010). Structure of the gating ring from the human large-conductance Ca(2+)-gated K(+) channel. *Nature* 466, 393–397. doi: 10.1038/nature09252

Xie, J., and McCobb, D. P. (1998). Control of alternative splicing of potassium channels by stress hormones. *Science* 280, 443–446. doi: 10.1126/science.280.5362.443

Zhao, W., and Wang, R. (2002). H(2)S-induced vasorelaxation and underlying cellular and molecular mechanisms. *Am. J. Physiol. Heart Circ. Physiol.* 283, H474–H480. doi: 10.1152/ajpheart.00013.2002

Zhao, Y., Wei, H., Kong, G., Shim, W., and Zhang, G. (2013). Hydrogen sulfide augments the proliferation and survival of human induced pluripotent stem cell-derived mesenchymal stromal cells through inhibition of BKCa. *Cytotherapy* 15, 1395–1405. doi: 10.1016/j.jcyt.2013.06.004

Zhou, X. B., Arntz, C., Kamm, S., Motejlek, K., Sausbier, U., Wang, G. X., et al. (2001). A molecular switch for specific stimulation of the BKCa channel by cGMP and cAMP kinase. *J. Biol. Chem.* 276, 43239–43245. doi: 10.1074/jbc.M104202200

Zhou, X.-B., Wulfsen, I., Utku, E., Sausbier, U., Sausbier, M., Wieland, T., et al. (2010). Dual role of protein kinase C on BK channel regulation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8005–8010. doi: 10.1073/pnas.0912029107

Zhou, X., Wulfsen, I., Korth, M., McClafferty, H., Lukowski, R., Shipston, M. J., et al. (2012). Palmitoylation and membrane association of STREX controls BK channel regulation by protein kinase C. *J. Biol. Chem.* 287, 32161–32171. doi: 10.1074/jbc.M112.386359

Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A., and Smith, G. M. (2009). *Mixed Effects Models and Extensions in Ecology with R*. New York, NY: Springer. doi: 10.1007/978-0-387-87458-6

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 July 2014; accepted: 16 October 2014; published online: 12 November 2014.

Citation: Sitdikova GF, Fuchs R, Kainz V, Weiger TM and Hermann A (2014) Phosphorylation of BK channels modulates the sensitivity to hydrogen sulfide (H_2S). Front. Physiol. ***5****:431. doi: 10.3389/fphys.2014.00431*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

REVIEW ARTICLE
published: 09 October 2014
doi: 10.3389/fphys.2014.00389

BK channel activators and their therapeutic perspectives

Bo H. Bentzen[1,2]*, Søren-Peter Olesen[1], Lars C. B. Rønn[3] and Morten Grunnet[2,3]

[1] Department of Biomedical Sciences, Faculty of Health Sciences, Danish Arrhythmia Research Centre, University of Copenhagen, Copenhagen, Denmark
[2] Acesion Pharma, Copenhagen, Denmark
[3] H. Lundbeck A/S, Copenhagen, Denmark

Edited by:
Thomas M. Weiger, University of Salzburg, Austria

Reviewed by:
Luis A. Pardo, Max-Planck-Institute of Experimental Medicine, Germany
Fabiana Silvia Scornik, University of Girona, Spain

***Correspondence:**
Bo H. Bentzen, Department of Biomedical Sciences, Faculty of Health Sciences, Danish Arrhythmia Research Centre, University of Copenhagen, Blegdamsvej 3B, DK-2200 Copenhagen, Denmark
e-mail: bobe@sund.ku.dk

The large conductance calcium- and voltage-activated K^+ channel (KCa1.1, BK, MaxiK) is ubiquitously expressed in the body, and holds the ability to integrate changes in intracellular calcium and membrane potential. This makes the BK channel an important negative feedback system linking increases in intracellular calcium to outward hyperpolarizing potassium currents. Consequently, the channel has many important physiological roles including regulation of smooth muscle tone, neurotransmitter release and neuronal excitability. Additionally, cardioprotective roles have been revealed in recent years. After a short introduction to the structure, function and regulation of BK channels, we review the small organic molecules activating BK channels and how these tool compounds have helped delineate the roles of BK channels in health and disease.

Keywords: KCa1.1, BK, MaxiK, *KCNMA1*, high-conductance calcium-activated potassium channels, activators, openers, pharmacology

INTRODUCTION

The large conductance calcium- and voltage-activated K^+ channel (BK, KCa1.1, MaxiK) channel is encoded by a single gene (*KCNMA1*, *SLO-1*). Structurally the channel consist of four pore-forming BK α-subunits, each with 7 transmembrane segments and an extracellular N-terminus. The channel monomer is composed of essentially two distinct modules, one being primarily responsible for voltage-sensing and the other for Ca^{2+}-sensing (Meera et al., 1997).

The BK channel is ubiquitously expressed in the body and represents a unique class of potassium channels, not only because of its high single channel conductance (Butler et al., 1993; Pallanck and Ganetzky, 1994) (∼250 pS measured in symmetrical K^+), but also because it can be activated by Ca^{2+} alone, membrane depolarization alone, or synergistically by both (Magleby, 2003). The ability to integrate changes in intracellular calcium and membrane potential makes the BK channel an important negative feedback system, linking changes in intracellular calcium to outward hyperpolarizing potassium currents. Consequently, the channel has many important roles, including regulation of neurotransmitter release, neuronal excitability and smooth muscle tone (Salkoff et al., 2006). In agreement with this experimental down-regulation of BK in mice is associated with erectile dysfunction, bladder over-activity, urinary incontinence (Meredith et al., 2004; Werner et al., 2005; Sprossmann et al., 2009) and hypertension (Sausbier et al., 2005). In addition, a gain-of-function mutation in *KCNMA1* has been reported to result in a syndrome of coexistent generalized epilepsy and paroxysmal dyskinesia in humans (Du et al., 2005); Gain-of-function mutations in the β1 subunit were associated with low prevalence of diastolic hypertension (Fernández-Fernández et al., 2004). However, the role of BK channels in epilepsy is not straight forward, as a loss of function mutation has also been associated with temporal lobe epilepsy, as described in detail in the paragraph "*BK channels in the central nervous system.*"

To serve its many diverse roles, and considering its wide tissue distribution, it is not surprising that BK activity is regulated by numerous mechanisms. On the transcriptional level, extensive alternative splicing gives rise to channels with altered Ca^{2+} sensitivity, channel kinetics, localization and hormone sensitivity (for review see Shipston, 2001). Moreover co-assembly with auxillary subunits including β1-4 and leucine-rich repeat-containing proteins (LRRC) changes the channels calcium- and voltage-sensitivity, as well as pharmacological properties (Knaus et al., 1994; McManus et al., 1995; Valverde et al., 1999; Wallner et al., 1999; Meera et al., 2000; Yan and Aldrich, 2010, 2012). On the post-translational level, channel activity is regulated by a number of endogenous mediators such as arachidonic acids, NO, pH (Avdonin et al., 2003), zinc (Hou et al., 2010), and phosphorylation of the channel (Yan et al., 2008) by protein kinase A, C, G, and CaMKII (Hou et al., 2009; Wang et al., 2013; Hosseinzadeh et al., 2014; Toro et al., 2014). Other modifications such as palmitoylation favor cell surface expression, while myristoylation appear to favor endocytosis (Jeffries et al., 2010; Alioua et al., 2011; Tian et al., 2012). Finally, BK channels have been found to localize to macro-molecular signaling complexes that also contain calcium channels (Grunnet and Kaufmann, 2004; Fakler and Adelman, 2008) or in close proximity to the IP3-receptors and ryanodine receptors (Nelson et al., 1995; Weaver et al., 2007). This co-localization to areas of high local calcium levels adds a regulatory level. Consequently, it is difficult to predict the activity of BK channels in a given cell; but because of their synergistic activation by voltage and calcium, one can assume that pharmacological activation of the channel will tend to potentiate the existing physiological regulatory role that BK channels play. Hence, under most physiological conditions, drugs that increase the open probability

of BK channels will promote an efflux of potassium ions upon increases in intracellular Ca^{2+}. This hyperpolarization of cell membranes leads to cellular responses such as decreased cell excitability and smooth muscle relaxation. Due to this, drugs that activate BK channels could represent a novel therapeutic strategy for treatment of certain types of epilepsy, bladder instability, erectile dysfunction, ischemic heart disease and chronic obstructive pulmonary disease. Despite intense academic and industrial focus, no drugs intentionally targeting BK channels are on the market and to the best of our knowledge only one drug (Andolast) is currently in clinical development.

BK CHANNEL ACTIVATORS

Many different chemical entities have been found to increase the activity of BK channels. Within these entities, differences in calcium dependency, subunit composition and drug binding sites have been found. Based on their origin and structure the chemical entities can be classified in: (A) Endogenous BK channel modulators and structural analogs; (B) Naturally occurring BK channel openers and structural analogs; (C) Synthetic BK channel openers. Only a handful of interesting examples will be provided. For a comprehensive review see Nardi and Olesen (2008).

ENDOGENOUS BK CHANNEL MODULATORS AND STRUCTURAL ANALOGS

Endogenous chemicals such as arachidonic acid and the metabolites of cytochrome P450, epoxygenase and lipoxygenase, have been found to increase BK channel activity and be important regulators of vascular tone (for review see Félétou, 2009; Hou et al., 2009). Likewise, other unsaturated free fatty acids such as the omega-3 docosahexaenoic acid have also been found to increase BK channel activity (Denson et al., 2000) in an β-subunit dependent manner (Hoshi et al., 2013a). *In vivo* studies involving omega-3 docosahexaenoic acid report lowering of blood pressure in anesthetized wild-type, but not in *Slo-1* knockout mice (Hoshi et al., 2013b). Direct activation of BK channels by the sex hormone 17β-estradiol has also been reported, with its effect being dependent on the presence of the β-1 subunit (Valverde et al., 1999). 17β-estradiol was later shown to protect cardiac cells from ischemia reperfusion injuries via a possible mitochondrial BK effect, which led the authors to speculate its possible contribution to the increased incidence of post-menopausal heart attack (Ohya et al., 2005). Likewise other steroid hormones also modulate BK channel activity in a β-subunit dependent manner (King et al., 2006). Later it was found that already marketed drugs such as the xenoestrogen Tamoxifen also displays BK channel activation (Dick et al., 2001). However, the binding site and mechanism of both estrogen and xenoestrogens are still largely unknown. Other drugs on the market targeting CNS excitability and smooth muscle contraction also display BK channel activating properties in the therapeutic concentration range. These include the antiepileptics zonisamide (Huang et al., 2007) and chlorzoxazone (Liu et al., 2003), along with the phosphodiesterase III inhibitor (cilostazol) approved for treatment of intermittent claudication (Wu et al., 2004).

Recently the first peptide BK channel activator was described. Human β-defensin 2 is an antimicrobial peptide that is important in the innate immune system. It was found to increase the open probability of the BK channel complex when applied in physiologically relevant concentrations, with the effect being dependent on two amino-acids in the β-1 loop. Interestingly the authors further demonstrated that this peptide was down-regulated in sera from hypertensive patients, and that infusion of human β-defensin 2 in monkeys significantly reduced blood pressure (Liu et al., 2013).

NATURALLY OCCURRING BK CHANNEL OPENERS AND STRUCTURAL ANALOGS

Natural occurring BK channel activators are found in herbs, roots, fungi, and leaves, and have been used in folk medicine for treatment of asthma and smooth muscle disorders (Nardi et al., 2003). Isolation of natural occurring compounds revealed several BK channel activators including DHS-I that was found to dramatically increase the open probability of smooth muscle BK channels when applied to the intracellular site (McManus et al., 1993). The effect like many openers was dependent on the presence of β-1 subunit (McManus et al., 1995).

SYNTHETIC BK CHANNEL OPENERS

A wealth of synthetic BK channel activators capable of increasing channel open probabilities have been synthesized by a number of companies. NeuroSearch was among the first to report on small molecule BK channel activators such as NS004 (Olesen et al., 1994a). Later, another benzimidazolone in the form of NS1619 was introduced (Olesen et al., 1994b), which turned out to be the most used tool compound for studying the functional effect of BK channels. NS1619 has been used to study the involvement and therapeutic potential in smooth muscle disorders such as pulmonary hypertension (Vang et al., 2010; Revermann et al., 2014), erectile dysfunction (Spektor et al., 2002; González-Corrochano et al., 2013; Király et al., 2013), bladder instability (Soder and Petkov, 2011; La Fuente et al., 2014) and shock-induced vascular hyperactivity (Hu et al., 2014) along with research into migraines (Lu et al., 2014), inflammatory pain (Akerman et al., 2010), and cytoprotection (Xu et al., 2002; Gáspár et al., 2009).

The binding site of NS1619 has not been established. However, a recent report on another BK channel activator, Cym04, found that the activating effects of both drugs were dependent on the S6/RCK (Regulator of Conductance for K^+) linker; the sensitivity to both drugs was lost in BK channel variants with a distinct S6/RCK linker sequence having two deletion mutations. Whether the linker serves as a direct binding site, or is merely important for indirect conformational changes that lead to activation induced by the drug, is not known (Gessner et al., 2012). This linker has previously been proposed to act as a spring that transmits the conformational changes in the RCK upon calcium binding to opening of the channel's gate (Jiang et al., 2002).

The use of NS1619 is however hampered by a relative poor potency, and many off target effects such as inhibition of calcium channels. Consequently, we generated the biarylthiourea NS11021 as a more selective and potent tool compound to study BK channel function. Like many other activators, NS11021 works by shifting the voltage-activation curve of the channel to more negative potentials. At the single channel level this is reflected in

an increased open probability (Bentzen et al., 2007). This compound has been found to reduce infarct sizes and increase cardiac performance after ischemia, to improve mitochondrial respiration and to protect isolated cardiomyocytes from reperfusion injuries (Bentzen et al., 2009; Aon et al., 2010; Borchert et al., 2013). With regard to studies on smooth muscle dysfunction, NS11021 has been found to enhance erectile responses in rats primarily through its BK activating effects (Kun et al., 2009), and to evoke a pronounced relaxation in small human penile arteries (Király et al., 2013), both equipotent to that induced by the phosphodiesterase type-5 inhibitor sildenafil. In the guinea pig bladder NS11021 decreased excitability and contractility of urinary bladder smooth muscle. This effect was antagonized by iberiotoxin, a selective toxin inhibitor of BK channels (Layne et al., 2010). In the CNS NS11021 has been used to establish a role of BK channels in migraine (Wulf-Johansson et al., 2010; Liu et al., 2014). More recently a new family of BK channel activators called the GoSlo-SR family was introduced. They are capable of shifting the voltage dependence of BK activation by more than −100 mV when applied at 10 μM to rabbit bladder smooth muscle cells (Roy et al., 2012), with newer derivatives producing the same effects at lower concentrations (Roy et al., 2014). The voltage-sensitive dye bis-(1, 3-dibutylbarbituric acid) trimethine oxonol [DiBAC4(3)] at submicromalor levels (Morimoto et al., 2007) produced a shift in the voltage-dependence of the BK channel only when β-1 and β-4 subunits were present, whereas saturating concentrations (30 μM), produced huge negative shifts by up to −300 mV in the voltage-dependent activation of BK channels, but this effect was neither dependent on calcium nor the presence of β-subunits (Scornik et al., 2013). Subunit dependent potency and efficacy was also recently demonstrated for a series of N-arylbenzamide, with loss of effect when β-1 subunits were co-expressed (Kirby et al., 2013). We recently reported a novel positive BK channel modulator, NS19504 identified in a HTS FLIPR screen (Nausch et al., 2014). This compound represent a novel chemical scaffold and was observed to activate endogenous BK channels in native smooth muscle cells from guinea pig bladder, and to reduce spontaneous contractions in bladder strips in an iberiotoxin-sensitive manner. This suggests NS19504 could serve as a tool to elucidate BK channel function in complex tissues.

Over the last decades a range of structurally distinct synthetic BK channel activators have been produced by different pharmaceutical companies. One of these was BMS204352 that showed neuroprotective effects in animal models (Cheney et al., 2001), and was advanced to phase III clinical trials for the treatment of acute ischemic stroke but failed. A related compound, BMS223131, was shown to relax smooth muscle (Boy et al., 2004) and studied clinically for overactive bladder, but not developed. Other newly synthesized small molecule BK channel activators include thioureas, tetrahydroquinolines, such as compound 36 (Gore et al., 2010) and compound Z (Ponte et al., 2012), terpenes and benzofuroindoles. These were intended mostly for the treatment of urinary incontinence, overactive bladder, erectile dysfunction and stroke, and some of the drugs entered phase I and II clinical trials (for a comprehensive review of synthetic BK channel activators please refer to Nardi and Olesen, 2008). To our knowledge the only BK channel activating drug still considered for clinical development is Andolast. It is neither selective nor potent, but according to the manufacture, Rottapharm, it has shown clinical efficacy and an acceptable safety profile in mild/moderate asthma. However, phase III clinical trials have not been commenced.

BK CHANNELS IN THE WORKING MYOCARDIUM

Following a coronary artery occlusion, early restoration of blood perfusion to the ischemic myocardium by reperfusion therapy is of most importance in order to salvage the heart (Keeley et al., 2003). Paradoxically, restoration of blood flow to an ischemic myocardium also causes a so-called reperfusion injury by which cardiac myocytes that were viable immediately before reperfusion die giving rise to an increased infarct size. Because infarct size is recognized as the major determinant of myocardial functional recovery and mortality, any therapy aimed at reducing reperfusion injuries would be appreciated (for review see Yellon and Hausenloy, 2007). In 1986 Murry and colleagues were the first to demonstrate such an intervention when they showed that four short cycles of ischemia interspersed by reperfusion before the sustained ischemic event resulted in a dramatic reduction in infarct size of ∼75% (Murry et al., 1986). The nature of this protective mechanism probably involves cardiac K^+ channels.

The role of BK channels in cardiomyocytes was for many years neglected because of their absence from the plasma membrane of cardiomyocytes.

However, in 2002 the group of Brian O'Rourke demonstrated the presence of mitochondrial BK channel in cardiomyocytes (Xu et al., 2002). Patch-clamp recordings on mitoplasts from isolated guinea pig cardiomyocytes revealed a voltage- and calcium-dependent potassium current, with a single channel conductance of 307 pS that could be blocked by charybdotoxin, thereby resembling the properties of the plasma membrane BK channel, oriented with its C-terminal facing the mitochondrial matrix. They further demonstrated that mitochondrial uptake of K^+ was blunted by charybdotoxin and iberiotoxin and accelerated by the BK channel activator NS1619. The molecular identity of the channel remained unknown, but a link to cardioprotection was made by demonstrating that NS1619 when administered prior to the ischemic event, protected isolated perfused rabbit hearts from global ischemia and reperfusion injury, and that this effect was blocked when the BK channel blocker paxilline was co-administered (Xu et al., 2002). Subsequent studies have confirmed that administration of NS1619 protects the heart from ischemia-reperfusion injury both in mice (Wang et al., 2004; Redel et al., 2008), rats (Cao et al., 2005; Gao et al., 2005) guinea pigs (Stowe et al., 2006), rabbits (Shi et al., 2007), dogs (Shintani et al., 2004), and in aged rats (Heinen et al., 2014). Likewise, we found that NS11021 protected the heart from ischemia-reperfusion injury when applied prior to, or immediately after ischemia and that this could be antagonized by paxilline (Bentzen et al., 2009).

Because most of the data addressing the role of mitochondrial BK channels rely on pharmacological tools, controversies exist about the importance of mitochondrial BK channels in cardioprotection, as it has been found that both the activators and inhibitors used display unspecific effects. NS1619 at higher

concentrations directly inhibits L-type Ca^{2+} channels in rat ventricular myocytes (Park et al., 2007), Ca^{2+}-activated chloride currents (Saleh et al., 2007), and voltage-activated Ca^{2+}, K^+, and Na^+ channels (Edwards et al., 1994; Olesen et al., 1994b; Holland et al., 1996). Moreover, Cancherini et al. demonstrated that NS1619 also display non-ion channel effects on mitochondria, which they claim could explain some of the cardioprotective effects of NS1619 (Cancherini et al., 2007). A study using the cardioprotective BK channel activator NS11021, found that in nanomolar concentration NS11021 displayed beneficial effects on mitochondria, whereas when the concentration was increased, unspecific effects not related to mitochondrial BK channels were observed (Aon et al., 2010). Interestingly, a methylated analog of NS11021 that is inactive on BK channel, and does not provide cardioprotection (Bentzen et al., 2010) still possesses the unspecific effects on mitochondria of NS11021. This supports the notion that the cardioprotection mediated by NS11021 is BK channel related and not caused by the unspecific effects of the compound (Aon et al., 2010).

With regards to the BK channel inhibitors used as tool compounds for studying cardioprotection, there is concern about the use paxilline as it has been found at higher concentrations to inhibit the sarco/endoplasmatic reticulum Ca^{2+}-ATPase (SERCA) (Bilmen et al., 2002). Moreover, the cardioprotective effects of the BK channel activator isoflurane were abolished by paxilline both in wild-type and in *KCNMA1*$^{-/-}$ hearts, arguing for unspecific effects of paxilline (100). In addition, this study also suggested a dispensable role for *KCNMA1* in both mitochondrial K^+ transport and cardioprotection. Instead, using *C. elegans* as a model system they found that the related K^+ channel *Slo-2* was required for mitochondrial K^+ transport and cardioprotection.

Recently a study by Singh et al. helped to settle the controversies about the pharmacology and role of mitochondrial BK channels in cardioprotection, as they describe the molecular identity of the mitochondrial BK channel. They found that the mitochondrial BK channel in the heart is encoded by a splice variant (VEDEC) of the plasma membrane *KCNMA1*, and that it holds a 50-aa splice insert which they report is essential for trafficking to the mitochondria. Moreover they also demonstrated that the cardioprotection offered by NS1619 was lost in *KCNMA1* KO mice (Singh et al., 2013), cementing the involvement of BK channels in the cardioprotection conferred by NS1619.

Additionally, Soltysinska et al. recently report electrophysiological evidence for a BK-mediated current of 190 pS in mitoplasts from wild-type but not *KCNMA1*$^{-/-}$ cardiomyocytes. Moreover, changes in reactive oxygen species (ROS) production and attenuated oxidative phosphorylation capacities in *KCNMA1*$^{-/-}$ cardiomyocytes were observed. This suggests a mitochondrial role of *KCNMA1* encoded BK channels in fine-tuning the oxidative state of the cell (Soltysinska et al., 2014).

The discrepancy between these two studies demonstrating a role of *KCNMA1* in mitochondria and cardioprotection and the earlier work by Wojtovich on *Slo-2* where they found *KCNMA1* to be dispensable cannot be easily resolved; it should be remembered that different genetic models and experimental procedures were utilized in conducting the research. Moreover, as the authors also state, their results do neither preclude the presence of *KCNMA1* in the mitochondria nor a role for *KCNMA1* channels in other protective paradigms (Wojtovich et al., 2011).

It is difficult to imagine how the mitochondrial BK channel can at all function considering the negative inner mitochondrial membrane potential in the range of −180 mV. However, the phosphorylation state of the channel, the presence of auxillary subunits such as β-1 (Ohya et al., 2005), β-4 (Piwonska et al., 2008; Fretwell and Dickenson, 2009; Skalska et al., 2009) and perhaps LRRCs, could allow for channel activation under physiological and pathophysiological conditions, although the role of LRRCs needs to be explored further. Moreover, considering the high single channel conductance and the large driving force for K^+ entry, a too high open probability would be detrimental for the mitochondria, as the influx of K^+ to the matrix would depolarize the mitochondria. Therefore, the channel needs to be present in low abundance and/or with a tightly controlled open probability so that channel activation does not dissipate the proton motive force and ionic homeostasis.

How opening of mitochondrial BK channels confer cardioprotection is largely unknown, but could take place by affecting one or more of the following parameters:

1. Mitochondrial calcium accumulation: K^+ influx leads to a partial depolarization of the inner membrane potential (Aon et al., 2010). This will reduce the driving force for calcium entry, thereby reducing calcium overload during ischemia (Sato et al., 2005; Stowe et al., 2006). Indeed Stowe et al. found that pre-treatment with the BK channel activation by NS1619 in isolated perfused guinea pig hearts attenuated mitochondrial calcium accumulation following an ischemic event (Stowe et al., 2006). Moreover Singh et al. recently demonstrated that pre-treatment with NS1619 increased the Ca^{2+} retention capacity of mitochondria, and that this effect was lost in *KCNMA1*$^{-/-}$ mice (Singh et al., 2013).
2. ROS production: Studies on isolated mitochondria from hearts and neurons have demonstrated a reduced ROS production following stimulation with NS1619, along with the putative activator of mitochondrial BK channels, CGS7184 (Heinen et al., 2007; Kulawiak et al., 2008). This finding was confirmed in isolated heart studies (Stowe et al., 2006). Moreover, using isolated cadiomyocyte mitochondria from *KCNMA1*$^{-/-}$ mice it was found that the absence of BK channels resulted in elevated post-anoxic ROS levels (Soltysinska et al., 2014).
3. ATP preservation: Opening the mitochondrial BK channel has been shown to improve mitochondrial energy production, likely via swelling of the mitochondrial matrix (Aon et al., 2010). Likewise, genetically engineered loss of BK channels have been found to attenuate oxidative phosphorylation capacity (Soltysinska et al., 2014).

Taken together, at reperfusion, the improved oxidative phosphorylation, reduced ROS and mitochondrial calcium levels would prevent activation of the mitochondrial permeability transition pore (MPTP), which would otherwise lead to a non-selective permeabilization of the inner mitochondrial membrane resulting in

mitochondrial collapse, termination of ATP synthesis and cell death (Szabo and Zoratti, 2014).

Indeed, experiments on mitochondria isolated from the brain have demonstrated that opening of MPTP is accelerated in the presence of iberiotoxin (Cheng et al., 2008).

The recent findings using genetically engineered models have clearly demonstrated that *KCNMA1* is important for cardioprotection and mitochondrial function. However, much exploration is still needed in order to understand the physiological role of mitochondrial BK channels and how they confer cardioprotection.

From a therapeutic perspective, the recent findings are encouraging and help to clarify the role of BK channel activators in cardioprotection. Considering the importance of BK channels in controlling vascular tone, and their role in intra-cardiac neurons (Pérez et al., 2013; Wojtovich et al., 2013) it is also important to understand how, and if these extra-cardiac BK channels contribute to cardioprotection. Somewhat surprising, and to the best of our knowledge, little research has been undertaken to explore the importance of BK channel mediated flow changes in cardioprotection. Future work using tissue specific knock-down of *KCNMA1* could shed light on this interesting aspect.

BK CHANNELS IN BLADDER FUNCTION

The BK channel α-pore forming subunit and auxillary β-1 and β-4 subunits are expressed in urinary bladder smooth muscle (UBSM) cells (Ohya et al., 2000; Ohi et al., 2001; Petkov et al., 2001; Werner et al., 2007; Chen and Petkov, 2009; Hristov et al., 2011), where BK channels have been shown to be important regulators of bladder contractility and excitability (reviewed in Petkov, 2014).

One line of studies has demonstrated that inhibition of BK channel function markedly affects bladder function. Thus, the BK channel blockers iberiotoxin and paxilline have been shown to increase contractility in detrusor smooth muscle strips from humans (Darblade et al., 2006; La Fuente et al., 2014), pigs (Buckner et al., 2002), guinea pigs (Heppner et al., 1997; Kobayashi et al., 2000; Mora and Suarez-Kurtz, 2005), rats (Uchida et al., 2005), and mice (Herrera et al., 2005) when stimulated electrically or pharmacologically.

In addition, support for a role of BK channels in bladder function has been provided by genetic mouse models. In bladder strips from BK deficient mice lacking the BK pore forming α-subunit, contractility has been found to be markedly increased in response to both cholinergic and purinergic stimulation. This is reflected in a lower frequency of electrical stimulation required to elicit a contraction (Thorneloe et al., 2005; Werner et al., 2007). *In vivo*, *KCNMA1*$^{-/-}$ mice without functional BK-expression show a pronounced increase in voiding frequency and the occurrence of non-voiding bladder contractions. The mice have increased average bladder pressure and decreased void volume (Meredith et al., 2004; Thorneloe et al., 2005).

Mice deficient in the BK subunit β-1, the predominant accessory subunit in smooth muscle cells show moderate hypertension (Brenner et al., 2000; Plüger et al., 2000). Bladder strips from these mice showed increased amplitude and decreased frequency of phasic contraction similar to strips from WT mice treated with iberiotoxin. However, an additional response to iberiotoxin was still present in strips from KO mice (Petkov et al., 2001). Thus, studies on BK β-1 KO mice support a role of BK and specifically the β-1 subunit in modulation of smooth muscle contractility in the cardiovascular system and bladder.

Taken together, there is considerable support that inhibition or block of BK function adversely affects bladder function, increasing contractility in mice *in vivo* and in isolated bladder preparations from several species including mice, guinea pigs, and humans. This could suggest that increased BK channel activity in the bladder may counteract excessive detrusor contractions in bladder overactivity. Indeed, in pharmacological studies, positive modulation of BK channels by means of small molecules including NS-8, NS1608, and NS1619, have been shown to at varying degrees to reduce contractility of bladder strips from rats, guinea pigs, pigs (Imai et al., 2001; Malysz et al., 2004; Mora and Suarez-Kurtz, 2005), and humans (La Fuente et al., 2014). In one study, NS8 and NS1619 reduced rat bladder and aorta contractility with approximately similar potency (Malysz et al., 2004); while in another study, using NS-8, a clear selectivity for rat bladder over portal vein was reported. This supports the notion of BK channels being an attractive target in overactive bladder and urgency urinary incontinence. NS-8 was also reported to increase bladder capacity in urethane-anaesthetized rats after duodenal administration (Nicot et al., 1992).

In detrusor myocytes, BK channels may be activated both by increases in internal Ca^{2+} mediated via voltage-dependent L-type calcium channels and transiently by ryanodine receptors mediating local "Ca^{2+} sparks" as shown by patch clamp in guinea pig and human myocytes (Ohi et al., 2001; Herrera and Nelson, 2002; Hristov et al., 2011; Malysz et al., 2013). BK channels are thereby thought to mediate a negative feedback to limit contractions of the detrusor smooth muscle (Herrera et al., 2000, 2005) and to regulate patterns of spontaneous phasic contractions. Thus, in human UBSM cells BK channel outward currents have been shown to be a major determinant of repolarization following action potentials. Moreover, BK channel activity is also an important regulator of the resting potential and spontaneous phasic contraction amplitude, as well as the amplitude of electric field stimulation-induced contractions in UBSM strips (Hristov et al., 2011).

Spontaneous non-voiding contractions have been associated with bladder overactivity (Brading, 1997; Andersson, 2010); Inhibition of spontaneous phasic contractions, with minimal effect on voiding contractions have been suggested as a favorable profile in myogenic bladder overactivity. Interestingly, decreased BK channel α- and β-subunit expression have been reported in tissue from prostate patient bladders with detrusor overactivity, when compared with patients with similar obstruction but without detrusor overactivity (Chang et al., 2010). Therefore, positive modulation of BK channels may represent a strategy to alleviate bladder overactivity and increased detrusor contractility. The BK channel modulators NS11021 and NS19504 have been studied in guinea pig strips, and shown to potently inhibit spontaneous phasic contractions with more modest effects on contractions induced by electric field stimulation (Layne et al., 2010; Nausch et al., 2014). This supports the potential of BK

openers to normalize detrusor function in bladder overactivity with minimal effect on voiding contractions.

BK channel openers have also been suggested as a strategy to alleviate bladder overactivity of neurogenic origin. Interestingly, decreased BK UBSM expression, increased contractility and increased excitability in cells from humans with neurogenic detrusor overactivity have been reported (Hristov et al., 2013). However, in a study of the BK opener NS1619, effect on spontaneous contractions was only observed in detrusor strips from normal subjects, not in strips from patients with neurogenic bladder overactivity (Oger et al., 2011). In another study using isolated human UBSM cells and strips from subjects without prior history of overactive bladder, NS1619 was found to inhibit myogenic and nerve-evoked contractions (Hristov et al., 2012).

The underlying pathophysiology in overactive bladder is often not well understood and may be multifactorial, depending on both myogenic and neurogenic factors (Hanna-Mitchell et al., 2013). In addition to being a major regulator of UBSM contractility and excitability, BK channels may also be important for bladder afferent nerve activity and urothelial function. However, the role of BK channels in specific pathophysiologies associated with bladder overactivity are not known. Regulation of BK channel activity is complex; in the bladder it is regulated by voltage levels, calcium levels, kinase activity and by the action of signal molecules involved in dysregulation of UBSM function (Hristov et al., 2014). It was recently reported that BK channel activity mediates PGE2 induced increase in spontaneous phasic contractions in detrusor strips (Parajuli et al., 2014a). BK channel activity has also been linked functionally to changes in Ca^{2+} signaling and detrusor contractility induced by cAMP (Xin et al., 2014) and muscarinergic receptor activation (Parajuli et al., 2014b).

BK channel openers have been advanced to clinical studies for bladder dysfunction, but have so far not reached the market. TA-1702 (structure not disclosed) has been in Phase I by GSK under license from Tanabe. Although no public announcements have been made about withdrawal, it does not currently appear in the company's development pipeline. Nippon-Shinyaku discontinued development of NS-8 for overactive bladder (Announced Jan 16, 2007) due to lack of efficacy in proof-of-concept study with Apogepha. NS-8 reached Phase II in Europe and Phase I in Japan. A number of new BK openers representing new chemical scaffolds and showing improved selectivity have been reported, and may be promising leads for future development within bladder dysfunction.

Taken together, there is strong support that positive modulators of BK may have beneficial effect in instable or overactive bladder, without compromising normal voiding functions. Further studies using recently discovered BK openers, that possess improved selectivity are needed to translate findings from animal models to the human bladder, and to elucidate the potential for BK modulators in bladder overactivity disorders of myogenic or neurogenic origin.

BK CHANNELS IN THE CENTRAL NERVOUS SYSTEM

BK channels are abundantly expressed in the central nervous system (CNS) with various functions being associated to these channels in neurons. The channels are expressed in specific neurons, with subcellular localizations in presynaptic terminals, soma and dendrites (Knaus et al., 1996). As mentioned previously BK channels are distinctive among ion channels, being gated by both voltage and intracellular Ca^{2+}. This gating mechanism, in combination with their close proximity or even physical coupling to voltage-gated Ca^{2+} channels, makes BK channels potentially important components in negative feed-back mechanisms (Marrion and Tavalin, 1998; Grunnet and Kaufmann, 2004). In a situation of excessive Ca^{2+} influx e.g., in pre-synaptic terminals and corresponding disproportionate transmitter release, activation of BK channels by incoming Ca^{2+} will counterbalance this effect by hyperpolarizing the membrane, and thereby shut off voltage-dependent Ca^{2+} influx. In other words, BK channels can be seen as an "emergency break," which prevents transmitter related hyperexcitability and concomitant cell toxicity. An example of this is observed in Purkinje cells in the cerebellum (Swensen and Bean, 2003; Womack and Khodakhah, 2004). From the somatic localization, BK channels are thought to exert their function by shaping the repolarization and thereby after-hyperpolarization (AHP) of action potentials. The after-hyperpolarization is divided into three phases; a fast (fAHP), an intermediate (mAHP) and a slow (sAHP) part. BK channels participate in the fast phase and have been especially well studied in the CA1 region of the hippocampus, where fAHP is inhibited by addition of TEA and the more selective BK inhibitor iberiotoxin (Storm, 1987). It should be mentioned that BK α-subunit knock-out mice are viable, but have several phenotypes that include CNS related functions. Among these are ataxia and high frequency hearing loss (Salkoff et al., 2006).

The localization of BK channels in Purkinje cells from the cerebellum points to the important role of BK in motor coordination, since Purkinje neurons are the sole output of the cerebellar cortex. As a consequence *KCNMA1* (−/−) knock-out mice have impaired motor coordination and ataxia (Sausbier et al., 2004). These results have later been consolidated in transgenic mice lacking BK channels exclusively in the Purkinje cells of the cerebellum (Chen et al., 2010). BK channel impact on locomotor impairments may also rely on the channel expression in basal ganglia, where prominent expression of BK channels has been demonstrated in substantia nigra, pars reticulate, globus pallidus, and entopeduncular nucleus. All these areas have a potential impact on the pathophysiology of tremors and ataxia (Sausbier et al., 2006).

An auditory phenotype of the BK knock-outs also points to a function of the channels in hearing. When knocking out the BK α-subunit, but not the β-subunit, a resulting progressive hearing loss is observed. Hearing loss at high frequencies is evident, but only after 8 weeks of age and onward. This point to irreversible progressive loss of cochlear outer hair cells. A similar phenotype can be observed by genetic deletion or pharmacological inhibition of the voltage-dependent potassium channel KCNQ4 (Jentsch, 2000; Rüttiger et al., 2004).

BK channels have also been associated to circadian rhythms. Circadian rhythms in mammals are driven by a central oscillator in the hypothalamic suprachiasmatic nucleus (SCN). This nucleus is constituted by relatively few (about 10,000) neurons that are characterized to oscillate in a 24 h rhythms. These cells

are unique in the sense that they can generate rhythms. The SCN neurons are central in understanding the pathophysiology of sleep and circadian diseases; these neurons are innervated from the retina and this projection is essential in setting the clock to the external light-dark regimen (entrainment). Entrainment maintains the internal rhythms of exactly 24 h.

BK channels have been suggested as a novel and attractive target for sleep and circadian diseases (Meredith et al., 2006). BK channels are expressed in the SCN, and the expression of *KCNMA1* (BK α-subunit) mRNA is regulated in a diurnal manner. Most interestingly, *KCNMA1* deficient mice have a larger amplitude in electrical activity of SCN neurons. The same mice had a weak rhythm in locomotor behavior. Taken together, these data suggest that BK modulators could influence the endogenous rhythm structure, and perhaps be useful in patients with circadian and sleep dysfunctions.

BK channels have also been associated to various epileptogenic phenotypes but the picture is not straight forward. In its simplest term, inhibition of BK channels should result in general depolarization and thereby hyperexcitability. Consequently it could be expected that epilepsy could be treated with pharmacological activation of BK channels. There is evidence of BK loss-of-function in relation to epilepsy, however to tamper the picture also BK gain-of-function has been linked to the disease. In preclinical models of inherited generalized tonic-clonic epilepsy and temporal lobe epilepsy (TLE) animals demonstrated a reduction in AHP. This decrease was suggested to be due to a reduction in Ca^{2+}-dependent K^+ conductance, that could be related to BK channels, but the exact nature of the specific conductance was not addressed by application of specific pharmacological tools (Verma-Ahuja et al., 1995). Also in a pharmacological TLE model, application of the muscarinic receptor agonist pilocarpine produced a down regulation of BK channels, especially in the hippocampus and cortex (Pacheco Otalora et al., 2008). The picture is blurred by the fact that BK channel gain-of-function, at least as a consequence of β4-subunit knock-out, that leads to an overall increase in BK activity, can result in TLE (Brenner et al., 2005). Reports in favor of increased BK activity as a cause for epilepsy, are observations that gain-of-function BK channels can result in generalized tonic-clonic seizures (Shruti et al., 2008). Target engagement in these findings was substantiated by application of paxilline that could suppress the epileptogenic phenotype (Sheehan et al., 2009).

When it comes to clinical evidence of BK channel functions, CNS is actually one of the only areas where reports exist. The first associations between BK activity and epilepsy in patients were concomitant, with the general notion of loss of BK channel activity as the underlying cause of hyperactivity and epilepsy. In TLE patients a reduction in fAHP and a down-regulation of BK channels was observed (N'Gouemo, 2011). Again the picture is distorted by the fact that human absence epilepsy and idiopathic generalized epilepsy are directly associated to a BK channel gain-of-function mutation (Du et al., 2005). The gain-of-function is a single amino acid substitution (D434G) in the BK α-subunit, that among other things, increases Ca^{2+} sensitivity and mean open time of the channel (Du et al., 2005). These observations are good examples of how the prediction of the physiological outcome of a certain ion channel mutation can be very difficult. The intuitive feeling will be that increasing a potassium conductance and thereby hyperpolarizing a cell membrane should dampen cellular excitability. However, in intact cell system this logic rational is challenged by the fact that hyperpolarization will result in more efficient release of Na^+ channels from inactivation, resulting in a higher number of Na^+ channels being available for activation in a subsequent action potential. The exact phenotype of a given ion channel mutation, will therefore always be the sum of the orchestrated activity of a number of different ion channels, in combination with other membrane proteins and the entire cellular machinery. When considering CNS it should also be kept in mind that the brain consists of both excitable and inhibitory neurons, and the exact interplay between neuronal circuits and cellular and subcellular positions of the mutated proteins will impact the final phenotype of any given mutation.

CONCLUDING REMARKS

It is now two decades since the first small molecule BK channel opener was reported. Over these years a wealth of BK channel openers, including naturally occurring and synthetic compounds have been discovered, and recently the first peptide opener of BK channels was disclosed (Liu et al., 2013). The structural diversity of these molecules is also reflected in their mode of action. Some compounds display β-subunit dependency (Valverde et al., 1999), whereas others are dependent upon intracellular calcium concentrations (Bentzen et al., 2007).

The pharmaceutical interest in BK channel activators has been spurred on by the last decade's biological research, reporting the important roles of BK channels in both health and disease. By integrating changes in intracellular calcium and membrane potential, the BK channel serves as an important negative feedback system, linking increases in intracellular calcium to outward hyperpolarizing potassium current. Using BK channel openers, evidence of the channels role and possible therapeutic potential has been established with respect to a number of indications, characterized by hyper excitability and smooth muscle dysfunction. Despite vast amounts of preclinical work and basic research, clinical evidence for the usefulness of BK channel openers is still missing and clinical development of BK channel activators has in large been discontinued and is to our knowledge currently not taking place except for Andolast. Translating preclinical findings to the clinic is tough, with failure being caused by many factors. However, the lack of potency and especially the poor selectivity of compounds toward off-targets are troublesome, as it can obscure preclinical proof-of-concept findings. Despite substantial amount of *in vitro* based BK pharmacology literature, it is striking how few *in vivo* evaluations have been published. Although only speculative, this could point to the challenges for *in vivo* applications of many BK activators, thereby also adding to the lack of successful clinical attempts to reveal the potential for BK activators. Therefore, a need for more potent, selective and *in vivo* applicable compounds is still warranted.

AUTHOR CONTRIBUTIONS

All authors contributed to the drafting, writing, revising, and approval of the manuscript.

ACKNOWLEDGMENT

This work was financially supported by The Danish National Research Foundation.

REFERENCES

Akerman, S., Holland, P. R., Lasalandra, M. P., and Goadsby, P. J. (2010). Inhibition of trigeminovascular dural nociceptive afferents by Ca(2+)-activated K(+) (MaxiK/BK(Ca)) channel opening. *Pain* 151, 128–136. doi: 10.1016/j.pain.2010.06.028

Alioua, A., Li, M., Wu, Y., Stefani, E., and Toro, L. (2011). Unconventional myristoylation of large-conductance Ca^{2+}-activated K^{+} channel (Slo1) via serine/threonine residues regulates channel surface expression. *Proc. Natl. Acad. Sci. U.S.A.* 108, 10744–10749. doi: 10.1073/pnas.1008863108

Andersson, K.-E. (2010). Detrusor myocyte activity and afferent signaling. *Neurourol. Urodyn*. 29, 97–106. doi: 10.1002/nau.20784

Aon, M. A., Cortassa, S., Wei, A.-C., Grunnet, M., and O'Rourke, B. (2010). Energetic performance is improved by specific activation of K+ fluxes through K(Ca) channels in heart mitochondria. *Biochim. Biophys. Acta* 1797, 71–80. doi: 10.1016/j.bbabio.2009.08.002

Avdonin, V., Tang, X. D., and Hoshi, T. (2003). Stimulatory action of internal protons on Slo1 BK channels. *Biophys. J.* 84, 2969–2980. doi: 10.1016/S0006-3495(03)70023-X

Bentzen, B. H., Andersen, R. W., Olesen, S.-P., Grunnet, M., and Nardi, A. (2010). Synthesis and characterisation of NS13558: a new important tool for addressing KCa1. 1 channel function *ex vivo*. *Naunyn Schmiedebergs Arch. Pharmacol*. 381, 271–283. doi: 10.1007/s00210-009-0456-2

Bentzen, B. H., Nardi, A., Calloe, K., Madsen, L. S., Olesen, S.-P., and Grunnet, M. (2007). The small molecule NS11021 is a potent and specific activator of Ca2+-activated big-conductance K+ channels. *Mol. Pharmacol*. 72, 1033–1044. doi: 10.1124/mol.107.038331

Bentzen, B. H., Osadchii, O., Jespersen, T., Hansen, R. S., Olesen, S.-P., and Grunnet, M. (2009). Activation of big conductance Ca2+-activated K+ channels (BK) protects the heart against ischemia–reperfusion injury. *Pflügers Arch.* 457, 979–988. doi: 10.1007/s00424-008-0583-5

Bilmen, J. G., Wootton, L. L., and Michelangeli, F. (2002). The mechanism of inhibition of the sarco/endoplasmic reticulum Ca2+ ATPase by paxilline. *Arch. Biochem. Biophys.* 406, 55–64. doi: 10.1016/S0003-9861(02)00240-0

Borchert, G. H., Hlaváčková, M., and Kolář, F. (2013). Pharmacological activation of mitochondrial BK(Ca) channels protects isolated cardiomyocytes against simulated reperfusion-induced injury. *Exp. Biol. Med. (Maywood)* 233–241. doi: 10.1177/1535370212474596

Boy, K. M., Guernon, J. M., Sit, S.-Y., Xie, K., Hewawasam, P., Boissard, C. G., et al. (2004). 3-Thio-quinolinone maxi-K openers for the treatment of erectile dysfunction. *Bioorg. Med. Chem. Lett*. 14, 5089–5093. doi: 10.1016/j.bmcl.2004.07.080

Brading, A. F. (1997). A myogenic basis for the overactive bladder. *Urology* 50(Suppl. 6A), 57–67. discussion: 68–73.

Brenner, R., Chen, Q. H., Vilaythong, A., Toney, G. M., Noebels, J. L., and Aldrich, R. W. (2005). BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat. Neurosci.* 8, 1752–1759. doi: 10.1038/nn1573

Brenner, R., Peréz, G. J., Bonev, A. D., Eckman, D. M., Kosek, J. C., Wiler, S. W., et al. (2000). Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature* 407, 870–876. doi: 10.1038/35038011

Buckner, S. A., Milicic, I., Daza, A. V., Coghlan, M. J., and Gopalakrishnan, M. (2002). Spontaneous phasic activity of the pig urinary bladder smooth muscle: characteristics and sensitivity to potassium channel modulators. *Br. J. Pharmacol.* 135, 639–648. doi: 10.1038/sj.bjp.0704499

Butler, A., Tsunoda, S., McCobb, D. P., Wei, A., and Salkoff, L. (1993). mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels. *Science* 261, 221–224. doi: 10.1126/science.7687074

Cancherini, D. V., Queliconi, B. B., and Kowaltowski, A. J. (2007). Pharmacological and physiological stimuli do not promote Ca(2+)-sensitive K+ channel activity in isolated heart mitochondria. *Cardiovasc. Res.* 73, 720–728. doi: 10.1016/j.cardiores.2006.11.035

Cao, C.-M., Xia, Q., Gao, Q., Chen, M., and Wong, T.-M. (2005). Calcium-activated potassium channel triggers cardioprotection of ischemic preconditioning. *J. Pharmacol. Exp. Ther.* 312, 644–650. doi: 10.1124/jpet.104.074476

Chang, S., Gomes, C. M., Hypolite, J. A., Marx, J., Alanzi, J., Zderic, S. A., et al. (2010). Detrusor overactivity is associated with downregulation of large-conductance calcium- and voltage-activated potassium channel protein. *Am. J. Physiol. Renal Physiol.* 298, F1416–F1423. doi: 10.1152/ajprenal.00595.2009

Chen, M., and Petkov, G. V. (2009). Identification of large conductance calcium activated potassium channel accessory beta4 subunit in rat and mouse bladder smooth muscle. *J. Urol.* 182, 374–381. doi: 10.1016/j.juro.2009.02.109

Chen, X., Kovalchuk, Y., Adelsberger, H., Henning, H. A., Sausbier, M., Wietzorrek, G., et al. (2010). Disruption of the olivo-cerebellar circuit by Purkinje neuron-specific ablation of BK channels. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12323–12328. doi: 10.1073/pnas.1001745107

Cheney, J. A., Weisser, J. D., Bareyre, F. M., Laurer, H. L., Saatman, K. E., Raghupathi, R., et al. (2001). The maxi-K channel opener BMS-204352 attenuates regional cerebral edema and neurologic motor impairment after experimental brain injury. *J. Cereb. Blood Flow Metab.* 21, 396–403. doi: 10.1097/00004647-200104000-00008

Cheng, Y., Gu, X. Q., Bednarczyk, P., Wiedemann, F. R., Haddad, G. G., and Siemen, D. (2008). Hypoxia increases activity of the BK-channel in the inner mitochondrial membrane and reduces activity of the permeability transition pore. *Cell. Physiol. Biochem.* 22, 127–136. doi: 10.1159/000149790

Darblade, B., Behr-Roussel, D., Oger, S., Hieble, J.-P., Lebret, T., Gorny, D., et al. (2006). Effects of potassium channel modulators on human detrusor smooth muscle myogenic phasic contractile activity: potential therapeutic targets for overactive bladder. *Urology* 68, 442–448. doi: 10.1016/j.urology.2006.03.039

Denson, D. D., Wang, X., Worrell, R. T., and Eaton, D. C. (2000). Effects of fatty acids on BK channels in GH(3) cells. *Am. J. Physiol. Cell Physiol.* 279, C1211–C1219.

Dick, G. M., Rossow, C. F., Smirnov, S., Horowitz, B., and Sanders, K. M. (2001). Tamoxifen activates smooth muscle BK channels through the regulatory beta 1 subunit. *J. Biol. Chem*. 276, 34594–34599. doi: 10.1074/jbc.M104689200

Du, W., Bautista, J. F., Yang, H., Diez-Sampedro, A., You, S.-A., Wang, L., et al. (2005). Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat. Genet.* 37, 733–738. doi: 10.1038/ng1585

Edwards, G., Niederste-Hollenberg, A., Schneider, J., Noack, T., and Weston, A. H. (1994). Ion channel modulation by NS1619, the putative BKCa channel opener, in vascular smooth muscle. *Br. J. Pharmacol.* 113, 1538–1547. doi: 10.1111/j.1476-5381.1994.tb17171.x

Fakler, B., and Adelman, J. P. (2008). Control of K(Ca) channels by calcium nano/microdomains. *Neuron* 59, 873–881. doi: 10.1016/j.neuron.2008.09.001

Félétou, M. (2009). Calcium-activated potassium channels and endothelial dysfunction: therapeutic options? *Br. J. Pharmacol.* 156, 545–562. doi: 10.1111/j.1476-5381.2009.00052.x

Fernández-Fernández, J. M., Tomás, M., Vázquez, E., Orio, P., Latorre, R., Sentí, M., et al. (2004). Gain-of-function mutation in the KCNMB1 potassium channel subunit is associated with low prevalence of diastolic hypertension. *J. Clin. Invest.* 113, 1032–1039. doi: 10.1172/JCI200420347

Fretwell, L., and Dickenson, J. M. (2009). Role of large-conductance Ca(2+) -activated potassium channels in adenosine A(1) receptor-mediated pharmacological preconditioning in H9c2 cells. *Eur. J. Pharmacol*. 618, 37–44. doi: 10.1016/j.ejphar.2009.07.008

Gao, Q., Zhang, S.-Z., Cao, C.-M., Bruce, I. C., and Xia, Q. (2005). The mitochondrial permeability transition pore and the Ca2+-activated K+ channel contribute to the cardioprotection conferred by tumor necrosis factor-alpha. *Cytokine* 32, 199–205. doi: 10.1016/j.cyto.2005.09.008

Gáspár, T., Domoki, F., Lenti, L., Katakam, P. V. G., Snipes, J. A., Bari, F., et al. (2009). Immediate neuronal preconditioning by NS1619. *Brain Res*. 1285, 196–207. doi: 10.1016/j.brainres.2009.06.008

Gessner, G., Cui, Y.-M., Otani, Y., Ohwada, T., Soom, M., Hoshi, T., et al. (2012). Molecular mechanism of pharmacological activation of BK channels. *Proc. Natl. Acad. Sci. U.S.A*. 109, 3552–3557. doi: 10.1073/pnas.1114321109

González-Corrochano, R., La Fuente, J., Cuevas, P., Fernández, A., Chen, M., Sáenz de Tejada, I., et al. (2013). Ca2+ -activated K+ channel (KCa) stimulation improves relaxant capacity of PDE5 inhibitors in human penile arteries and recovers the reduced efficacy of PDE5 inhibition in diabetic erectile dysfunction. *Br. J. Pharmacol.* 169, 449–461. doi: 10.1111/bph.12143

Gore, V. K., Ma, V. V., Yin, R., Ligutti, J., Immke, D., Doherty, E. M., et al. (2010). Structure-activity relationship (SAR) investigations of tetrahydroquinolines as BKCa agonists. *Bioorg. Med. Chem. Lett.* 20, 3573–3578. doi: 10.1016/j.bmcl.2010.04.125

Grunnet, M., and Kaufmann, W. A. (2004). Coassembly of big conductance Ca2+-activated K+ channels and L-type voltage-gated Ca2+ channels in rat brain. *J. Biol. Chem.* 279, 36445–36453. doi: 10.1074/jbc.M402254200

Hanna-Mitchell, A. T., Kashyap, M., Chan, W. V., Andersson, K.-E., and Tannenbaum, C. (2013). Pathophysiology of idiopathic overactive bladder and the success of treatment: a systematic review from ICI-RS. *Neurourol. Urodyn.* 33, 611–617. doi: 10.1002/nau.22582

Heinen, A., Aldakkak, M., Stowe, D. F., Rhodes, S. S., Riess, M. L., Varadarajan, S. G., et al. (2007). Reverse electron flow-induced ROS production is attenuated by activation of mitochondrial Ca2+-sensitive K+ channels. *Am. J. Physiol. Heart Circ. Physiol.* 293, H1400–H1407. doi: 10.1152/ajpheart.00198.2007

Heinen, A., Ströthoff, M., Schmidt, A., Stracke, N., Behmenburg, F., Bauer, I., et al. (2014). Pharmacological options to protect the aged heart from ischemia and reperfusion injury by targeting the PKA-BKCa signaling pathway. *Exp. Gerontol.* 56, 99–105. doi: 10.1016/j.exger.2014.03.029

Heppner, T. J., Bonev, A. D., and Nelson, M. T. (1997). Ca(2+)-activated K+ channels regulate action potential repolarization in urinary bladder smooth muscle. *Am. J. Physiol.* 273(1 Pt 1), C110–C117.

Herrera, G. M., Etherton, B., Nausch, B., and Nelson, M. T. (2005). Negative feedback regulation of nerve-mediated contractions by KCa channels in mouse urinary bladder smooth muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 289, R402–R409. doi: 10.1152/ajpregu.00488.2004

Herrera, G. M., Heppner, T. J., and Nelson, M. T. (2000). Regulation of urinary bladder smooth muscle contractions by ryanodine receptors and BK and SK channels. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 279, R60–R68.

Herrera, G. M., and Nelson, M. T. (2002). Differential regulation of SK and BK channels by Ca(2+) signals from Ca(2+) channels and ryanodine receptors in guinea-pig urinary bladder myocytes. *J. Physiol.* 541(Pt 2), 483–492. doi: 10.1113/jphysiol.2002.017707

Holland, M., Langton, P. D., Standen, N. B., and Boyle, J. P. (1996). Effects of the BKCa channel activator, NS1619, on rat cerebral artery smooth muscle. *Br. J. Pharmacol.* 117, 119–129. doi: 10.1111/j.1476-5381.1996.tb15163.x

Hoshi, T., Tian, Y., Xu, R., Heinemann, S. H., and Hou, S. (2013a). Mechanism of the modulation of BK potassium channel complexes with different auxiliary subunit compositions by the omega-3 fatty acid DHA. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4822–4827. doi: 10.1073/pnas.1222003110

Hoshi, T., Wissuwa, B., Tian, Y., Tajima, N., Xu, R., Bauer, M., et al. (2013b). Omega-3 fatty acids lower blood pressure by directly activating large-conductance Ca^{2+}-dependent K^{+} channels. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4816–4821. doi: 10.1073/pnas.1221997110

Hosseinzadeh, Z., Almilaji, A., Honisch, S., Pakladok, T., Liu, G., Bhavsar, S. K., et al. (2014). Upregulation of the large conductance voltage- and Ca2+-activated K+ channels by Janus kinase 2. *Am. J. Physiol. Cell Physiol.* 306, C1041–C1049. doi: 10.1152/ajpcell.00209.2013

Hou, S., Heinemann, S. H., and Hoshi, T. (2009). Modulation of BKCa channel gating by endogenous signaling molecules. *Physiology (Bethesda)* 24, 26–35. doi: 10.1152/physiol.00032.2008

Hou, S., Vigeland, L. E., Zhang, G., Xu, R., Li, M., Heinemann, S. H., et al. (2010). Zn2+ activates large conductance Ca2+-activated K+ channel via an intracellular domain. *J. Biol. Chem.* 285, 6434–6442. doi: 10.1074/jbc.M109.069211

Hristov, K. L., Afeli, S. A. Y., Parajuli, S. P., Cheng, Q., Rovner, E. S., and Petkov, G. V. (2013). Neurogenic detrusor overactivity is associated with decreased expression and function of the large conductance voltage- and Ca(2+)-activated K(+) channels. *PLoS ONE* 8:e68052. doi: 10.1371/journal.pone.0068052

Hristov, K. L., Chen, M., Kellett, W. F., Rovner, E. S., and Petkov, G. V. (2011). Large-conductance voltage- and Ca2+-activated K+ channels regulate human detrusor smooth muscle function. *Am. J. Physiol. Cell Physiol.* 301, C903–C912. doi: 10.1152/ajpcell.00495.2010

Hristov, K. L., Parajuli, S. P., Soder, R. P., Cheng, Q., Rovner, E. S., and Petkov, G. V. (2012). Suppression of human detrusor smooth muscle excitability and contractility via pharmacological activation of large conductance Ca2+-activated K+ channels. *Am. J. Physiol. Cell Physiol.* 302, C1632–C1641. doi: 10.1152/ajpcell.00417.2011

Hristov, K. L., Smith, A. C., Parajuli, S. P., Malysz, J., and Petkov, G. V. (2014). Large-conductance voltage- and Ca2+-activated K+ channel regulation by protein kinase C in guinea pig urinary bladder smooth muscle. *Am. J. Physiol. Cell Physiol.* 306, C460–C470. doi: 10.1152/ajpcell.00325.2013

Hu, Y., Yang, G., Xiao, X., Liu, L., and Li, T. (2014). Bkca opener, NS1619 pretreatment protects against shock-induced vascular hyporeactivity through PDZ-Rho GEF-RhoA-Rho kinase pathway in rats. *J. Trauma Acute Care Surg.* 76, 394–401. doi: 10.1097/TA.0b013e3182aa2d98

Huang, C.-W., Huang, C.-C., and Wu, S.-N. (2007). Activation by zonisamide, a newer antiepileptic drug, of large-conductance calcium-activated potassium channel in differentiated hippocampal neuron-derived H19-7 cells. *J. Pharmacol. Exp. Ther.* 321, 98–106. doi: 10.1124/jpet.106.116954

Imai, T., Okamoto, T., Yamamoto, Y., Tanaka, H., Koike, K., Shigenobu, K., et al. (2001). Effects of different types of K+ channel modulators on the spontaneous myogenic contraction of guinea-pig urinary bladder smooth muscle. *Acta Physiol. Scand.* 173, 323–333. doi: 10.1046/j.1365-201X.2001.00908.x

Jeffries, O., Geiger, N., Rowe, I. C. M., Tian, L., McClafferty, H., Chen, L., et al. (2010). Palmitoylation of the S0-S1 linker regulates cell surface expression of voltage- and calcium-activated potassium (BK) channels. *J. Biol. Chem.* 285, 33307–33314. doi: 10.1074/jbc.M110.153940

Jentsch, T. J. (2000). Neuronal KCNQ potassium channels: physiology and role in disease. *Nat. Rev. Neurosci.* 1, 21–30. doi: 10.1038/35036198

Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T., and MacKinnon, R. (2002). Crystal structure and mechanism of a calcium-gated potassium channel. *Nature* 417, 515–522. doi: 10.1038/417515a

Keeley, E. C., Boura, J. A., and Grines, C. L. (2003). Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: a quantitative review of 23 randomised trials. *Lancet* 361, 13–20. doi: 10.1016/S0140-6736(03)12113-7

King, J. T., Lovell, P. V., Rishniw, M., Kotlikoff, M. I., Zeeman, M. L., and McCobb, D. P. (2006). Beta2 and beta4 subunits of BK channels confer differential sensitivity to acute modulation by steroid hormones. *J. Neurophysiol.* 95, 2878–2888. doi: 10.1152/jn.01352.2005

Király, I., Pataricza, J., Bajory, Z., Simonsen, U., Varro, A., Papp, J. G., et al. (2013). Involvement of large-conductance Ca(2+) -activated K(+) channels in both nitric oxide and endothelium-derived hyperpolarization-type relaxation in human penile small arteries. *Basic Clin. Pharmacol. Toxicol.* 113, 19–24. doi: 10.1111/bcpt.12059

Kirby, R. W., Martelli, A., Calderone, V., McKay, N. G., and Lawson, K. (2013). Large conductance Ca(2+)-activated K(+) channel (BKCa) activating properties of a series of novel N-arylbenzamides: channel subunit dependent effects. *Bioorg. Med. Chem.* 21, 4186–4191. doi: 10.1016/j.bmc.2013.05.003

Knaus, H. G., Folander, K., Garcia-Calvo, M., Garcia, M. L., Kaczorowski, G. J., Smith, M., et al. (1994). Primary sequence and immunological characterization of beta-subunit of high conductance Ca(2+)-activated K+ channel from smooth muscle. *J. Biol. Chem.* 269, 17274–17278.

Knaus, H. G., Schwarzer, C., Koch, R. O., Eberhart, A., Kaczorowski, G. J., Glossmann, H., et al. (1996). Distribution of high-conductance Ca(2+)-activated K+ channels in rat brain: targeting to axons and nerve terminals. *J. Neurosci.* 16, 955–963.

Kobayashi, H., Adachi-Akahane, S., and Nagao, T. (2000). Involvement of BK(Ca) channels in the relaxation of detrusor muscle via beta-adrenoceptors. *Eur. J. Pharmacol.* 404, 231–238. doi: 10.1016/S0014-2999(00)00606-3

Kulawiak, B., Kudin, A. P., Szewczyk, A., and Kunz, W. S. (2008). BK channel openers inhibit ROS production of isolated rat brain mitochondria. *Exp. Neurol.* 212, 543–547. doi: 10.1016/j.expneurol.2008.05.004

Kun, A., Matchkov, V. V., Stankevicius, E., Nardi, A., Hughes, A. D., Kirkeby, H. J., et al. (2009). NS11021, a novel opener of large-conductance Ca(2+)-activated K(+) channels, enhances erectile responses in rats. *Br. J. Pharmacol.* 158, 1465–1476. doi: 10.1111/j.1476-5381.2009.00404.x

La Fuente, J. M., Fernández, A., Cuevas, P., González-Corrochano, R., Chen, M. X., and Angulo, J. (2014). Stimulation of large-conductance calcium-activated potassium channels inhibits neurogenic contraction of human bladder from patients with urinary symptoms and reverses acetic acid-induced bladder hyperactivity in rats. *Eur. J. Pharmacol.* 735, 68–76. doi: 10.1016/j.ejphar.2014.03.060

Layne, J. J., Nausch, B., Olesen, S.-P., and Nelson, M. T. (2010). BK channel activation by NS11021 decreases excitability and contractility of urinary bladder smooth muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 298, R378–R384. doi: 10.1152/ajpregu.00458.2009

Liu, H., Xin, T., He, W., Li, F., and Su, Z.-Q. (2014). Myelinated Ah-type trigeminal ganglion neurons in female rats: neuroexcitability, chemosensitivity to histamine, and potential clinical impact. *Neurosci. Lett.* 567, 74–79. doi: 10.1016/j.neulet.2014.03.039

Liu, R., Zhang, Z., Liu, H., Hou, P., Lang, J., Wang, S., et al. (2013). Human β-defensin 2 is a novel opener of Ca2+-activated potassium channels and

induces vasodilation and hypotension in monkeys. *Hypertension* 62, 415–425. doi: 10.1161/HYPERTENSIONAHA.111.01076

Liu, Y. C., Lo, Y. K., and Wu, S. N. (2003). Stimulatory effects of chlorzoxazone, a centrally acting muscle relaxant, on large conductance calcium-activated potassium channels in pituitary GH3 cells. *Brain Res.* 959, 86–97. doi: 10.1016/S0006-8993(02)03730-7

Lu, R., Lukowski, R., Sausbier, M., Zhang, D. D., Sisignano, M., Schuh, C.-D., et al. (2014). BKCa channels expressed in sensory neurons modulate inflammatory pain in mice. *Pain* 155, 556–565. doi: 10.1016/j.pain.2013.12.005

Magleby, K. L. (2003). Gating mechanism of BK (Slo1) channels: so near, yet so far. *J. Gen. Physiol.* 121, 81–96. doi: 10.1085/jgp.20028721

Malysz, J., Buckner, S. A., Daza, A. V., Milicic, I., Perez-Medrano, A., and Gopalakrishnan, M. (2004). Functional characterization of large conductance calcium-activated K+ channel openers in bladder and vascular smooth muscle. *Naunyn Schmiedebergs Arch. Pharmacol.* 369, 481–489. doi: 10.1007/s00210-004-0920-y

Malysz, J., Rovner, E. S., and Petkov, G. V. (2013). Single-channel biophysical and pharmacological characterizations of native human large-conductance calcium-activated potassium channels in freshly isolated detrusor smooth muscle cells. *Pflügers Arch.* 465, 965–975. doi: 10.1007/s00424-012-1214-8

Marrion, N. V., and Tavalin, S. J. (1998). Selective activation of Ca2+-activated K+ channels by co-localized Ca2+ channels in hippocampal neurons. *Nature* 395, 900–905. doi: 10.1038/27674

McManus, O. B., Harris, G. H., Giangiacomo, K. M., Feigenbaum, P., Reuben, J. P., Addy, M. E., et al. (1993). An activator of calcium-dependent potassium channels isolated from a medicinal herb. *Biochemistry (Mosc.)* 32, 6128–6133. doi: 10.1021/bi00075a002

McManus, O. B., Helms, L. M., Pallanck, L., Ganetzky, B., Swanson, R., and Leonard, R. J. (1995). Functional role of the beta subunit of high conductance calcium-activated potassium channels. *Neuron* 14, 645–650. doi: 10.1016/0896-6273(95)90321-6

Meera, P., Wallner, M., Song, M., and Toro, L. (1997). Large conductance voltage- and calcium-dependent K+ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0-S6), an extracellular N terminus, and an intracellular (S9-S10) C terminus. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14066–14071. doi: 10.1073/pnas.94.25.14066

Meera, P., Wallner, M., and Toro, L. (2000). A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca2+-activated K+ channel resistant to charybdotoxin and iberiotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5562–5567. doi: 10.1073/pnas.100118597

Meredith, A. L., Thorneloe, K. S., Werner, M. E., Nelson, M. T., and Aldrich, R. W. (2004). Overactive bladder and incontinence in the absence of the BK large conductance Ca2+-activated K+ channel. *J. Biol. Chem.* 279, 36746–36752. doi: 10.1074/jbc.M405621200

Meredith, A. L., Wiler, S. W., Miller, B. H., Takahashi, J. S., Fodor, A. A., Ruby, N. F., et al. (2006). BK calcium-activated potassium channels regulate circadian behavioral rhythms and pacemaker output. *Nat. Neurosci.* 9, 1041–1049. doi: 10.1038/nn1740

Mora, T. C., and Suarez-Kurtz, G. (2005). Effects of NS1608, a BK(Ca) channel agonist, on the contractility of guinea-pig urinary bladder *in vitro*. *Br. J. Pharmacol.* 144, 636–641. doi: 10.1038/sj.bjp.0706034

Morimoto, T., Sakamoto, K., Sade, H., Ohya, S., Muraki, K., and Imaizumi, Y. (2007). Voltage-sensitive oxonol dyes are novel large-conductance Ca2+-activated K+ channel activators selective for beta1 and beta4 but not for beta2 subunits. *Mol. Pharmacol.* 71, 1075–1088. doi: 10.1124/mol.106.031146

Murry, C. E., Jennings, R. B., and Reimer, K. A. (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74, 1124–1136. doi: 10.1161/01.CIR.74.5.1124

N'Gouemo, P. (2011). Targeting BK (big potassium) channels in epilepsy. *Expert Opin. Ther. Targets* 15, 1283–1295. doi: 10.1517/14728222.2011.620607

Nardi, A., Calderone, V., Chericoni, S., and Morelli, I. (2003). Natural modulators of large-conductance calcium-activated potassium channels. *Planta Med.* 69, 885–892. doi: 10.1055/s-2003-45095

Nardi, A., and Olesen, S.-P. (2008). BK channel modulators: a comprehensive overview. *Curr. Med. Chem.* 15, 1126–1146. doi: 10.2174/092986708784221412

Nausch, B., Rode, F., Jorgensen, S., Nardi, A., Korsgaard, M. P. G., Hougaard, C., et al. (2014). NS19504: a novel BK channel activator with relaxing effect on bladder smooth muscle spontaneous phasic contractions. *J. Pharmacol. Exp. Ther.* 350, 520-530. doi: 10.1124/jpet.113.212662

Nelson, M. T., Cheng, H., Rubart, M., Santana, L. F., Bonev, A. D., Knot, H. J., et al. (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science* 270, 633–637. doi: 10.1126/science.270.5236.633

Nicot, A., Berod, A., and Rostene, W. (1992). Distribution of preproneurotensin/neuromedin N mRNA in the young and adult rat forebrain. *Ann. N.Y. Acad. Sci.* 668, 361–364. doi: 10.1111/j.1749-6632.1992.tb27377.x

Oger, S., Behr-Roussel, D., Gorny, D., Bernabé, J., Comperat, E., Chartier-Kastler, E., et al. (2011). Effects of potassium channel modulators on myogenic spontaneous phasic contractile activity in human detrusor from neurogenic patients. *BJU Int.* 108, 604–611. doi: 10.1111/j.1464-410X.2010.09935.x

Ohi, Y., Yamamura, H., Nagano, N., Ohya, S., Muraki, K., Watanabe, M., et al. (2001). Local Ca(2+) transients and distribution of BK channels and ryanodine receptors in smooth muscle cells of guinea-pig vas deferens and urinary bladder. *J. Physiol.* 534(Pt 2), 313–326. doi: 10.1111/j.1469-7793.2001.t01-3-00313.x

Ohya, S., Kimura, S., Kitsukawa, M., Muraki, K., Watanabe, M., and Imaizumi, Y. (2000). SK4 encodes intermediate conductance Ca2+-activated K+ channels in mouse urinary bladder smooth muscle cells. *Jpn. J. Pharmacol.* 84, 97–100. doi: 10.1254/jjp.84.97

Ohya, S., Kuwata, Y., Sakamoto, K., Muraki, K., and Imaizumi, Y. (2005). Cardioprotective effects of estradiol include the activation of large-conductance Ca(2+)-activated K(+) channels in cardiac mitochondria. *Am. J. Physiol. Heart Circ. Physiol.* 289, H1635–H1642. doi: 10.1152/ajpheart.00016.2005

Olesen, S. P., Munch, E., Moldt, P., and Drejer, J. (1994b). Selective activation of Ca(2+)-dependent K+ channels by novel benzimidazolone. *Eur. J. Pharmacol.* 251, 53–59. doi: 10.1016/0014-2999(94)90442-1

Olesen, S. P., Munch, E., Wätjen, F., and Drejer, J. (1994a). NS004–an activator of Ca(2+)-dependent K+ channels in cerebellar granule cells. *Neuroreport* 5, 1001–1004. doi: 10.1097/00001756-199404000-00037

Pacheco Otalora, L. F., Hernandez, E. F., Arshadmansab, M. F., Francisco, S., Willis, M., Ermolinsky, B., et al. (2008). Down-regulation of BK channel expression in the pilocarpine model of temporal lobe epilepsy. *Brain Res.* 1200, 116–131. doi: 10.1016/j.brainres.2008.01.017

Pallanck, L., and Ganetzky, B. (1994). Cloning and characterization of human and mouse homologs of the Drosophila calcium-activated potassium channel gene, slowpoke. *Hum. Mol. Genet.* 3, 1239–1243. doi: 10.1093/hmg/3.8.1239

Parajuli, S. P., Hristov, K. L., Cheng, Q., Malysz, J., Rovner, E. S., and Petkov, G. V. (2014b). Functional link between muscarinic receptors and large-conductance Ca(2+)-activated K (+) channels in freshly isolated human detrusor smooth muscle cells. *Pflugers Arch.* doi: 10.1007/s00424-014-1537-8

Parajuli, S. P., Provence, A., and Petkov, G. V. (2014a). Prostaglandin E2 excitatory effects on guinea pig urinary bladder smooth muscle: a novel regulatory mechanism mediated by large-conductance voltage- and Ca(2+)-activated K(+) channels. *Eur. J. Pharmacol.* 738C, 179–185. doi: 10.1016/j.ejphar.2014.05.042

Park, W. S., Kang, S. H., Son, Y. K., Kim, N., Ko, J.-H., Kim, H. K., et al. (2007). The mitochondrial Ca2+-activated K+ channel activator, NS1619 inhibits L-type Ca2+ channels in rat ventricular myocytes. *Biochem. Biophys. Res. Commun.* 362, 31–36. doi: 10.1016/j.bbrc.2007.07.057

Pérez, G. J., Desai, M., Anderson, S., and Scornik, F. S. (2013). Large-conductance calcium-activated potassium current modulates excitability in isolated canine intracardiac neurons. *Am. J. Physiol. Cell Physiol.* 304, C280–C286. doi: 10.1152/ajpcell.00148.2012

Petkov, G. V. (2014). Central role of the BK channel in urinary bladder smooth muscle physiology and pathophysiology. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 307, R571–R584. doi: 10.1152/ajpregu.00142.2014

Petkov, G. V., Bonev, A. D., Heppner, T. J., Brenner, R., Aldrich, R. W., and Nelson, M. T. (2001). Beta1-subunit of the Ca2+-activated K+ channel regulates contractile activity of mouse urinary bladder smooth muscle. *J. Physiol.* 537(Pt 2), 443–452. doi: 10.1111/j.1469-7793.2001.00443.x

Piwonska, M., Wilczek, E., Szewczyk, A., and Wilczynski, G. M. (2008). Differential distribution of Ca2+-activated potassium channel beta4 subunit in rat brain: immunolocalization in neuronal mitochondria. *Neuroscience* 153, 446–460. doi: 10.1016/j.neuroscience.2008.01.050

Plüger, S., Faulhaber, J., Fürstenau, M., Löhn, M., Waldschütz, R., Gollasch, M., et al. (2000). Mice with disrupted BK channel beta1 subunit gene feature abnormal Ca(2+) spark/STOC coupling and elevated blood pressure. *Circ. Res.* 87, E53–E60. doi: 10.1161/01.RES.87.11.e53

Ponte, C. G., McManus, O. B., Schmalhofer, W. A., Shen, D.-M., Dai, G., Stevenson, A., et al. (2012). Selective, direct activation of high-conductance,

calcium-activated potassium channels causes smooth muscle relaxation. *Mol. Pharmacol.* 81, 567–577. doi: 10.1124/mol.111.075853

Redel, A., Lange, M., Jazbutyte, V., Lotz, C., Smul, T. M., Roewer, N., et al. (2008). Activation of mitochondrial large-conductance calcium-activated K+ channels via protein kinase A mediates desflurane-induced preconditioning. *Anesth. Analg.* 106, 384–391. doi: 10.1213/ane.0b013e318160650f

Revermann, M., Neofitidou, S., Kirschning, T., Schloss, M., Brandes, R. P., and Hofstetter, C. (2014). Inhalation of the BK(Ca)-opener NS1619 attenuates right ventricular pressure and improves oxygenation in the rat monocrotaline model of pulmonary hypertension. *PLoS ONE* 9:e86636. doi: 10.1371/journal.pone.0086636

Roy, S., Large, R. J., Akande, A. M., Kshatri, A., Webb, T. I., Domene, C., et al. (2014). Development of GoSlo-SR-5-69, a potent activator of large conductance Ca2+-activated K+ (BK) channels. *Eur. J. Med. Chem.* 75, 426–437. doi: 10.1016/j.ejmech.2014.01.035

Roy, S., Morayo Akande, A., Large, R. J., Webb, T. I., Camarasu, C., Sergeant, G. P., et al. (2012). Structure-activity relationships of a novel group of large-conductance Ca(2+)-activated K(+) (BK) channel modulators: the GoSlo-SR family. *Chem. Med. Chem.* 7, 1763–1769. doi: 10.1002/cmdc.201200321

Rüttiger, L., Sausbier, M., Zimmermann, U., Winter, H., Braig, C., Engel, J., et al. (2004). Deletion of the Ca2+-activated potassium (BK) alpha-subunit but not the BKbeta1-subunit leads to progressive hearing loss. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12922–12927. doi: 10.1073/pnas.0402660101

Saleh, S. N., Angermann, J. E., Sones, W. R., Leblanc, N., and Greenwood, I. A. (2007). Stimulation of Ca2+-gated Cl- currents by the calcium-dependent K+ channel modulators NS1619 [1, 3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one] and isopimaric acid. *J. Pharmacol. Exp. Ther.* 321, 1075–1084. doi: 10.1124/jpet.106.118786

Salkoff, L., Butler, A., Ferreira, G., Santi, C., and Wei, A. (2006). High-conductance potassium channels of the SLO family. *Nat. Rev. Neurosci.* 7, 921–931. doi: 10.1038/nrn1992

Sato, T., Saito, T., Saegusa, N., and Nakaya, H. (2005). Mitochondrial Ca2+-activated K+ channels in cardiac myocytes: a mechanism of the cardioprotective effect and modulation by protein kinase A. *Circulation* 111, 198–203. doi: 10.1161/01.CIR.0000151099.15706.B1

Sausbier, M., Arntz, C., Bucurenciu, I., Zhao, H., Zhou, X.-B., Sausbier, U., et al. (2005). Elevated blood pressure linked to primary hyperaldosteronism and impaired vasodilation in BK channel-deficient mice. *Circulation* 112, 60–68. doi: 10.1161/01.CIR.0000156448.74296.FE

Sausbier, M., Hu, H., Arntz, C., Feil, S., Kamm, S., Adelsberger, H., et al. (2004). Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+-activated K+ channel deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9474–9478. doi: 10.1073/pnas.0401702101

Sausbier, U., Sausbier, M., Sailer, C. A., Arntz, C., Knaus, H.-G., Neuhuber, W., et al. (2006). Ca2+ -activated K+ channels of the BK-type in the mouse brain. *Histochem. Cell Biol.* 125, 725–41. doi: 10.1007/s00418-005-0124-7

Scornik, F. S., Bucciero, R. S., Wu, Y., Selga, E., Bosch Calero, C., Brugada, R., et al. (2013). DiBAC4(3) hits a "sweet spot" for the activation of arterial large-conductance Ca^{2+}-activated potassium channels independently of the β1-subunit. *Am. J. Physiol. Heart Circ. Physiol.* 304, H1471–H1482. doi: 10.1152/ajpheart.00939.2012

Sheehan, J. J., Benedetti, B. L., and Barth, A. L. (2009). Anticonvulsant effects of the BK-channel antagonist paxilline. *Epilepsia* 50, 711–720. doi: 10.1111/j.1528-1167.2008.01888.x

Shi, Y., Jiang, M. T., Su, J., Hutchins, W., Konorev, E., and Baker, J. E. (2007). Mitochondrial big conductance KCa channel and cardioprotection in infant rabbit heart. *J. Cardiovasc. Pharmacol.* 50, 497–502. doi: 10.1097/FJC.0b013e318137991d

Shintani, Y., Node, K., Asanuma, H., Sanada, S., Takashima, S., Asano, Y., et al. (2004). Opening of Ca2+-activated K+ channels is involved in ischemic preconditioning in canine hearts. *J. Mol. Cell. Cardiol.* 37, 1213–1218. doi: 10.1016/j.yjmcc.2004.09.012

Shipston, M. J. (2001). Alternative splicing of potassium channels: a dynamic switch of cellular excitability. *Trends Cell Biol.* 11, 353–358. doi: 10.1016/S0962-8924(01)02068-2

Shruti, S., Clem, R. L., and Barth, A. L. (2008). A seizure-induced gain-of-function in BK channels is associated with elevated firing activity in neocortical pyramidal neurons. *Neurobiol. Dis.* 30, 323–330. doi: 10.1016/j.nbd.2008.02.002

Singh, H., Lu, R., Bopassa, J. C., Meredith, A. L., Stefani, E., and Toro, L. (2013). MitoBK(Ca) is encoded by the Kcnma1 gene, and a splicing sequence defines its mitochondrial location. *Proc. Natl. Acad. Sci. U.S.A.* 110, 10836–10841. doi: 10.1073/pnas.1302028110

Skalska, J., Bednarczyk, P., Piwońska, M., Kulawiak, B., Wilczynski, G., Dołowy, K., et al. (2009). Calcium ions regulate K^+ uptake into brain mitochondria: the evidence for a novel potassium channel. *Int. J. Mol. Sci.* 10, 1104–1120. doi: 10.3390/ijms10031104

Soder, R. P., and Petkov, G. V. (2011). Large conductance Ca2+ -activated K+ channel activation with NS1619 decreases myogenic and neurogenic contractions of rat detrusor smooth muscle. *Eur. J. Pharmacol.* 670, 252–259. doi: 10.1016/j.ejphar.2011.08.013

Soltysinska, E., Bentzen, B. H., Barthmes, M., Hattel, H., Thrush, A. B., Harper, M.-E., et al. (2014). KCNMA1 Encoded Cardiac BK Channels Afford Protection against Ischemia-Reperfusion Injury. *PLoS ONE* 9:e103402. doi: 10.1371/journal.pone.0103402

Spektor, M., Rodriguez, R., Rosenbaum, R. S., Wang, H.-Z., Melman, A., and Christ, G. J. (2002). Potassium channels and human corporeal smooth muscle cell tone: further evidence of the physiological relevance of the Maxi-K channel subtype to the regulation of human corporeal smooth muscle tone *in vitro*. *J. Urol.* 167, 2628–2635. doi: 10.1016/S0022-5347(05)65049-5

Sprossmann, F., Pankert, P., Sausbier, U., Wirth, A., Zhou, X.-B., Madlung, J., et al. (2009). Inducible knockout mutagenesis reveals compensatory mechanisms elicited by constitutive BK channel deficiency in overactive murine bladder. *FEBS J.* 276, 1680–1697. doi: 10.1111/j.1742-4658.2009.06900.x

Storm, J. F. (1987). Action potential repolarization and a fast after-hyperpolarization in rat hippocampal pyramidal cells. *J. Physiol.* 385, 733–759.

Stowe, D. F., Aldakkak, M., Camara, A. K. S., Riess, M. L., Heinen, A., Varadarajan, S. G., et al. (2006). Cardiac mitochondrial preconditioning by Big Ca2+-sensitive K+ channel opening requires superoxide radical generation. *Am. J. Physiol. Heart Circ. Physiol.* 290, H434–H440. doi: 10.1152/ajpheart.00763.2005

Swensen, A. M., and Bean, B. P. (2003). Ionic mechanisms of burst firing in dissociated Purkinje neurons. *J. Neurosci.* 23, 9650–9663.

Szabo, I., and Zoratti, M. (2014). Mitochondrial channels: ion fluxes and more. *Physiol. Rev.* 94, 519–608. doi: 10.1152/physrev.00021.2013

Thorneloe, K. S., Meredith, A. L., Knorn, A. M., Aldrich, R. W., and Nelson, M. T. (2005). Urodynamic properties and neurotransmitter dependence of urinary bladder contractility in the BK channel deletion model of overactive bladder. *Am. J. Physiol. Renal Physiol.* 289, F604–F610. doi: 10.1152/ajprenal.00060.2005

Tian, L., McClafferty, H., Knaus, H.-G., Ruth, P., and Shipston, M. J. (2012). Distinct acyl protein transferases and thioesterases control surface expression of calcium-activated potassium channels. *J. Biol. Chem.* 287, 14718–14725. doi: 10.1074/jbc.M111.335547

Toro, L., Li, M., Zhang, Z., Singh, H., Wu, Y., and Stefani, E. (2014). MaxiK channel and cell signalling. *Pflügers Arch. Eur. J. Physiol.* 466, 875–886. doi: 10.1007/s00424-013-1359-0

Uchida, H., Shishido, K., Nomiya, M., and Yamaguchi, O. (2005). Involvement of cyclic AMP-dependent and -independent mechanisms in the relaxation of rat detrusor muscle via beta-adrenoceptors. *Eur. J. Pharmacol.* 518, 195–202. doi: 10.1016/j.ejphar.2005.06.029

Valverde, M. A., Rojas, P., Amigo, J., Cosmelli, D., Orio, P., Bahamonde, M. I., et al. (1999). Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit. *Science* 285, 1929–1931. doi: 10.1126/science.285.5435.1929

Vang, A., Mazer, J., Casserly, B., and Choudhary, G. (2010). Activation of endothelial BKCa channels causes pulmonary vasodilation. *Vascul. Pharmacol.* 53, 122–129. doi: 10.1016/j.vph.2010.05.001

Verma-Ahuja, S., Evans, M. S., and Pencek, T. L. (1995). Evidence for decreased calcium dependent potassium conductance in hippocampal CA3 neurons of genetically epilepsy-prone rats. *Epilepsy Res.* 22, 137–144. doi: 10.1016/0920-1211(95)00040-2

Wallner, M., Meera, P., and Toro, L. (1999). Molecular basis of fast inactivation in voltage and Ca2+-activated K+ channels: a transmembrane beta-subunit homolog. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4137–4142. doi: 10.1073/pnas.96.7.4137

Wang, X., Yin, C., Xi, L., and Kukreja, R. C. (2004). Opening of Ca2+-activated K+ channels triggers early and delayed preconditioning against I/R injury independent of NOS in mice. *Am. J. Physiol. Heart Circ. Physiol.* 287, H2070–H2077. doi: 10.1152/ajpheart.00431.2004

Wang, Z., Subramanya, A. R., Satlin, L. M., Pastor-Soler, N. M., Carattino, M. D., and Kleyman, T. R. (2013). Regulation of large-conductance Ca2+-activated K+ channels by WNK4 kinase. *Am. J. Physiol. Cell Physiol.* 305, C846–C853. doi: 10.1152/ajpcell.00133.2013

Weaver, A. K., Olsen, M. L., McFerrin, M. B., and Sontheimer, H. (2007). BK channels are linked to inositol 1, 4, 5-triphosphate receptors via lipid rafts: a novel mechanism for coupling [Ca(2+)](i) to ion channel activation. *J. Biol. Chem.* 282, 31558–31568. doi: 10.1074/jbc.M702866200

Werner, M. E., Knorn, A.-M., Meredith, A. L., Aldrich, R. W., and Nelson, M. T. (2007). Frequency encoding of cholinergic- and purinergic-mediated signaling to mouse urinary bladder smooth muscle: modulation by BK channels. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292, R616–R624. doi: 10.1152/ajpregu.00036.2006

Werner, M. E., Zvara, P., Meredith, A. L., Aldrich, R. W., and Nelson, M. T. (2005). Erectile dysfunction in mice lacking the large-conductance calcium-activated potassium (BK) channel. *J. Physiol.* 567(Pt 2), 545–556. doi: 10.1113/jphysiol.2005.093823

Wojtovich, A. P., Nadtochiy, S. M., Urciuoli, W. R., Smith, C. O., Grunnet, M., Nehrke, K., et al. (2013). A non-cardiomyocyte autonomous mechanism of cardioprotection involving the SLO1 BK channel. *Peer J.* 1:e48. doi: 10.7717/peerj.48

Wojtovich, A. P., Sherman, T. A., Nadtochiy, S. M., Urciuoli, W. R., Brookes, P. S., and Nehrke, K. (2011). SLO-2 is cytoprotective and contributes to mitochondrial potassium transport. *PLoS ONE* 6:e28287. doi: 10.1371/journal.pone.0028287

Womack, M. D., and Khodakhah, K. (2004). Dendritic control of spontaneous bursting in cerebellar Purkinje cells. *J. Neurosci.* 24, 3511–3521. doi: 10.1523/JNEUROSCI.0290-04.2004

Wu, S.-N., Liu, S.-I., and Huang, M.-H. (2004). Cilostazol, an inhibitor of type 3 phosphodiesterase, stimulates large-conductance, calcium-activated potassium channels in pituitary GH3 cells and pheochromocytoma PC12 cells. *Endocrinology* 145, 1175–1184. doi: 10.1210/en.2003-1430

Wulf-Johansson, H., Amrutkar, D. V., Hay-Schmidt, A., Poulsen, A. N., Klaerke, D. A., Olesen, J., et al. (2010). Localization of large conductance calcium-activated potassium channels and their effect on calcitonin gene-related peptide release in the rat trigemino-neuronal pathway. *Neuroscience* 167, 1091–102. doi: 10.1016/j.neuroscience.2010.02.063

Xin, W., Li, N., Cheng, Q., and Petkov, G. V. (2014). BK channel-mediated relaxation of urinary bladder smooth muscle: a novel paradigm for phosphodiesterase type 4 regulation of bladder function. *J. Pharmacol. Exp. Ther.* 349, 56–65. doi: 10.1124/jpet.113.210708

Xu, W., Liu, Y., Wang, S., McDonald, T., Van Eyk, J. E., Sidor, A., et al. (2002). Cytoprotective role of Ca2+- activated K+ channels in the cardiac inner mitochondrial membrane. *Science* 298, 1029–1033. doi: 10.1126/science.1074360

Yan, J., and Aldrich, R. W. (2010). LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. *Nature* 466, 513–516. doi: 10.1038/nature09162

Yan, J., and Aldrich, R. W. (2012). BK potassium channel modulation by leucine-rich repeat-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7917–7922. doi: 10.1073/pnas.1205435109

Yan, J., Olsen, J. V., Park, K.-S., Li, W., Bildl, W., Schulte, U., et al. (2008). Profiling the phospho-status of the BKCa channel alpha subunit in rat brain reveals unexpected patterns and complexity. *Mol Cell Proteomics* 7, 2188–2198. doi: 10.1074/mcp.M800063-MCP200

Yellon, D. M., and Hausenloy, D. J. (2007). Myocardial reperfusion injury. *N. Engl. J. Med.* 357, 1121–1135. doi: 10.1056/NEJMra071667

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 July 2014; accepted: 19 September 2014; published online: 09 October 2014.

*Citation: Bentzen BH, Olesen S-P, Rønn LCB and Grunnet M (2014) BK channel activators and their therapeutic perspectives. Front. Physiol. **5**:389. doi: 10.3389/fphys.2014.00389*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

MINI REVIEW ARTICLE
published: 06 October 2014
doi: 10.3389/fphys.2014.00381

Calcium-activated potassium channels in ischemia reperfusion: a brief update

Jean-Yves Tano*[1,2]* and *Maik Gollasch*[1,2]

[1] *Experimental and Clinical Research Center, Charité University Medicine - Max Delbrück Center (MDC) for Molecular Medicine, Berlin, Germany*
[2] *Nephrology/Intensive Care Section, Charité University Medicine, Berlin, Germany*

Edited by:
Alex M. Dopico, The University of Tennessee Health Science Center, USA

Reviewed by:
Anthony Lewis, University of Portsmouth, UK
Geoff Abbott, University of California, Irvine, USA

****Correspondence:***
Jean-Yves Tano and Maik Gollasch, Experimental and Clinical Research Center, Lindenberger Weg 80, 13125 Berlin, Germany
e-mail: jean-yves.tano@charite.de; maik.gollasch@charite.de

Ischemia and reperfusion (IR) injury constitutes one of the major causes of cardiovascular morbidity and mortality. The discovery of new therapies to block/mediate the effects of IR is therefore an important goal in the biomedical sciences. Dysfunction associated with IR involves modification of calcium-activated potassium channels (K_{Ca}) through different mechanisms, which are still under study. Respectively, the K_{Ca} family, major contributors to plasma membrane calcium influx in cells and essential players in the regulation of the vascular tone are interesting candidates. This family is divided into two groups including the large conductance (BK_{Ca}) and the small/intermediate conductance (SK_{Ca}/IK_{Ca}) K^+ channels. In the heart and brain, these channels have been described to offer protection against IR injury. BK_{Ca} and SK_{Ca} channels deserve special attention since new data demonstrate that these channels are also expressed in mitochondria. More studies are however needed to fully determine their potential use as therapeutic targets.

Keywords: ischemia-reperfusion, K_{Ca} channels, potassium channels, cardiovascular

INTRODUCTION

The proper function of the vasculature requires an intricate balance between plasma membrane ion channels embedded in the endothelium and smooth muscle cells (Luksha et al., 2009). In this regard, the calcium-activated potassium channels (K_{Ca}) exert a great influence in this process (Brayden and Nelson, 1992; Félétou, 2009). These potassium channels possess high sensitivity to intracellular calcium as well as to changes in membrane voltage (Yang et al., 2012). Vascular dysfunction, which is a characteristic trait of several pathophysiological problems such as ischemia-reperfusion (IR) injury, is usually associated with a breakdown of mechanisms in the endothelium or smooth muscle cells. Many of these mechanisms involve the contribution of ion channels including the K_{Ca}. Due to their importance in the regulation of the vascular tone, the plasma membrane K_{Ca} channels have been under scrutiny to resolve vascular dysfunction. Consequently, their role in IR injury has been uncovered with the use of pharmacological tools and more recently with animal models. Our objective in this mini-review is to highlight the observed beneficial effect of K_{Ca} channels under IR conditions.

STRUCTURE AND FUNCTION OF K_{Ca} CHANNELS

On the basis of structure, the K_{Ca} family of potassium channels comprises two groups (Wei et al., 2005). Due to sequence similarity in the pore region and in the C-terminal bound calmodulin Ca^{2+} sensing domain, the small-conductance (SK_{Ca}1, 2, 3) and intermediate conductance (IK_{Ca}1) belong to the same subgroup (Wei et al., 2005). The large-conductance BK_{Ca}, Slo3, Slack, and Slick are also grouped together although Slo3, Slack, and Slick are insensitive to internal calcium (Wei et al., 2005) (see **Table 1**: for simplicity, only the Ca^{2+} activated potassium channels are shown). In contrast to the other members of the family, the BK_{Ca} channels are unique in that they are not only calcium but also markedly voltage sensitive and that calcium binds directly at a specific domain within the protein structure (Wei et al., 1994; Schreiber and Salkoff, 1997). BK_{Ca} channels can be in complex with several modulatory subunits (**Figure 1**) that greatly modify the channel kinetics and voltage/Ca^{2+} sensitivities: β1–β4 have two transmembrane domains, while leucine-rich repeat-containing proteins LRRC26, LRRC38, LRRC52, and LRRC55 are single pass membrane proteins with LRRC26 being the most potent activator producing a negative shift of approximately 140 mV of the voltage dependence of activation (Yan and Aldrich, 2010, 2012; Singh et al., 2012). LRRC26 is a functional BK Channel auxiliary γ subunit in arterial smooth muscle (Evanson et al., 2014). SK_{Ca} and IK_{Ca} channels, however, are very sensitive to changes in $[Ca^{2+}]_i$(submicromolar), whose activation of the channels depends on the binding to a constitutively attached calmodulin (Burnham et al., 2002; Bychkov et al., 2002). SK_{Ca} and IK_{Ca} are expressed predominantly in the endothelial cells whereas BK_{Ca} can be found in greater numbers in the smooth muscle cells (Yang et al., 2012). In the vasculature, these channels contribute predominantly in the regulation of the vascular tone.

SK_{Ca} and IK_{Ca} in the endothelium facilitate the endothelial-derived hyperpolarizing factor mediated relaxation (EDHF) and more recently were found to be important for nitric oxide release (Doughty et al., 1999; McNeish et al., 2006; Stankevicius et al., 2006; Absi et al., 2007; Brähler et al., 2009). At least in mice, the EDHF response is caused by hydrogen peroxide, but not by cytochrome P450 eicosanoids (Hercule et al., 2009). In effect,

Table 1 | Nomenclature of the calcium-activated potassium channels and their described participation in IR injury.

IUPHAR Name	Common name	HGNC	Role in IR injury
$K_{Ca}1.1$	Slo, Slo1, BK	*KCNMA1*	Heart: Protection Brain: Protection
$K_{Ca}2.1$ $K_{Ca}2.2$ $K_{Ca}2.3$	SK_{Ca}, $SK_{Ca}2$	*KCNN1* *KCNN2* *KCNN3*	Heart: Protection Brain: Protection
$K_{Ca}3.1$	IK_{Ca}, $IK_{Ca}1$	*KCNN4*	Heart: Protection Brain: Protection

All of the channels seem to provide protection against injury in the heart and the brain whether administered pre- or post-ischemia. Abbreviations: IUPHAR, International Union of Pharmacology; HGNC, HUGO Gene Nomenclature Committee; IR, Ischemia-Reperfusion.

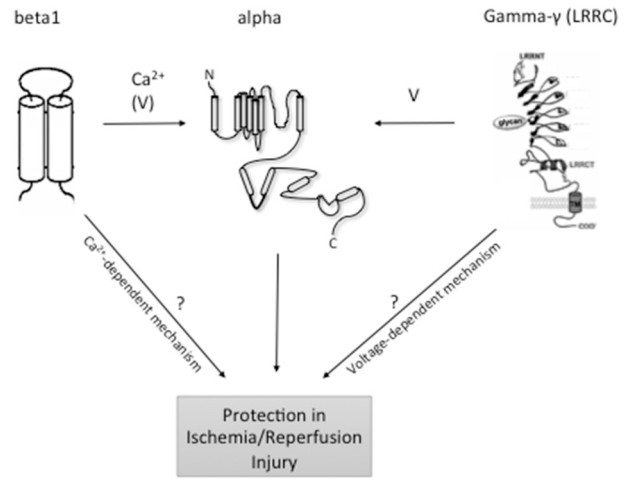


FIGURE 1 | Topology of BK_{Ca} and modulatory subunits. At the plasma membrane, the N-terminus of BK_{Ca} α-subunits is extracellular, and the C-terminus is intracellular. Orientation in organelles is unknown. S0–S4 transmembrane domains are involved in voltage sensing. The S5–S6 linker lines the K+ selective pore. Four α-subunits are needed to form a functional channel. β1–β4 subunits have two transmembrane domains. N- and C-termini are facing the same side of the membrane. γ (LRRC)-subunits have a single transmembrane domain. N- and C-termini face opposite sides of the membrane. ß subunits have a major impact on the intracellular Ca^{2+} sensitivity of the channels, whereas γ subunits have major effects on BK_{Ca} channel voltage sensitivity to different degrees. Following reperfusion, an exacerbated accumulation of $[Ca^{2+}]_i$ mainly in the mitochondria, along with a significant increase in ROS and inflammation may result in cellular death. At least, mitoBK_{Ca} channels play a protective role against IR injury through thus far unclear mechanisms. LRRC exhibit tissue-specific expression although which individual cell types express LRRC proteins is unclear. Organ- and organelle-specific deletion of BK_{Ca} LRRC and ß subunits may clarify the role of $[Ca^{2+}]_i$ accumulation vs. membrane potential in the protective effects of BK_{Ca} channels in IR.

following an increase in $[Ca^{2+}]_i$ in endothelial cells, SK_{Ca}, and IK_{Ca} channels open, causing membrane hyperpolarization. Local calcium (Ca^{2+}) signals ("sparklets") generated through cooperative opening of individual TRPV4 channels within a four-channel cluster can open plasma membrane IK_{Ca} and SK_{Ca} channels to cause vasodilation (Sonkusare et al., 2012). The hyperpolarization in turn leads to the electrical coupling of the endothelium and smooth muscle cells through myoendothelial gap junctions and vasorelaxation (Félétou, 2009). In parallel, opening of these channels can cause activation of the inward rectifier Kir2.1 channels and/or the Na^+/K^+ ATPase on the smooth muscle cells, another important mechanism in the EDHF-mediated relaxation (Edwards et al., 1998). The coupling of the SK_{Ca} and IK_{Ca} channels activation to NO release is currently under study and involves several different mechanisms discussed extensively in Dalsgaard et al. (2010).

In arterial smooth muscle cells, BK_{Ca} channels are involved in regulation of the vascular tone primarily through hyperpolarization and limitation of calcium influx through $Ca_v1.2$ L-type Ca^{2+} channels (Brayden and Nelson, 1992; Sausbier et al., 2005; Yang et al., 2012). Calcium sparks generated by opening of ryanodine receptors (RyR) in the sarcoplasmic reticulum serve as local elementary Ca^{2+} signals to open plasma membrane BK_{Ca} channels to induce membrane hyperpolarization and relaxation (Nelson et al., 1995; Gollasch et al., 1998; Essin et al., 2007), including in human vessels (Fürstenau et al., 2000). The accessory beta1 subunit of the BK_{Ca} channel plays an important role in calcium spark/BK channel coupling (Brenner et al., 2000; Plüger et al., 2000). Calcium sparks are possibly generated by opening of RyR2 (Essin and Gollasch, 2009; Vaithianathan et al., 2010), but not by RyR3 (Löhn et al., 2001). In addition, BK_{Ca} channels can contribute to endothelium-dependent vasorelaxation through activation by NO and EDHF (Bolotina et al., 1994; Weston et al., 2005; Hou et al., 2009). Interestingly, new studies have demonstrated the activation of BK channels by other gasotransmitters, notably carbon monoxide (CO) and hydrogen sulfide (H_2S) (Dong et al., 2007; Chai et al., 2014) although see Telezhkin et al. (2010).

In view of their prominent role in the regulation of the vascular tone, the likelihood of involvement of these channels in IR—a condition where mechanisms underlying vasorelaxation are compromised and where gasotransmitters have been shown to play a protective role—is very high (Murphy and Steenbergen, 2008; Luksha et al., 2009; Dalsgaard et al., 2010; Eltzschig and Eckle, 2011). Recent studies have better defined the role of BK_{Ca} in IR, however the picture concerning SK_{Ca} and IK_{Ca} remains still cloudy.

SK_{Ca} AND IK_{Ca} IN ISCHEMIA-REPERFUSION

A primary mechanism involved in IR injury is the exacerbation of intracellular calcium, which causes damages in the tissue (discussed in more detail in Eltzschig and Eckle (2011), Tano and Gollasch (2014). Limited studies have looked at the role of SK_{Ca} and IK_{Ca} in IR injury. Yang et al. recently demonstrated a decrease in endothelial IK_{Ca} and SK_{Ca} currents as well as IK_{Ca} protein content, associated with a decreased EDHF-mediated relaxation following 60 min ischemia and 30 min reoxygenation in pig arteries (Yang et al., 2011). This study suggests that these channels are important in the protection of the endothelium against IR injury. A more recent and rigorous study looking at isolated guinea pig hearts also found protection against IR injury through SK_{Ca} channels (Stowe et al., 2006). In this study, DCEBIO, an

SK_{Ca}, and IK_{Ca} channel activator, caused a 2-fold increase in left ventricular pressure as well as a 2.5 fold decrease in infarct size when administered for 10 min, 20 min before IR. This effect is, however, blocked by NS8593, an SK_{Ca} blocker, suggesting that these channels are responsible for the protection. Interestingly and most importantly, the authors isolate and purify novel mSK_{Ca} channels from the inner mitochondrial membrane of cardiac cell and suggest that DCEBIO mediates its cardioprotection through these channels (Stowe et al., 2006), by improving mitochondrial bioenergetics (Stowe et al., 2013).

In the brain, a few studies have also demonstrated a protective role of SK_{Ca} and a more ambiguous role for IK_{Ca}. In mice undergoing cardiac arrest/cardiopulmonary resuscitation (CA/CPR) and global cerebral ischemia, $SK_{Ca}2$ channels are responsible for the protection of the CA1 neurons against ischemic injury (Allen et al., 2011). Similarly to the study in the heart, pre-stimulation of $SK_{Ca}2$ with 1-EBIO diminished significantly the adverse effects of CA/CPR, an effect, which could be reversed with administration of apamin (a specific SK_{Ca} blocker). In addition, $SK_{Ca}2$ electrophysiological activity was reduced during CA/CPR in association with an increased synaptic $SK_{Ca}2$ channels internalization. Interestingly, post-treatment with 1-EBIO was able to also blunt the effects of CA/CPR (Allen et al., 2011). In the parenchymal arterioles, both SK_{Ca} and IK_{Ca} were shown to play a protective role on the basal tone and pressure reactivity following IR (Cipolla et al., 2009). Blockade of these channels in the parenchymal arterioles induced a significant increase in the basal tone, which was preserved following IR injury when compared to control animals. Furthermore, the authors suggest that EDHF act as a substitute for NO in the parenchymal arterioles due to the fact that NO responsiveness is significantly decreased after IR (Cipolla et al., 2009). Finally, a recent study demonstrated that inhibition of IK_{Ca} with the blocker TRAM-34 reduces infarct size and other neurological deficits in rats when administered as soon as 12 h after middle cerebral artery occlusion (Chen et al., 2011). The mechanism suggested for the protective actions of this drug is through the reduced activation of microglial cells, which is more noticeable with a higher dose (40 mg/Kg) of TRAM-34 (Chen et al., 2011).

Since the studies described in this review represent the only few published on this topic, one can see that much more work is required to properly decipher the role of these important channels in IR injury. It is especially difficult to understand the role of these channels since most of these studies take very different pharmacological approaches, notably pre-, and post-administration of inhibitors or blockers in conjunction with IR. Moreover, the use of available knockout mouse models of these channels would bring the scientific community closer to this goal. The prominent trend, however, seems to be a protective effect of these channels in the heart and the brain (see **Table 1**), which is also evident for BK_{Ca} channels.

BK_{Ca} IN ISCHEMIA-REPERFUSION

The combination of pharmacological tools and knockout mouse models has suggested a protective role of BK_{Ca} against IR injury. The use of pharmacological activators such as NS1619 and NS11021 suggested BK_{Ca} channels as cardioprotective following IR (Shintani et al., 2004; Shi et al., 2007; Bentzen et al., 2009). This notion was recently confirmed with the use of the *Kcnma*1 knockout mouse where the cardioprotective effects of these channels were lost (Wojtovich et al., 2013). Furthermore, Woodman et al. determined the effects of tetraethylammonium (TEA, 1 mM—a potent blocking concentration for BK_{Ca} channels (see Nelson, 1993) to coronary arteries from dogs subjected to IR. TEA significantly shifted the concentration response curve of the ischemic vessels to acetylcholine to the right, though without decreasing the maximal relaxation (Chan and Woodman, 1999). The authors concluded that EDHF may be the factor responsible for activation of BK_{Ca} channels (Chan and Woodman, 1999). However, the data have to be interpreted with caution since a number of other K+ channels are sensitive to TEA, within this range of concentration, e.g., Kv1.1, Kv1.3, and Kv1.6 (Al-Sabi et al., 2013), KCNQ1, KCNQ2, KCNQ4, KCNQ2 + KCNQ3 (Hadley et al., 2000). In skeletal muscle arterioles from patients undergoing cardiopulmonary bypass, Feng et al. observed activation of the BK_{Ca} channels (Feng et al., 2009). In addition, treatment with iberiotoxin (a specific BK_{Ca} blocker) improved the myogenic tone significantly associated with a reduced microvessel internal diameter in these patients. The molecular mechanisms of the protective effects of BK_{Ca} channels in IR may involve direct effects of hypoxia on BK_{Ca} channel gating, without involvement of soluble intracellular components (Lewis et al., 2002). Sensitivity to hypoxia is conferred by a highly conserved motif within an alternatively spliced cysteine-rich insert, the stress-regulated exon (STREX), within the intracellular C-terminus of the channel (McCartney et al., 2005). Recent studies using *Kcnma*1 knockout mice suggest that activation of cardiomyocyte BK_{Ca} channels in mitochondria ($mitoBK_{Ca}$) is one mechanism that protects the heart against IR injury (Singh et al., 2013; Tano and Gollasch, 2014). It is possible that sulfhydryl groups of the channel protein play a critical role in this process (Sitdikova et al., 2010; Liu et al., 2012).

The *Kcnma1* knockout mouse was also used to study BK_{Ca} channels in the brain. These channels offered protection and reduced infarct size in a middle cerebral artery occlusion model (Liao et al., 2010). Interestingly, Gu et al. found that unlike healthy brain cells, glioma $mitoBK_{Ca}$ channels, but not plasma membrane BK channels are oxygen sensitive (Gu et al., 2014). These findings may explain why tumor cells are resistant to hypoxia. On the other hand, discovery of this mechanism of tumor tolerance may have important clinical implications for the development of novel therapies in oncology.

CONCLUSION

The K_{Ca} play an essential function in the endothelium and arterial smooth muscle where they participate actively in the regulation of the myogenic tone. Disruption of this process as well as others such as NO formation in IR injury provides a reason to study a potential involvement of these channels in IR. Thus far, the consensus points toward a protective role of these channels against IR injury, although much more remains unknown, notably the mechanisms underlying this protection. The use of gene knockout mouse models, especially for the SK_{Ca}, and IK_{Ca} would be of great help in answering these questions. Also, the very recent discovery

of BK_{Ca} channel auxiliary γ subunits, such as LRRC26, LRRC38, LRRC52, and LRRC55 (Yan and Aldrich, 2010, 2012), may help to design experimental protocols to clarify the role of excess calcium vs. plasma/mito membrane potential in the protective BK_{Ca} function in IR injury (**Figure 1**). In this regard, targeting BK_{Ca} β subunits but not γ subunits is expected to affect IR injury if excess calcium plays a key role in this process. Future studies are necessary to address the composition of functional BK_{Ca} channels in the organs and organelles of interest (mitochondria) and to study their role in IR using genetically engineered BK_{Ca} subunit deficient animal models.

ACKNOWLEDGMENTS

The Deutsche Forschungsgemeinschaft (DFG) and the Alexander von Humboldt foundation support the work of the authors.

REFERENCES

Absi, M., Burnham, M. P., Weston, A. H., Harno, E., Rogers, M., and Edwards, G. (2007). Effects of methyl beta-cyclodextrin on EDHF responses in pig and rat arteries; association between SK(Ca) channels and caveolin-rich domains. *Br. J. Pharmacol.* 151, 332–340. doi: 10.1038/sj.bjp.0707222

Allen, D., Nakayama, S., Kuroiwa, M., Nakano, T., Palmateer, J., Kosaka, Y., et al. (2011). SK2 channels are neuroprotective for ischemia-induced neuronal cell death. *J. Cereb. Blood Flow Metab.* 31, 2302–2312. doi: 10.1038/jcbfm.2011.90

Al-Sabi, A., Kaza, S. K., Dolly, J. O., and Wang, J. (2013). Pharmacological characteristics of Kv1.1- and Kv1.2-containing channels are influenced by the stoichiometry and positioning of their α subunits. *Biochem. J.* 454, 101–108. doi: 10.1042/BJ20130297

Bentzen, B. H., Osadchii, O., Jespersen, T., Hansen, R. S., Olesen, S.-P., and Grunnet, M. (2009). Activation of big conductance Ca(2+)-activated K (+) channels (BK) protects the heart against ischemia-reperfusion injury. *Pflugers Arch.* 457, 979–988. doi: 10.1007/s00424-008-0583-5

Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J., and Cohen, R. A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 368, 850–853. doi: 10.1038/368850a0

Brähler, S., Kaistha, A., Schmidt, V. J., Wölfle, S. E., Busch, C., Kaistha, B. P., et al. (2009). Genetic deficit of SK3 and IK1 channels disrupts the endothelium-derived hyperpolarizing factor vasodilator pathway and causes hypertension. *Circulation* 119, 2323–2332. doi: 10.1161/CIRCULATIONAHA.108.846634

Brayden, J. E., and Nelson, M. T. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* 256, 532–535. doi: 10.1126/science.1373909

Brenner, R., Peréz, G. J., Bonev, A. D., Eckman, D. M., Kosek, J. C., Wiler, S. W., et al. (2000). Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature* 407, 870–876. doi: 10.1038/35038011

Burnham, M. P., Bychkov, R., Félétou, M., Richards, G. R., Vanhoutte, P. M., Weston, A. H., et al. (2002). Characterization of an apamin-sensitive small-conductance Ca(2+)-activated K(+) channel in porcine coronary artery endothelium: relevance to EDHF. *Br. J. Pharmacol.* 135, 1133–1143. doi: 10.1038/sj.bjp.0704551

Bychkov, R., Burnham, M. P., Richards, G. R., Edwards, G., Weston, A. H., Félétou, M., et al. (2002). Characterization of a charybdotoxin-sensitive intermediate conductance Ca2+-activated K+ channel in porcine coronary endothelium: relevance to EDHF. *Br. J. Pharmacol.* 137, 1346–1354. doi: 10.1038/sj.bjp.0705057

Chai, Q., Lu, T., Wang, X. L., and Lee, H. C. (2014). Hydrogen sulfide impairs shear stress-induced vasodilation in mouse coronary arteries. *Pflügers Arch.* doi: 10.1007/s00424-014-1526-y. [Epub ahead of print].

Chan, E. C., and Woodman, O. L. (1999). Enhanced role for the opening of potassium channels in relaxant responses to acetylcholine after myocardial ischaemia and reperfusion in dog coronary arteries. *Br. J. Pharmacol.* 126, 925–932. doi: 10.1038/sj.bjp.0702376

Chen, Y.-J., Raman, G., Bodendiek, S., O'Donnell, M. E., and Wulff, H. (2011). The KCa3.1 blocker TRAM-34 reduces infarction and neurological deficit in a rat model of ischemia/reperfusion stroke. *J. Cereb. Blood Flow Metab.* 31, 2363–2374. doi: 10.1038/jcbfm.2011.101

Cipolla, M. J., Smith, J., Kohlmeyer, M. M., and Godfrey, J. A. (2009). SKCa and IKCa Channels, myogenic tone, and vasodilator responses in middle cerebral arteries and parenchymal arterioles: effect of ischemia and reperfusion. *Stroke* 40, 1451–1457. doi: 10.1161/STROKEAHA.108.535435

Dalsgaard, T., Kroigaard, C., and Simonsen, U. (2010). Calcium-activated potassium channels - a therapeutic target for modulating nitric oxide in cardiovascular disease? *Expert Opin. Ther. Targets* 14, 825–837. doi: 10.1517/14728222.2010.500616

Dong, D.-L., Zhang, Y., Lin, D.-H., Chen, J., Patschan, S., Goligorsky, M. S., et al. (2007). Carbon monoxide stimulates the Ca^{2+}-activated big conductance K channels in cultured human endothelial cells. *Hypertension* 50, 643–651. doi: 10.1161/HYPERTENSIONAHA.107.096057

Doughty, J. M., Plane, F., and Langton, P. D. (1999). Charybdotoxin and apamin block EDHF in rat mesenteric artery if selectively applied to the endothelium. *Am. J. Physiol.* 276, H1107–H1112.

Edwards, G., Dora, K. A., Gardener, M. J., Garland, C. J., and Weston, A. H. (1998). K+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature* 396, 269–272. doi: 10.1038/24388

Eltzschig, H. K., and Eckle, T. (2011). Ischemia and reperfusion–from mechanism to translation. *Nat. Med.* 17, 1391–1401. doi: 10.1038/nm.2507

Essin, K., and Gollasch, M. (2009). Role of ryanodine receptor subtypes in initiation and formation of calcium sparks in arterial smooth muscle: comparison with striated muscle. *J. Biomed. Biotechnol.* 2009:135249. doi: 10.1155/2009/135249

Essin, K., Welling, A., Hofmann, F., Luft, F. C., Gollasch, M., and Moosmang, S. (2007). Indirect coupling between Cav1.2 channels and ryanodine receptors to generate Ca2+ sparks in murine arterial smooth muscle cells. *J. Physiol.* 584, 205–219. doi: 10.1113/jphysiol.2007.138982

Evanson, K. W., Bannister, J. P., Leo, M. D., and Jaggar, J. H. (2014). LRRC26 is a functional BK channel auxiliary γ subunit in arterial smooth muscle cells. *Circ. Res.* 115, 423–431. doi: 10.1161/CIRCRESAHA.115.303407

Félétou, M. (2009). Calcium-activated potassium channels and endothelial dysfunction: therapeutic options? *Br. J. Pharmacol.* 156, 545–562. doi: 10.1111/j.1476-5381.2009.00052.x

Feng, J., Liu, Y., Khabbaz, K. R., Sodha, N. R., Osipov, R. M., Hagberg, R., et al. (2009). Large conductance calcium-activated potassium channels contribute to the reduced myogenic tone of peripheral microvasculature after cardiopulmonary bypass1. *J. Surg. Res.* 157, 123–128. doi: 10.1016/j.jss.2009.03.043

Fürstenau, M., Löhn, M., Ried, C., Luft, F. C., Haller, H., and Gollasch, M. (2000). Calcium sparks in human coronary artery smooth muscle cells resolved by confocal imaging. *J. Hypertens.* 18, 1215–1222. doi: 10.1097/00004872-200018090-00007

Gollasch, M., Wellman, G. C., Knot, H. J., Jaggar, J. H., Damon, D. H., Bonev, A. D., et al. (1998). Ontogeny of local sarcoplasmic reticulum Ca2+ signals in cerebral arteries: Ca2+ sparks as elementary physiological events. *Circ. Res.* 83, 1104–1114. doi: 10.1161/01.RES.83.11.1104

Gu, X. Q., Pamenter, M. E., Siemen, D., Sun, X., and Haddad, G. G. (2014). Mitochondrial but not plasmalemmal BK channels are hypoxia-sensitive in human glioma. *Glia* 62, 504–513. doi: 10.1002/glia.22620

Hadley, J. K., Noda, M., Selyanko, A. A., Wood, I. C., Abogadie, F. C., and Brown, D. A. (2000). Differential tetraethylammonium sensitivity of KCNQ1-4 potassium channels. *Br. J. Pharmacol.* 129, 413–415. doi: 10.1038/sj.bjp.0703086

Hercule, H. C., Schunck, W.-H., Gross, V., Seringer, J., Leung, F. P., Weldon, S. M., et al. (2009). Interaction between P450 eicosanoids and nitric oxide in the control of arterial tone in mice. *Arterioscler. Thromb. Vasc. Biol.* 29, 54–60. doi: 10.1161/ATVBAHA.108.171298

Hou, S., Heinemann, S. H., and Hoshi, T. (2009). Modulation of BKCa channel gating by endogenous signaling molecules. *Physiology (Bethesda)* 24, 26–35. doi: 10.1152/physiol.00032.2008

Lewis, A., Peers, C., Ashford, M. L. J., and Kemp, P. J. (2002). Hypoxia inhibits human recombinant large conductance, Ca(2+)-activated K(+) (maxi-K) channels by a mechanism which is membrane delimited and Ca(2+) sensitive. *J. Physiol.* 540, 771–780. doi: 10.1113/jphysiol.2001.013888

Liao, Y., Kristiansen, A.-M., Oksvold, C. P., Tuvnes, F. A., Gu, N., Rundén-Pran, E., et al. (2010). Neuronal Ca2+-activated K+ channels limit brain infarction and promote survival. *PLoS ONE* 5:e15601. doi: 10.1371/journal.pone.0015601

Liu, Y., Kalogeris, T., Wang, M., Zuidema, M. Y., Wang, Q., Dai, H., et al. (2012). Hydrogen sulfide preconditioning or neutrophil depletion attenuates ischemia-reperfusion-induced mitochondrial dysfunction in rat small

intestine. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302, G44–G54. doi: 10.1152/ajpgi.00413.2010

Löhn, M., Jessner, W., Fürstenau, M., Wellner, M., Sorrentino, V., Haller, H., et al. (2001). Regulation of calcium sparks and spontaneous transient outward currents by RyR3 in arterial vascular smooth muscle cells. *Circ. Res.* 89, 1051–1057. doi: 10.1161/hh2301.100250

Luksha, L., Agewall, S., and Kublickiene, K. (2009). Endothelium-derived hyperpolarizing factor in vascular physiology and cardiovascular disease. *Atherosclerosis* 202, 330–344. doi: 10.1016/j.atherosclerosis.2008.06.008

McCartney, C. E., McClafferty, H., Huibant, J.-M., Rowan, E. G., Shipston, M. J., and Rowe, I. C. M. (2005). A cysteine-rich motif confers hypoxia sensitivity to mammalian large conductance voltage- and Ca-activated K (BK) channel alpha-subunits. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17870–17876. doi: 10.1073/pnas.0505270102

McNeish, A. J., Sandow, S. L., Neylon, C. B., Chen, M. X., Dora, K. A., and Garland, C. J. (2006). Evidence for involvement of both IKCa and SKCa channels in hyperpolarizing responses of the rat middle cerebral artery. *Stroke* 37, 1277–1282. doi: 10.1161/01.STR.0000217307.71231.43

Murphy, E., and Steenbergen, C. (2008). Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol. Rev.* 88, 581–609. doi: 10.1152/physrev.00024.2007

Nelson, M. T. (1993). Ca(2+)-activated potassium channels and ATP-sensitive potassium channels as modulators of vascular tone. *Trends Cardiovasc. Med.* 3, 54–60. doi: 10.1016/1050-1738(93)90037-7

Nelson, M. T., Cheng, H., Rubart, M., Santana, L. F., Bonev, A. D., Knot, H. J., et al. (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science* 270, 633–637. doi: 10.1126/science.270.5236.633

Plüger, S., Faulhaber, J., Fürstenau, M., Löhn, M., Waldschütz, R., Gollasch, M., et al. (2000). Mice with disrupted BK channel beta1 subunit gene feature abnormal Ca(2+) spark/STOC coupling and elevated blood pressure. *Circ. Res.* 87, E53–E60. doi: 10.1161/01.RES.87.11.e53

Sausbier, M., Arntz, C., Bucurenciu, I., Zhao, H., Zhou, X.-B., Sausbier, U., et al. (2005). Elevated blood pressure linked to primary hyperaldosteronism and impaired vasodilation in BK channel-deficient mice. *Circulation* 112, 60–68. doi: 10.1161/01.CIR.0000156448.74296.FE

Schreiber, M., and Salkoff, L. (1997). A novel calcium-sensing domain in the BK channel. *Biophys. J.* 73, 1355–1363. doi: 10.1016/S0006-3495(97)78168-2

Shi, Y., Jiang, M. T., Su, J., Hutchins, W., Konorev, E., and Baker, J. E. (2007). Mitochondrial big conductance KCa channel and cardioprotection in infant rabbit heart. *J. Cardiovasc. Pharmacol.* 50, 497–502. doi: 10.1097/FJC.0b013e318137991d

Shintani, Y., Node, K., Asanuma, H., Sanada, S., Takashima, S., Asano, Y., et al. (2004). Opening of Ca2+-activated K+ channels is involved in ischemic preconditioning in canine hearts. *J. Mol. Cell. Cardiol.* 37, 1213–1218. doi: 10.1016/j.yjmcc.2004.09.012

Singh, H., Lu, R., Bopassa, J. C., Meredith, A. L., Stefani, E., and Toro, L. (2013). MitoBK(Ca) is encoded by the Kcnma1 gene, and a splicing sequence defines its mitochondrial location. *Proc. Natl. Acad. Sci. U.S.A.* 110, 10836–10841. doi: 10.1073/pnas.1302028110

Singh, H., Stefani, E., and Toro, L. (2012). Intracellular BK(Ca) (iBK(Ca)) channels. *J. Physiol.* 590, 5937–5947. doi: 10.1113/jphysiol.2011.215533

Sitdikova, G. F., Weiger, T. M., and Hermann, A. (2010). Hydrogen sulfide increases calcium-activated potassium (BK) channel activity of rat pituitary tumor cells. *Pflugers Arch.* 459, 389–397. doi: 10.1007/s00424-009-0737-0

Sonkusare, S. K., Bonev, A. D., Ledoux, J., Liedtke, W., Kotlikoff, M. I., Heppner, T. J., et al. (2012). Elementary Ca2+ signals through endothelial TRPV4 channels regulate vascular function. *Science* 336, 597–601. doi: 10.1126/science.1216283

Stankevicius, E., Lopez-Valverde, V., Rivera, L., Hughes, A., Mulvany, M. J., and Simonsen, U. (2006). Combination of Ca2+ -activated K+ channel blockers inhibits acetylcholine-evoked nitric oxide release in rat superior mesenteric artery. *Br. J. Pharmacol.* 149, 560–572. doi: 10.1038/sj.bjp.0706886

Stowe, D. F., Aldakkak, M., Camara, A. K. S., Riess, M. L., Heinen, A., Varadarajan, S. G., et al. (2006). Cardiac mitochondrial preconditioning by Big Ca2+-sensitive K+ channel opening requires superoxide radical generation. *Am. J. Physiol. Heart Circ. Physiol.* 290, H434–H440. doi: 10.1152/ajpheart.00763.2005

Stowe, D. F., Gadicherla, A. K., Zhou, Y., Aldakkak, M., Cheng, Q., Kwok, W.-M., et al. (2013). Protection against cardiac injury by small Ca(2+)-sensitive K(+) channels identified in guinea pig cardiac inner mitochondrial membrane. *Biochim. Biophys. Acta* 1828, 427–442. doi: 10.1016/j.bbamem.2012.08.031

Tano, J.-Y., and Gollasch, M. (2014). Hypoxia and ischemia-reperfusion: a BiK contribution? *Am. J. Physiol. Heart Circ. Physiol.* 307, H811–H817. doi: 10.1152/ajpheart.00319.2014

Telezhkin, V., Brazier, S. P., Cayzac, S. H., Wilkinson, W. J., Riccardi, D., and Kemp, P. J. (2010). Mechanism of inhibition by hydrogen sulfide of native and recombinant BKCa channels. *Respir. Physiol. Neurobiol.* 172, 169–178. doi: 10.1016/j.resp.2010.05.016

Vaithianathan, T., Narayanan, D., Asuncion-Chin, M. T., Jeyakumar, L. H., Liu, J., Fleischer, S., et al. (2010). Subtype identification and functional characterization of ryanodine receptors in rat cerebral artery myocytes. *Am. J. Physiol. Cell Physiol.* 299, C264–C278. doi: 10.1152/ajpcell.00318.2009

Wei, A. D., Gutman, G. A., Aldrich, R., Chandy, K. G., Grissmer, S., and Wulff, H. (2005). International Union of Pharmacology. LII. Nomenclature and molecular relationships of calcium-activated potassium channels. *Pharmacol. Rev.* 57, 463–472. doi: 10.1124/pr.57.4.9

Wei, A., Solaro, C., Lingle, C., and Salkoff, L. (1994). Calcium sensitivity of BK-type KCa channels determined by a separable domain. *Neuron* 13, 671–681. doi: 10.1016/0896-6273(94)90034-5

Weston, A. H., Félétou, M., Vanhoutte, P. M., Falck, J. R., Campbell, W. B., and Edwards, G. (2005). Bradykinin-induced, endothelium-dependent responses in porcine coronary arteries: involvement of potassium channel activation and epoxyeicosatrienoic acids. *Br. J. Pharmacol.* 145, 775–784. doi: 10.1038/sj.bjp.0706256

Wojtovich, A. P., Nadtochiy, S. M., Urciuoli, W. R., Smith, C. O., Grunnet, M., Nehrke, K., et al. (2013). A non-cardiomyocyte autonomous mechanism of cardioprotection involving the SLO1 BK channel. *PeerJ* 1:e48. doi: 10.7717/peerj.48

Yan, J., and Aldrich, R. W. (2010). LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. *Nature* 466, 513–516. doi: 10.1038/nature09162

Yan, J., and Aldrich, R. W. (2012). BK potassium channel modulation by leucine-rich repeat-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7917–7922. doi: 10.1073/pnas.1205435109

Yang, Q., Huang, J.-H., Man, Y.-B., Yao, X.-Q., and He, G.-W. (2011). Use of intermediate/small conductance calcium-activated potassium-channel activator for endothelial protection. *J. Thorac. Cardiovasc. Surg.* 141, 501–510, 510.e1. doi: 10.1016/j.jtcvs.2010.04.005

Yang, Q., Underwood, M. J., and He, G.-W. (2012). Calcium-activated potassium channels in vasculature in response to ischemia-reperfusion. *J. Cardiovasc. Pharmacol.* 59, 109–115. doi: 10.1097/FJC.0b013e318210fb4b

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 July 2014; accepted: 13 September 2014; published online: 06 October 2014.

*Citation: Tano J-Y and Gollasch M (2014) Calcium-activated potassium channels in ischemia reperfusion: a brief update. Front. Physiol. **5**:381. doi: 10.3389/fphys.2014.00381*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

REVIEW ARTICLE
published: 29 September 2014
doi: 10.3389/fphys.2014.00373

BK_{Ca} channel dysfunction in neurological diseases

Prosper N'Gouemo*

Department of Pediatrics and Interdisciplinary Program in Neuroscience, Georgetown University Medical Center, Washington, DC, USA

Edited by:
Thomas M. Weiger, University of Salzburg, Austria

Reviewed by:
Brad S. Rothberg, Temple University School of Medicine, USA
Jose Bargas, Universidad Nacional Autónoma de México, Mexico

***Correspondence:**
Prosper N'Gouemo, Department of Pediatrics and Interdisciplinary Program in Neuroscience, Georgetown University Medical Center, 3900 Reservoir Rd, NW, Washington, DC 20057, USA
e-mail: pn@georgetown.edu

The large conductance, Ca^{2+}-activated K^+ channels (BK_{Ca}, $K_{Ca1.1}$) are expressed in various brain neurons where they play important roles in regulating action potential duration, firing frequency and neurotransmitter release. Membrane potential depolarization and rising levels of intracellular Ca^{2+} gated BK_{Ca} channels, which in turn results in an outward K^+ flux that re/hyperpolarizes the membrane. The sensitivity of BK_{Ca} channels to Ca^{2+} provides an important negative-feedback system for Ca^{2+} entry into brain neurons and suppresses repetitive firing. Thus, BK_{Ca} channel loss-of-function gives rise to neuronal hyperexcitability, which can lead to seizures. Evidence also indicates that BK_{Ca} channels can facilitate high-frequency firing (gain-of-function) in some brain neurons. Interestingly, both gain-of-function and loss-of-function mutations of genes encoding for various BK_{Ca} channel subunits have been associated with the development of neuronal excitability disorders, such as seizure disorders. The role of BK_{Ca} channels in the etiology of some neurological diseases raises the possibility that these channels can be used as molecular targets to prevent and suppress disease phenotypes.

Keywords: autism, alcohol withdrawal seizures, epilepsy, gain-of-function, loss-of-function

BK_{Ca} CHANNELS AND NEURONAL EXCITABILITY

Intrinsic membrane properties play an important role in the control of neuronal activity in the central nervous system (CNS). Alterations of intrinsic membrane properties can contribute to diseases of neuronal excitability such as epilepsy. Potassium (K^+) channels in particular are well known for their role in the regulation of membrane excitability due to their ability to stabilize the membrane potential. Compelling evidence indicates that K^+ channels are critical molecular determinants for seizure generation and epileptogenesis. One particular type of K^+ channel, the large conductance, Ca^{2+}-activated K^+ channel (BK_{Ca}, $K_{Ca1.1}$) is considered to be one of the intrinsic molecular determinants for the control of neuronal excitability in the CNS. Unlike other K^+ channels, BK_{Ca} channels are activated by both voltage and elevated levels of intracellular Ca^{2+}, resulting in large K^+ conductances which in turn re/hyperpolarizes the membrane. The sensitivity of BK_{Ca} channels to Ca^{2+} provides an important negative feedback for Ca^{2+} entry into brain neurons; thus, BK_{Ca} channels may serve as a link between membrane depolarization and Ca^{2+} signaling to provide a rapid response to reduce or prevent neuronal hyperexcitability.

BK_{Ca} channels are tetramers of four α subunits, which form the ion channel pore, and four regulatory β (β1–4) subunits that are expressed in various tissues, including the brain (Pallanek and Genetzky, 1994; Jiang et al., 1999). BK_{Ca} channels can also be regulated by acidification (Brelidze and Magleby, 2004; Hou et al., 2008), ethanol (Liu et al., 2008), protein kinase phosphorylation (Tian et al., 2001; Zhou et al., 2010), ubiquitination (Liu et al., 2014) and palmitoylation (Shipston, 2013; Zhou et al., 2012). Of particular importance, protein S-palmitoylation (or palmitoylation) and ubiquitination control the cell surface expression and activity of BK_{Ca}, thereby critically contributing to BK_{Ca} channel functions (Shipston, 2013; Liu et al., 2014). Notably, the palmitoylation of BK_{Ca} channel β subunits promotes the exit of the pore-forming α subunit from the endoplasmic reticulum and promotes BK_{Ca} channel surface expression (Chen et al., 2013). The BK_{Ca} channel α subunit is encoded by the *Slo*1 gene, which can be subjected to splicing to produce channels with different functional properties and sensitivity to Ca^{2+}; including the STREX (stress-axis hormone-regulated exon) channels (Xie and McCobb, 1998; Chen et al., 2005). Expression profiling studies have reported that BK_{Ca} channel α subunits are broadly expressed in the CNS (Chang et al., 1997; Wanner et al., 1999; Sausbier et al., 2006). The regulatory BK_{Ca} channel β1 and β4 subunits are also expressed in the brain, whereas the β2 and β3 subunits are nearly absent in the brain (Tseng-Crank et al., 1996). BK_{Ca} channels are predominantly located at the axon and presynaptic terminals, associated with glutamatergic synapses in hippocampus and cortex and GABAergic synapses in the cerebellum (Knaus et al., 1996; Hu et al., 2001; Misonou et al., 2006; Martire et al., 2010). These channels are usually found in close proximity to N-methyl-D-asparte receptors (Isaacson and Murphy, 2001) and voltage-gated Ca^{2+} channels (Ca_V), including $Ca_V1.2$, $Ca_V2.2$, and $Ca_V2.1$ in the CNS (Marrion and Tavalin, 1998; Grunnet and Kaufmann, 2004). During an action potential (AP), both membrane depolarization and elevated intracellular Ca^{2+} can activate BK_{Ca} channels, which in turn contribute to AP fast repolarization, generate the fast component of the afterhyperpolarization (fAHP) and reduce Ca^{2+} influx via inactivation of Ca_V channels. Prominently, AP repolarization and fAHP significantly contribute to AP shape and duration. By controlling the AP shape and duration, BK_{Ca} channels can regulate neuronal excitability and some Ca^{2+} transients that underlie the release of neurotransmitter at presynaptic terminals.

The mechanisms underlying the inhibitory and excitatory role of BK_{Ca} channels are complex (**Figure 1**). Functional studies have reported that the activation of BK_{Ca} channels is hyperpolarizing; thus the resulting net effect on membrane excitability is inhibitory. However, evidence suggests that the activation of BK_{Ca} channels can also facilitate high-frequency firing in some brain neurons, including CA1 pyramidal cells of the hippocampus (Gu et al., 2007). In physiological conditions, BK_{Ca} channels activate slowly during an AP, allowing intracellular Ca^{2+} to activate Ca^{2+}-dependent conductances such as the small conductance Ca^{2+}-activated K^+ (SK_{Ca}) channels, thereby inhibiting repetitive firing. The inhibitory effect following the activation of BK_{Ca} channels may result from a delay in the development of an AP spike or decrease in fAHP conductances. Altered extracellular K^+ levels can modify the cell membrane potential to persistently depolarized values that may lead to paroxysmal discharges (Lebovitz, 1996). Interestingly, conversion from regular firing into burst firing upon the elevation of extracellular K^+ has been observed in hippocampal slices (Jensen et al., 1994; Jensen and Yaari, 1997). Blockade of BK_{Ca} channels also can inhibit neuronal firing because the resulting AP broadening can allow the activation of slow-onset voltage-gated K^+ channels, such as small SK_{Ca} channels and delayed rectifier K^+ channels. The resulting K^+ currents associated with an increased inactivation of voltage-gated Na^+ (Na_V) channels could slow the depolarization during an interspike interval. Further, excitation following the activation of upregulated BK_{Ca} channels may result from their role in the generation of fast spike repolarization and fAHP, which would favor a reduced activation of SK_{Ca} channels and delayed rectifier K^+ channels and would indirectly facilitate the recovery of Na_V from inactivation (Gu et al., 2007). The upregulation of BK_{Ca} channels may cause large increase in extracellular K^+, which in turn reduces the driving force for inhibitory K^+ currents leading to enhanced neuronal excitability. The activation of BK_{Ca} channels can reduce neurotransmitter (GABA) release by shortening the duration of depolarization to allow Ca^{2+} entry via Ca_V channels, resulting in enhanced neuronal excitability (Hu et al., 2001; Raffaelli et al., 2004). There is also a possibility that the inhibitory and excitatory action of BK_{Ca} channels may be age dependent. Indeed, smaller BK_{Ca} channel currents were recorded in pyramidal neurons of the prefrontal cortex in developing animals compared with adolescent and adult animals (Ksiazek et al., 2013). Multiple lines of evidence indicate that a lower availability and/or expression of BK_{Ca} channels may contribute to the broadening of APs during repetitive firing (Shao et al., 1999; Faber and Sah, 2003). Therefore, the lower availability of BK_{Ca} channels in young animals may facilitate neuronal activity during this developmental stage. Given the relevance of BK_{Ca} channels in the control of neuronal excitability, these channels have been implicated in the pathophysiology of several neurological disorders associated with altered neuronal excitability, including seizure disorders.

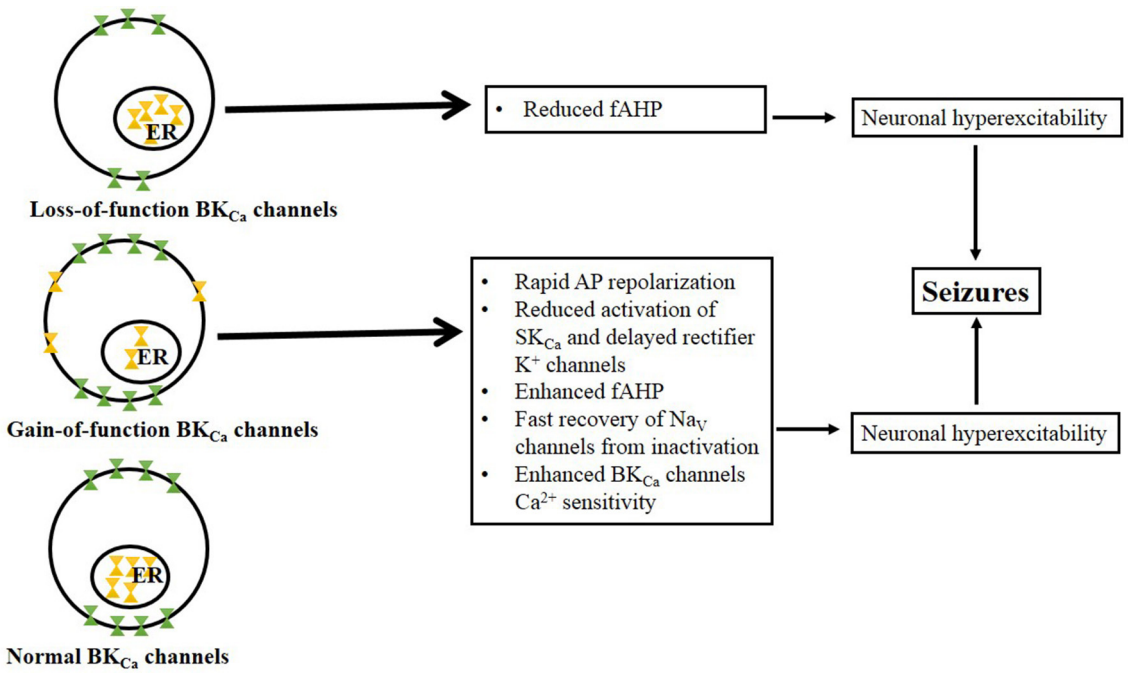


FIGURE 1 | Proposed mechanisms associated with BK_{Ca} loss-of-function and gain-of-function channels. BK_{Ca} channel loss-of-function occurs when there is low abundance of the channel at the membrane surface but no change in the BK_{Ca} channel number in the endoplasmic reticulum (ER, note that ubiquitination prevent channels from trafficking to the cell surface). Potential mechanisms underlying neuronal hyperexcitability following BK_{Ca} channels loss-of-function include reduced fAHP conductances. BK_{Ca} channel gain-of-function is characterized by the release of ubiquitinated BK_{Ca} channels from the ER and their insertion into the membrane surface (Liu et al., 2014). Thus, impairing ubiquitination may lead to overexpression of BK_{Ca} channels relative to control conditions. Potential mechanisms underlying neuronal hyperexcitability following BK_{Ca} channels gain-of-function include: rapid AP repolarization that would favor reduced activation of SK_{Ca} and delayed rectifier K^+ channels as well as facilitated the rate of recovery of Na_V channels from inactivation.

BK_{Ca} CHANNEL LOSS-OF-FUNCTION HYPOTHESIS

BK_{Ca} CHANNEL LOSS-OF-FUNCTION AND ENHANCED NEURONAL EXCITABILITY IN SEIZURE DISORDERS

Epilepsy consists of a group of chronic neurological disorders characterized by spontaneous and recurrent seizures. These seizures result from aberrant neuronal excitability associated with abnormal connections in the brain. Because the activation of BK_{Ca} channels limits the depolarization-induced bursting activity in neurons, it is assumed that a loss-of-function in BK_{Ca} channels will promote neuronal hyperexcitability, which can lead to seizures. Accordingly, reduced fAHP conductances were found in dentate gyrus granule cells obtained from patients suffering from temporal lobe epilepsy (Williamson et al., 1993). Similarly, idiopathic generalized epilepsy (mostly typical absence epilepsy) in humans has been associated with a single nucleotide deletion in exon 4 (delA750) of the *KCNMB3* gene encoding for BK_{Ca}channel β3 subunit (Lorenz et al., 2007). When expressed in a heterologous system, this mutation (BK_{Ca} channel β3b-V4 subunit isoform) exhibited BK_{Ca} channel loss-of-function, characterized by fast inactivation kinetics (Hu et al., 2003). The mutated *KCNMB3* gene also has been found in patients with dup(3q) syndrome with seizures (Riazi et al., 1999).

BK_{Ca} channel loss-of-function has also been implicated in the pathophysiology of animal models of seizures and epilepsy. A transient loss of fAHP conductances was found in subicular neurons following a kindling model of epileptogenesis (Behr et al., 2000). In the genetically epilepsy-prone rat (GEPR), an inherited model of generalized tonic-clonic epilepsy, reduced fAHP conductances were reported in CA3 neurons of the hippocampus (Verma-Ahuja et al., 1995). Similarly, in preliminary experiments, we found that the current density of BK_{Ca} channels is significantly reduced in inferior colliculus (IC) neurons, the site of seizure initiation in this model. However, no significant change was observed in the abundance of BK_{Ca} channel α subunit proteins in IC neurons of the GEPR (N'Gouemo et al., 2009). Similarly, the expression of BK_{Ca} channel α subunit was not altered in the dentate gyrus of the Krushinskii-Molodkina rat, a model of inherited epilepsy (Savina et al., 2014). Nevertheless, the protein expression of BK_{Ca} channel β4 subunits was elevated in the dentate gyrus of the Krushinskii-Moslodkina rat (Savina et al., 2014). The upregulation of β4 subunit is consistent with loss-of-function because this subunit inhibits BK_{Ca} channel activity (Brenner et al., 2005). In a model of alcohol withdrawal seizures, BK_{Ca} channel loss-of-function was reported and characterized by reduced current density, decreased channel conductance and lower protein abundance of BK_{Ca} channel α subunit in IC neurons (N'Gouemo and Morad, 2014). However, these changes outlasted the finite period of alcohol withdrawal seizure susceptibility, suggesting that BK_{Ca} channel loss-of-function in IC neurons was associated with the long-term effects of alcohol withdrawal hyperexcitability. Whether BK_{Ca} channels in IC neurons play an important role in the pathogenesis of alcohol withdrawal seizures remains to be determined. In a pilocarpine post-status epilepticus model, a downregulation of BK_{Ca} channel α subunit mRNA and protein was found in the cortex and hippocampus, consistent with a loss-of-function of BK_{Ca} channels associated with seizure generation (Pacheco Otalora et al., 2008; Ermolinsky et al., 2011). Further analysis revealed that the remaining BK_{Ca} channels in the dentate gurus were essentially made of the BK_{Ca} channel STREX splice variant instead of the ZERO variant (Ermolinsky et al., 2011). Interestingly, inserting the STREX splice variant shifts the conductance/voltage relation of BK_{Ca} channels to the left so that the channels are active at more physiological Ca^{2+} and voltage levels (Shipston, 2013). However, elevated intracellular Ca^{2+} is associated with seizure activity and epileptogenesis (Sanabria et al., 2001; Raza et al., 2004), suggesting an altered function of the remaining STREX BK_{Ca} channels in the pilocarpine model.

BK_{Ca} CHANNEL LOSS-OF-FUNCTION AND ENHANCED NEURONAL EXCITABILITY IN AUTISM SPECTRUM DISORDERS

Autism spectrum disorders (ASD) are a heterogeneous group of genetic neurodevelopmental disorders characterized by impairment of social communication and behavioral problems. Interestingly, studies have reported a co-occurrence of ASD and epilepsy (Deykin and MacMahon, 1979). The prevalence of epilepsy and associated electroencephalogram abnormalities in ASD significantly exceeded that of the normal population (Tuchman and Rapin, 1997). The higher incidence of epileptiform electroencephalogram abnormalities was also reported in children with ASD without epilepsy (Tuchman and Rapin, 1997). Thus, autism may be classified as a disorder of neuronal excitability, suggesting a potential role for ion channels in the etiology of ASD. ASD-linked ion channels of interest include BK_{Ca} channels. A mutation in the *KCNAM1* gene, which encodes for the α subunit of BK_{Ca} channels, has been reported in some ASD patients with epilepsy (Laumonnier et al., 2006). The mutated *KCNAM1* gene also causes haploinsufficiency in ASD patients, suggesting a potential role of BK_{Ca} channels in the pathogenesis of ASD (Laumonnier et al., 2006). When expressed in a heterologous system, this mutation exhibits reduced BK_{Ca} channel currents consistent with a loss-of-function (Laumonnier et al., 2006). Whether the downregulation of BK_{Ca} channels directly contributes to the pathogenesis of autism-epilepsy phenotype remains unknown.

BK_{Ca} CHANNEL LOSS-OF-FUNCTION AND REDUCED NEURONAL EXCITABILITY IN SEIZURE DISORDERS

Evidence shows that pharmacological blockade of BK_{Ca} channels can trigger seizures and status epilepticus, providing compelling evidence that BK_{Ca} channel loss-of-function can contribute to epileptogenesis (Young et al., 2003). However, mice lacking BK_{Ca} channel α (and β1) subunits do not exhibit spontaneous seizures, consistent with no change or reduced CNS excitability (Sausbier et al., 2004). Thus, the elevated seizure susceptibility observed in animal models cannot be explained solely by a downregulation of BK_{Ca} channel α subunits. Notably, evidence shows that BK_{Ca} channels can be subjected to ubiquitination by $CRL4A^{CRBN}$ and are therefore retained in the endoplasmic reticulum and prevented from trafficking to the cell surface. Deregulation of this control mechanism results in enhanced activity of neuronal BK_{Ca} channels and epileptogenesis (Liu et al., 2014). Notably, the

cereblon (CRBN) co-localizes with BK_{Ca} channels in brain neurons and regulate their surface expression (Jo et al., 2005). The CRBN gene is highly expressed in the hippocampus, consistent with its role in the pathogenesis of limbic seizures (Liu et al., 2014).

BK_{Ca} CHANNEL GAIN-OF-FUNCTION HYPOTHESIS

BK_{Ca} CHANNEL GAIN-OF-FUNCTION AND ENHANCED NEURONAL EXCITABILITY IN SEIZURE DISORDERS

Although BK_{Ca} channels are thought to reduce neuronal firing, evidence indicates that the gain-of-function of these channels can contribute to bursting activity and epileptogenesis. Indeed, upregulation of the α subunit and downregulation of the β4 subunit of BK_{Ca} channels were found in the dentate gyrus neurons of Krushinskii-Molodkin rats subjected to audiogenic kindling, which induced enhanced seizure severity (Savina et al., 2014). These findings are consistent with the BK_{Ca} channel gain-of-function associated with enhanced seizure severity because the β4 subunit inhibits BK_{Ca} channel activity. Notably, genetic deletion of the β4 subunit of BK_{Ca} channels facilitates the development of pilocarpine-induced seizures that are associated with gain-of-function of BK_{Ca} channels, as characterized by elevated cell-surface expression of BK_{Ca} channels, enhanced Ca^{2+} sensitivity to BK_{Ca} channels, larger currents and high-frequency firing in the dentate gyrus of the hippocampus (Brenner et al., 2005; Shruti et al., 2012).

BK_{Ca} channel gain-of-function has also been found in human epilepsy. Accordingly, in a family of patients suffering from generalized epilepsy (mostly absence epilepsy) and paroxysmal dyskinesia, a missense mutation (D434G) in exon 10 of the *KCNMA1* gene that encodes the BK_{Ca} channel α subunit has been found (Du et al., 2005). When expressed in a heterologous system, this mutation gave rise to gain-of-function of BK_{Ca} channel currents characterized by larger currents, elevated open channel probability and enhanced Ca^{2+} sensitivity to BK_{Ca} channels (Du et al., 2005; Wang et al., 2009; Yang et al., 2010). The D434G mutation gain-of-function was potentiated in the presence of β1, β2, and β4 subunits of BK_{Ca} channels (Díez-Sampedro et al., 2006; Lee and Cui, 2009). Notably, a polymorphism in the β4 subunit has been associated with human epilepsy (Cavalleri et al., 2007). These findings suggest that D434G mutation-induced changes in BK_{Ca} channels contribute to neuronal hyperexcitability and lead to generalized seizures and paroxysmal dyskinesia.

BK_{Ca} CHANNEL GAIN-OF-FUNCTION AND REDUCED NEURONAL EXCITABILITY IN SEIZURE DISORDERS

BK_{Ca} channels are found in excitatory neurons located in several brain sites, including the hippocampus, where they may promote high-frequency firing (Gu et al., 2007). Blockade of BK_{Ca} channels in these brain sites may reduce or suppress neuronal hyperexcitability. Consistent with this hypothesis, the blockade of BK_{Ca} channels suppressed pentylenetetrazole-induced epileptiform activity as well as spontaneous bursting activity in cortical neurons obtained from EL mouse, an inherited model of epilepsy (Jin et al., 2000). Similarly, picrotoxin-induced generalized tonic-clonic seizures give rise to BK_{Ca} channel gain-of-function characterized by elevated currents and high-frequency firing in somatosensory (barrel) cortical neurons of pre-sensitized animals (Shruti et al., 2008). Accordingly, the blockade of BK_{Ca} channels suppressed these picrotoxin-induced generalized tonic-clonic seizures (Sheehan et al., 2009). Thus, picrotoxin-induced seizure pre-sensitization may cause a maladaptive regulation (e.g., exit from the endoplasmic reticulum) of BK_{Ca} channels in brain neurons. In a fly model of ethanol intoxication/withdrawal, a blockade of *Slo1* gene neural promoter prevented the occurrence of ethanol-induced enhancement of electrographical seizure susceptibility, suggesting BK_{Ca} channel gain-of-function in the pathogenesis of alcohol withdrawal seizures (Ghezzi et al., 2012). However, this report raises some controversy with a rodent model of alcohol withdrawal seizures (N'Gouemo and Morad, 2014).

CONCLUSION

The role of BK_{Ca} channels in the pathophysiology of diseases of neuronal excitability is complex, in part because the activity of these channels can be regulated by many metabolic factors that alter neuronal excitability, including phosphorylation and acidification. Compelling evidence suggests that BK_{Ca} channel loss-of-function and gain-of-function can both contribute to neuronal hyperexcitability that leads to enhanced seizure susceptibility. The identification of BK_{Ca} channel subunit mutations has been critical in determining the role of these channels in etiology and mechanisms for epileptogenesis and seizure generation, raising the possibility that BK_{Ca} channels may represent potential molecular targets for seizure suppression.

ACKNOWLEDGMENTS

This work was supported by the NIH Public Health Service Grant AA020073. The author would like to thank Dr. Gholam Motamedi for helpful discussions and critical reading.

REFERENCES

Behr, J., Gloveli, T., and Heinemann, U. (2000). Kindling induces a transient suppression of afterhyperpolarization in rat subicular neurons. *Brain Res.* 867, 259–264. doi: 10.1016/S0006-8993(00)02324-6

Brelidze, T. I., and Magleby, K. L. (2004). Protons block BK channels by competitive inhibition with K^+ and contribute to the limits of unitary currents at high voltages. *J. Gen. Physiol.* 123, 305–319. doi: 10.1085/jgp.200308951

Brenner, R., Chen, Q. H., Vilaythong, A., Toney, G. M., Noebels, J. L., and Aldrich, R. W. (2005). BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat. Neurosci.* 28, 1752–1759. doi: 10.1038/nn1573

Cavalleri, G. L., Weale, M. E., and Shianna, K. V. (2007). Multicentre search for genetic susceptibility loci in sporadic epilepsy syndrome and seizure types: a case-control study. *Lancet Neurol.* 6, 970–980. doi: 10.1016/S1474-4422(07)70247-8

Chang, C. P., Dworetzky, S. I., Wang, J., and Goldstein, M. E. (1997). Differential expression of the alpha and beta subunits of the large-conductance calcium-activated potassium channel; implication for channel diversity. *Brain Res. Mol. Brain Res.* 45, 33–40. doi: 10.1016/S0169-328X(96)00230-6

Chen, L., Bi, D., Tian, L., McClafferty, H., Steeb, F., Ruth, P., et al. (2013). Palmitoylation of the β4-subunit regulates surface expression of large conductance calcium-activated potassiumchannel splice variants. *J. Biol. Chem.* 288, 13136–13144. doi: 10.1074/jbc.M113.461830

Chen, L., Tian, L., MacDonald, S. H., McClafferty, H., Hammond, M. S., Huibant, J. M., et al. (2005). Functionally diverse complement of large conductance calcium- and voltage-activated potassium channel (BK) alpha-subunits generated from a single site of splicing. *J. Biol. Chem.* 280, 33599–33609. doi: 10.1074/jbc.M505383200

Deykin, E. Y., and MacMahon, B. (1979). The incidence of seizures among children with autistic symptoms. *Am. J. Psychiatry* 136, 1310–1312.

Díez-Sampedro, A., Silverman, W. R., Bautista, J. F., and Richerson, G. B. (2006). Mechanism of increased open probability by a mutation of the BK channel. *J. Neurophysiol.* 96, 1507–1516. doi: 10.1152/jn.00461.2006

Du, W., Bautista, J. F., Yang, H., Diez-Sampedro, A., You, S. A., Wang, L., et al. (2005). Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat. Genet.* 37, 733–738. doi: 10.1038/ng1585

Ermolinsky, B., Skinner, F., Garcia, I., Arshadmansab, M. F., Otalora, L. F., Zarei, M. M., et al. (2011). Upregulation of STREX splice variant of the large conductance Ca^{2+}-activated potassium (BK) channel in a rat model of mesial temporal lobe epilepsy. *Neurosci. Res.* 69, 73–80. doi: 10.1016/j.neures.2010.09.011

Faber, E. S., and Sah, P. (2003). Ca^{2+}-activated K^+ (BK) channel inactivation contributes to spike broadening during repetitive firing in the rat lateral amygdala. *J. Physiol.* 552(Pt 2), 483–497. doi: 10.1113/jphysiol.2003.050120

Ghezzi, A., Krishnan, H. R., and Atkinson, N. S. (2012). Susceptibility to ethanol withdrawal seizures is produced by BK channel gene expression. *Addict. Biol.* doi: 10.1111/j.1369-1600.2012.00465.x

Grunnet, M., and Kaufmann, W. A. (2004). Coassembly of big conductance Ca^{2+}-activated K^+ channels and L-type voltage-gated Ca^{2+} channels in rat brain. *J. Biol. Chem.* 279, 36445–36453.

Gu, N., Vervaeke, K., and Storm, J. F. (2007). BK potassium channels facilitate high-frequency firing and cause early frequency adaptation in rat CA1 hippocampal pyramidal cells. *J. Physiol.* 580, 859–882. doi: 10.1113/jphysiol.2006.126367

Hou, S., Xu, R., Heinemann, S. H., and Hoshi, T. (2008). Reciprocal regulation of the Ca^{2+} and H^+ sensitivity in the SLO1 BK channel conferred by the RCK1 domain. *Nat. Struct. Mol. Biol.* 15, 403–410. doi: 10.1038/nsmb.1398

Hu, H., Shao, L. R., Chavoshy, S., Gu, N., Trieb, M., Behrens, R., et al. (2001). Presynaptic Ca^{2+}-activated K^+ channels in glutamatergic hippocampal terminals and their role in spike repolarization and regulation of transmitter release. *J. Neurosci.* 21, 9585–9597.

Hu, S., Labuda, M. Z., Pandolfo, M., Goss, G. G., McDermid, H. E., and Ali, D. W. (2003). Variants of the *KCNMB3* regulatory subunit of maxi BK channels affect channel inactivation. *Physiol. Genomics* 15,191–198.

Isaacson, J. S., and Murphy, G. J. (2001). Glutamate-mediated extrasynaptic inhibition: direct coupling of NMDA receptors to Ca^{2+}-activated K^+ channels. *Neuron* 31, 1027–1034. doi: 10.1016/S0896-6273(01)00428-7

Jensen, M. S., Azouz, R., and Yaari, Y. (1994). Variant firing patterns in rat hippocampal pyramidal cells modulated by extracellular potassium. *J. Neurophysiol.* 71, 831–839.

Jensen, M. S., and Yaari, Y. (1997). Role of intrinsic burst firing, potassium accumulation, and electrical coupling in the elevated potassium model of hippocampal epilepsy. *J. Neurophysiol.* 77, 1224–1233.

Jiang, Z., Wallner, M., Meera, P., and Toro, L. (1999). Human and rodent MaxiK channel beta-subunit genes: cloning and characterization. *Genomics* 55, 57–67. doi: 10.1006/geno.1998.5627

Jin, W., Sugaya, A., Tsuda, T., Ohguchi, H., and Sugaya, E. (2000). Relationship between large conductance calcium-activated potassium channel and bursting activity. *Brain Res.* 860, 21–28. doi: 10.1016/S0006-8993(00)01943-0

Jo, S., Lee, K.-H., Song, S., Jung, Y.-K., and Park, C.-S. (2005). Identification and functional characterization of cereblon as a binding protein for large-conductance calcium-activated potassium channel in rat brain. *J. Neurochem.* 94, 1212–1224. doi: 10.1111/j.1471-4159.2010.06938.x

Knaus, H. G., Schwarzer, C., Koch, R. O., Eberhart, A., Kaczorowski, J. G., Glossmann, H., et al. (1996). Distribution of high-conductance Ca^{2+}-activated K^+ channels in rat brain: targeting to axons and nerve terminals. *J. Neurosci.*16, 955–963.

Ksiazek, A., Ladno, W., Szulczyk, B., Grzelka, K., and Szulczyk, P. (2013). Properties of BK-type Ca^{++}-dependent K^+ channel currents in medial prefrontal cortex pyramidal neurons in rats of different ages. *Front. Cell. Neurosci.* 7:185. doi: 10.3389/fncel.2013.00185

Laumonnier, F., Roger, S., Guérin, P., Molinari, F., M'rad, R., Cahard, D., et al. (2006). Association of a functional deficit of the BKCa channel, a synaptic regulator of neuronal excitability, with autism and mental retardation. *Am. J. Psychiatry* 163, 1622–1629.

Lebovitz, R. M. (1996). Quantitative examination of dynamic interneuronal coupling via single-spike extracellular potassium ion transients. *J. Theor. Biol.* 180, 11–25.

Lee, U. S., and Cui, J. (2009). β-subunit-specific modulations of BK channels function by a mutation associated with epilepsy and dyskinesia. *J. Physiol.* 587, 1481–1498. doi: 10.1113/jphysiol.2009.169243

Liu, J., Vaithianathan, T., Manivannan, K., Parrill, A., and Dopico, A. M. (2008). Ethanol modulates BK_{Ca} channels by acting as an adjuvant of calcium. *Mol. Pharmacol.* 74, 628–640. doi: 10.1124/mol.108.048694

Liu, J., Ye, J., Zou, X., Xu, Z., Feng, Y., Zou, X., et al. (2014). CRL4A(CRBN) E3 ubiquitin ligase restricts BK channel activity and prevents epileptogenesis. *Nat. Commun.* 5, 3924. doi: 10.1038/ncomms4924

Lorenz, S., Heils, A., Kasper, J. M., and Sander, T. (2007). Allelic association of a truncation mutation of the KCNMB3 gene with idiopathic generalized epilepsy. *Am. J. Med. Genet.* 144B, 10–13. doi: 10.1002/ajmg.b.30369

Marrion, N. V., and Tavalin, S. J. (1998). Selective activation of Ca^{2+}-activated K^+ channels by co-localized Ca^{2+} channels in hippocampal neurons. *Nature* 395, 900–905.

Martire, M., Barrese, V., D'Amico, M., Iannotti, F. A., Pizzarelli, R., Samengo, I., et al. (2010). Pre-synaptic BK channels selectively control glutamate versus GABA release from cortical and hippocampal nerve terminals. *J. Neurochem.* 115, 411–422. doi: 10.1111/j.1471-4159.2010.06938.x

Misonou, H., Menegola, M., Buchwalder, L., Park, E. W., Meredith, A., Rhodes, K. J., et al. (2006). Immunolocalization of the Ca^{2+}-activated K^+ channel Slo1 in axons and nerve terminals of mammalian brain and cultured neurons. *J. Comp. Neurol.* 496, 289–302. doi: 10.1002/cne.20931

N'Gouemo, P., and Morad, M. (2014). Alcohol withdrawal is associated with a downregulation of large-conductance Ca^{2+}-activated K^+ channels in rat inferior colliculus neurons. *Psychopharmacology* 231, 2009–2018. doi: 10.1007/s00213-013-3346-8

N'Gouemo, P., Yasuda, R. P., and Faingold, C. L. (2009). Protein expression of small conductance calcium-activated potassium channels is altered in inferior colliculus neurons of the genetically epilepsy-prone rat. *Brain Res.* 1270, 107–111. doi: 10.1016/j.brainres.2009.02.034

Pacheco Otalora, L. F., Hernandez, E. F., Arshadmansab, M. F., Francisco, S., Willis, M., Ermolinsky, B., et al. (2008). Down-regulation of BK channel expression in the pilocarpine model of temporal lobe epilepsy. *Brain Res.* 1200, 116–131. doi: 10.1016/j.brainres.2008.01.017

Pallanek, L., and Genetzky, B. (1994). Cloning and characterization of human and mouse homologs of the *Drosophila* calcium-activated potassium channels gene *slowpoke*. *Hum. Mol. Genet.* 3, 1239–1243. doi: 10.1093/hmg/3.8.1239

Raffaelli, G., Saviane, C., Mohajerani, M. H., Perazani, P., and Cherubini, E. (2004). BK potassium channels control transmitter release at CA3-CA3 synapses in rat hippocampus. *J. Physiol.* 557, 147–157. doi: 10.1113/jphysiol.2004.062661

Raza, M., Blair, R. E., Sombati, S., Carter, D. S., Deshpande, L. S., and DeLorenzo, R. J. (2004). Evidence that injury-induced changes in hippocampal neuronal calcium dynamics during epileptogenesis cause acquired epilepsy. *Proc. Natl. Acad. Sci. U.S.A.* 101, 17522–17527. doi: 10.1073/pnas.0408155101

Riazi, M. A., Brinkman-Mills, P., Jonhson, A., Naylor, S. L., Minoshima, S., Shimizu, N., et al. (1999). Identification of a putative regulatory subunit of a calcium-activated potassium channel in the dup(3q) syndrome region and a related sequence on 22q11.2. *Genomics* 62, 90–94. doi: 10.1006/geno.1999.5975

Sanabria, E. R. G., Su, H., and Yaari, Y. (2001). Initiation of network bursts by Ca^{2+}-dependent intrinsic bursting in the rat pilocarpine model of temporal lobe epilepsy. *J. Physiol.* 532, 205–216. doi: 10.1111/j.1469-7793.2001.0205g.x

Sausbier, M., Hu, H., Arntz, C., Feil, S., Kamm, S., Adelsberger, H., et al. (2004). Cerebellar ataxia and Purkinje cell dysfunction caused by Ca^{2+}-activated K^+ channel deficiency. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9474–9478. doi: 10.1073/pnas.0401702101

Sausbier, U., Sausbier, M., Sailer, C. A., Arntz, C., Kauss, H. G., Neuhuber, W., et al. (2006). Ca^{2+} -activated K^+ channels of the BK-type in the mouse brain. *Histochem. Cell. Biol.* 125, 725–741. doi: 10.1007/s00418-005-0124-7

Savina, T. A., Levin, S. G., Poletaeva, I. I., Fedotova, I. B., and Shchipakina, T. G. (2014). Audiogenic kindling changes the subunit composition of BK-channels in dentate gyrus of Krushinskii-Molodkina rats. *Biochem. (Moscow) Suppl. Ser. A Membr. Cell Biol.* 8, 111–115. doi: 10.1134/S1990747813050164

Shao, L. R., Halvorsrud, R., Borg-Graham, L., and Storm, J. F. (1999). The role of BK-type Ca^{2+}-dependent K^+ channels in spike broadening during repetitive firing in rat hippocampal pyramidal cells. *J. Physiol.* 521, 135–146. doi: 10.1111/j.1469-7793.1999.00135.x

Sheehan, J. J., Benedetti, B. L., and Barth, A. L. (2009). Anticonvulsant effects of the BK-channel antagonist paxilline. *Epilepsia* 50, 711–720. doi: 10.1111/j.1528-1167.2008.01888.x

Shipston, M. J. (2013). Regulation of large conductance calcium- and voltage-activated potassium (BK) channels by S-palmitoylation. *Biochem. Soc. Trans.* 41, 67–71. doi: 10.1042/BST20120226

Shruti, S., Urban-Ciecko, J., Fitzpatrick, J. A., Brenner, R., Bruchez, M. P., and Barth, A. L. (2012). The brain-specific beta4 subunit downregulates BK channel cell surface expression. *PLoS ONE* 7:e33429. doi: 10.1371/journal.pone.0033429

Shruti, S., Clem, R. L., and Barth, A. L. (2008). A seizure-induced gain-of-function in BK channels is associated with elevated firing activity in neocortical pyramidal neurons. *Neurobiol. Dis.* 30, 323–330. doi: 10.1016/j.nbd.2008.02.002

Tian, L., Duncan, R. R., Hammond, M. S., Coghill, L. S., Wen, H., Rusinova, R., et al. (2001). Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J. Biol. Chem*. 276, 7717–7720. doi: 10.1074/jbc.C000741200

Tseng-Crank, J., Godinot, N., Johansen, T. E., Ahring, P. K., Strobaek, D., Mertz, R., et al. (1996). Cloning, expression, and distribution of a Ca^{2+}-activated K^+ channel beta-subunit from human brain. *Proc. Natl. Acad. Sci. U.S.A.* 93, 9200–9205. doi: 10.1073/pnas.93.17.9200

Tuchman, R. F., and Rapin, I. (1997). Regression in pervasive developmental disorders: seizures and epileptiform electroencephalogram correlates. *Pediatrics* 99, 560–566.

Verma-Ahuja, S., Evans, M. S., and Espinosa, J. A. (1995). Evidence of increased excitability in GEPR hippocampus preceding development of seizure susceptibility. *Epilepsy Res.* 31, 161–173. doi: 10.1016/S0920-1211(98)00027-8

Wang, B., Rothberg, B. S., and Brenner, R. (2009). Mechanism of increased BK channel activation from channel mutation that causes epilepsy. *J. Gen. Physiol.* 133, 283–294. doi: 10.1085/jgp.200810141

Wanner, S. G., Koch, R. O., Koschak, A., Trieb, M., Garcia, M. L., Kaczorowski, G. J., et al. (1999). High-conductance calcium-activated potassium channels in rat brain: pharmacology, distribution and subunit composition. *Biochemistry* 38, 5392–5400. doi: 10.1021/bi983040c

Williamson, A., Spencer, D. D., and Shepherd, G. N. (1993). Comparison between membrane and synaptic properties of the human and rodent granule cells. *Brain Res.* 622, 194–202. doi: 10.1016/0006-8993(93)90819-9

Xie, J., and McCobb, D. P. (1998). Control of alternative splicing of potassium channels by stress hormones. *Science* 280, 443–446.

Yang, J., Krishnamoorthy, G., Saxena, A., Zhang, G., Shi, J., Yang, H., et al. (2010). An epilepsy/dyskinesia-associated mutation enhances BK channel activation by potentiating Ca^{2+} sensing. *Neuron* 66, 871–883. doi: 10.1016/j.neuron.2010.05.009

Young, K. L., Villar, D., Carson, T. L., Ierman, P. M., Moore, R. A., and Bottoff, M. R. (2003). Tremorgenic mycotoxin intoxication with penitrem A and roquefortine in two dogs. *J. Am. Vet. Med. Assoc.* 222, 52–53. doi: 10.2460/javma.2003.222.52

Zhou, X.-B., Wulfsen, I., Utku, E., Sausbier, M., Wieland, T., Ruth, P., et al. (2010). Dual role of protein kinase C on BK channels regulation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8005–8010. doi: 10.1073/pnas.0912029107

Zhou, X., Wulfsen, I., Korth, M., McClafferty, H., Lukowski, R., Shipston, M. J., et al. (2012). Palmitoylation and membrane association of the stress axis regulated insert (STREX) controls BK channel regulation by protein kinase C. *J. Biol. Chem.* 287, 32161–32171. doi: 10.1074/jbc.M112.386359

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 July 2014; accepted: 10 September 2014; published online: 29 September 2014.

Citation: N'Gouemo P (2014) BK_{Ca} channel dysfunction in neurological diseases. Front. Physiol. ***5****:373. doi: 10.3389/fphys.2014.00373*

This article was submitted to Membrane Physiology and Membrane Biophysics, a section of the journal Frontiers in Physiology.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on