REGULATORY MECHANISMS OF CA^{2+}-ACTIVATED ION CHANNELS AND THEIR IMPACTS ON PHYSIOLOGICAL/ PATHOPHYSIOLOGICAL FUNCTIONS

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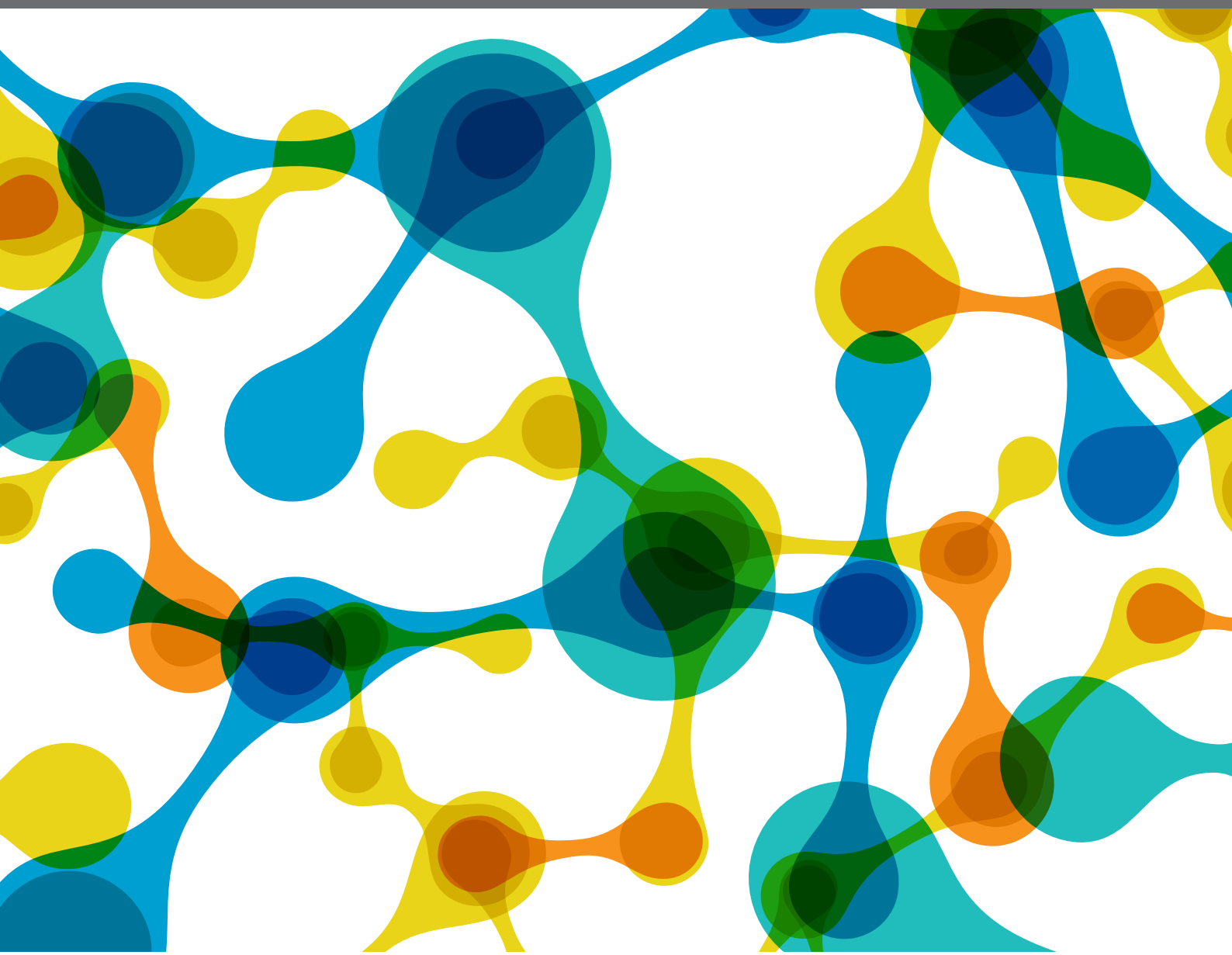

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REGULATORY MECHANISMS OF CA^{2+}-ACTIVATED ION CHANNELS AND THEIR IMPACTS ON PHYSIOLOGICAL/PATHOPHYSIOLOGICAL FUNCTIONS

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EDITORIAL
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Editorial: Regulatory Mechanisms of Ca^{2+} Activated Ion Channels and Their Impact on Physiological/ Pathophysiological Function

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Editorial on the Research Topic

Regulatory Mechanisms of Ca^{2+} Activated Ion Channels and Their Impacts on Physiological/ Pathophysiological Functions

The main goal of our Research Topic was to identify novel ionic, cellular and tissue level mechanisms that can regulate Ca^{2+}-activated K^+ channel expression and function. An important secondary goal was to summarize current knowledge concerning the consequences of changes in channel activity for initiation and progression of human diseases, and related targets for drug discovery. We also recognized that to achieve these goals it would be necessary to present current knowledge of Ca^{2+}-activated ion channel expression and function in intracellular organelles.

We were therefore very pleased with the engagement of the international community in our Research Topic. This resulted in the submission, peer review, and acceptance of 11 very informative reviews or original research articles. The predominant focus that emerged was on the large conductance Ca^{2+}-activated K^+ (BK) channels. Individual contributions focused on the ways in which BK channels form and then function as essential macromolecular signaling complexes, brought about (in part) by their alpha subunits being significant targets for pre- and posttranslational modifications (Sancho and Kyle). This functional theme is taken up and extended in the review by Shah et al., as they provide a succinct summary of what is now known about key aspects of spatially localized or nanodomain signaling complexes that have BK channels as their central element. These complexes can act as an electrophysiological negative feedback regulator for cellular membrane potential and/or agonist-induced changes in intracellular Ca^{2+}. An interesting extension of this recent knowledge concerning spatially localized channel expression and related signaling is provided by the clear and concise review by González-Sanabria et al. These authors synthesize and present current knowledge concerning key aspects of the functions of BK channels that are localized to the inner mitochondrial membrane, as well as the outer membrane of the nuclear envelope. When combined, these contributions provide an up-to-date and readable platform of knowledge for beginning to consider the role of BK channels in specific chronic disease pathophysiology; as well as in regulation of cellular homeostasis paradigms (detection and regulation of transient and maintained hypoxia) that need to be recognized and understood as an essential first step for clinical management. For example, the review by Lu and Lee provides a timely summary of the ways in which down regulation of BK channel expression can contribute to vascular dysfunction in the setting of a debilitating chronic disease, diabetes mellitus. The review contributed by Ochoa et al., builds on the fundamental

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mechanisms through which BK channels can function, both in essential physiological signaling and when dysregulated, to drive progressive pathophysiological sequalae. These authors first focus on hypoxic regulation of BK channels. They summarize basic mechanisms by which BK channels sense cellular oxygen levels, and then undergo hypoxia-induced changes that are specific to either the alpha or one of the beta or gamma subunits of the channel complex. This review concludes with a succinct account of the ways in which the BK channel signaling complex can contribute to the initiation or progression of a number of chronic diseases (including bronchial asthma or obstructive pulmonary disease including sleep apnea). Importantly, the more broadly based review contributed by Jackson reminds the reader of the very broad range of Ca^{2+}-activated, or Ca^{2+}-dependent, ion channels that are very widely expressed in the mammalian arteriolar vascular system. This review also provides a quite firm reminder that although the most insightful mechanistic studies of ion channel function require a singular focus and a reductionist experimental design; nevertheless, systems level integrative approaches are an absolute requirement for yielding advances in the understanding of essential physiological paradigms, such as the regulation of myogenic tone.

No current summary of Ca^{2+}-activated K^+ channel functionality would be considered to be complete in the absence of presentation of key aspects of what is known about the roles of these channels in neurophysiology. Two articles submitted to this Research Topic provide clear accounts of the roles of Ca^{2+}-activated K^+ channels. Interestingly, both emphasize the role of the integrated functional roles of at least two types of Ca^{2+}-activated K^+ channels in producing CNS phenotypes. McNally et al., provided novel information concerning the ways in which BK channels exhibit significant regulation that is driven by circadian clocks. This important insight is put in context of physiology of the suprachiasmatic nucleus, in part by the authors pointing out that a singular focus on the roles of only BK channels does not suffice. Instead, the contributions of a distinct subset of Ca^{2+}-activated K^+ channels (intermediate conductance) and activation of functionally linked Ca^{2+} channels must be included. For different reasons, and in a separate context, the clear and concise material offered up by Sahu and Turner summarizes the roles of three different Ca^{2+}-activated K^+ channels in hippocampal pyramidal neurons. These authors remind readers of the broadly based importance of being able to fully understand the Ca^{2+}-dependent slow afterhyperpolarization phase of the electrophysiological phenotype of CA1 neurons in the hippocampus. Based on their own comprehensive studies Sahu and Turner point out that advances in understanding the function of these neurons requires detailed knowledge of: 1) the profile of changes in intracellular Ca^{2+}, 2) spatially localized expression levels of all three subtypes of the Ca^{2+}-activated K^+ channel family, 3) as well as molecular and microanatomical information revealing the ways in which these integral membrane proteins are localized in nanodomains and are linked by molecular chaperones to form signaling complexes. The detailed contribution by Cui returns the emphasis to not only molecular features; but in fact, to atomistic structures. This manuscript illustrates the utility of this approach, when attempting to link some identified BK channel mutations to well recognized neurological disorders.

Our Research Topic was fortunate to receive two papers that provide timely insights into the roles of Ca^{2+}-activated Cl^- channels. The first (Le et al.) continues our molecular emphasis when presenting known properties of a Ca^{2+}-activated Cl^- channel denoted TMEM16. Le et al., summarize its major structural features and then focus on its unique gating and regulatory mechanisms. Importantly, this review also establishes that this channel isoform can exhibit specialized enzymatic (flipase) activity. Finally, Wray et al. and her colleagues provide an impactful review that presents some of the major roles of Ca^{2+}-activated Cl^- channels in key physiological functions of both myometrial and vascular smooth muscles. This Research Topic thus achieves its primary goals while also implicitly making it clear that in this broad, complex and quickly advancing field "more and perhaps the best is yet to come".

AUTHOR CONTRIBUTIONS

YS, SO, and WG wrote and edited the paper.

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ORIGINAL RESEARCH
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Contributions of $Ca_V1.3$ Channels to Ca^{2+} Current and Ca^{2+}-Activated BK Current in the Suprachiasmatic Nucleus

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Daily regulation of Ca^{2+}– and voltage-activated BK K^+ channel activity is required for action potential rhythmicity in the suprachiasmatic nucleus (SCN) of the hypothalamus, the brain's circadian clock. In SCN neurons, BK activation is dependent upon multiple types of Ca^{2+} channels in a circadian manner. Daytime BK current predominantly requires Ca^{2+} influx through L-type Ca^{2+} channels (LTCCs), a time when BK channels are closely coupled with their Ca^{2+} source. Here we show that daytime BK current is resistant to the Ca^{2+} chelator BAPTA. However, at night when LTCCs contribute little to BK activation, BK current decreases by a third in BAPTA compared to control EGTA conditions. In phase with this time-of-day specific effect on BK current activation, LTCC current is larger during the day. The specific Ca^{2+} channel subtypes underlying the LTCC current in SCN, as well as the subtypes contributing the Ca^{2+} influx relevant for BK current activation, have not been identified. SCN neurons express two LTCC subtypes, $Ca_V1.2$ and $Ca_V1.3$. While a role for $Ca_V1.2$ channels has been identified during the night, $Ca_V1.3$ channel modulation has also been suggested to contribute to daytime SCN action potential activity, as well as subthreshold Ca^{2+} oscillations. Here we characterize the role of $Ca_V1.3$ channels in LTCC and BK current activation in SCN neurons using a global deletion of CACNA1D in mouse ($Ca_V1.3$ KO). $Ca_V1.3$ KO SCN neurons had a 50% reduction in the daytime LTCC current, but not total Ca^{2+} current, with no difference in Ca^{2+} current levels at night. During the day, $Ca_V1.3$ KO neurons exhibited oscillations in membrane potential, and most neurons, although not all, also had BK currents. Changes in BK current activation were only detectable at the highest voltage tested. These data show that while $Ca_V1.3$ channels contribute to the daytime Ca^{2+} current, this does not translate into a major effect on the daytime BK current. These data suggest that BK current activation does not absolutely require $Ca_V1.3$ channels and may therefore also depend on other LTCC subtypes, such as $Ca_V1.2$.

Keywords: CACNA1D, KCNMA1, BK channel, L-type Ca^{2+} channel, circadian rhythm, Ca^{2+}-activated K^+ channel

INTRODUCTION

The suprachiasmatic nucleus (SCN) is the central circadian clock of the brain in mammals (Hastings et al., 2018; Harvey et al., 2020). Membrane signaling *via* ion channels regulates the daily patterning of action potential activity and neurotransmitter release, which is critical for the expression of circadian behavioral rhythms. Calcium is an important regulator of SCN signaling (Ikeda, 2004; Hastings et al., 2018; McNally et al., 2020), and SCN neurons have several different Ca^{2+} influx pathways, including voltage-gated Ca^{2+} channels (Pennartz et al., 2002; Cloues and Sather, 2003; Harvey et al., 2020; McNally et al., 2020). Of the channels involved in SCN signaling, L-type Ca^{2+} channels (LTCCs) are abundantly expressed (Nahm et al., 2005; Harvey et al., 2020).

LTCC currents are generated by the Ca_V1 family of Ca^{2+} channel subtypes (Lipscombe et al., 2004; Striessnig et al., 2006). $Ca_V1.1$ is the skeletal muscle LTCC, while $Ca_V1.2$ (α1C, CACNA1C) and $Ca_V1.3$ (α1D, CACNA1D) are the predominant subtypes in neurons and are sensitive to inhibition by dihydropyridines such as nimodipine (Lipscombe et al., 2004; Striessnig et al., 2006). $Ca_V1.2$ plays a wide variety of roles in neuronal excitability and cell signaling, regulating action potential activity, secretion, and transcription (Striessnig et al., 2006), but global genetic deletion of $Ca_V1.2$ is lethal due to cardiac dysfunction (Seisenberger et al., 2000). $Ca_V1.3$ is also expressed in variety of neurons and neuroendocrine cells, often co-expressed with $Ca_V1.2$, but differs from $Ca_V1.2$ in its activation at lower voltages (Striessnig et al., 2006). A global deletion of $Ca_V1.3$ channels is viable and results in deafness and cardiac arrhythmias (Platzer et al., 2000; Zhang et al., 2005). SCN neurons express both $Ca_V1.2$ and $Ca_V1.3$ channels (Nahm et al., 2005; Huang et al., 2012; Kim et al., 2015; Cheng et al., 2018).

SCN LTCC current is diurnally modulated, with higher current during the day that decreases at night (Pennartz et al., 2002; Whitt et al., 2018; McNally et al., 2020). Correlated with the change in current magnitude, inhibition of LTCC current with nimodipine decreases SCN firing selectively during the day (Pennartz et al., 2002; Whitt et al., 2018; McNally et al., 2020). One mechanism for the effect on SCN firing is *via* LTCC-dependent activation of Ca^{2+}-activated BK K^+ channels (Plante et al., 2021). During the day, LTCCs are responsible for the majority of BK current activation (Jackson et al., 2004; Whitt et al., 2018; Plante et al., 2021). However, at night, their role is reduced, and BK current activation relies primarily on release of Ca^{2+} from intracellular stores (Whitt et al., 2018; Plante et al., 2021). Either $Ca_V1.2$ or $Ca_V1.3$ could contribute to the LTCC current that drives daytime activation of the BK current, as both channel subtypes are known to activate BK currents produced by BK channel variants from SCN (Plante et al., 2021) or within other excitable cells (Roberts et al., 1990; Wisgirda and Dryer, 1994; Prakriya and Lingle, 1999; Sun et al., 2003; Grunnet and Kaufmann, 2004; Berkefeld et al., 2006; Marcantoni et al., 2010; Hou et al., 2016; Bellono et al., 2017; Vivas et al., 2017; Plante et al., 2021). Little is known about the specific role of $Ca_V1.2$ on SCN firing, but $Ca_V1.2$ expression is higher at night and mouse knockouts display altered nighttime phase-shifting behavior (Schmutz et al., 2014). In contrast, $Ca_V1.3$ channels have been proposed to regulate firing rate, Ca^{2+} oscillations, and histamine signaling during the day (Huang et al., 2012; Kim et al., 2015, 2016). Current pharmacological tools cannot effectively distinguish between these LTCC subtypes, necessitating the use of transgenic mouse lines to address the relative contributions of these channel subtypes to neuronal excitability.

In this study, we focused on investigating a role for $Ca_V1.3$ channels in BK current activation, based on their proposed roles in SCN during the day and their regulation of spontaneous firing in other cell types *via* BK channel activation (Vandael et al., 2010; Bellono et al., 2017). Using a $Ca_V1.3$ knockout mouse line ($Ca_V1.3$ KO, also called $\alpha1D^{-/-}$, $CACNA1D^{-/-}$)(Platzer et al., 2000), whole-cell Ca^{2+} and BK currents were recorded from day and night SCN neurons in acute brain slices. The results reveal that $Ca_V1.3$ channels contribute to the daytime LTCC current in SCN neurons, as well as BK current activation.

MATERIALS AND METHODS

Mice

Experimental mice were 3–6 week old male and female wildtype (WT) C57BL/6J and $Ca_V1.3$ WT and KO littermates produced from heterozygous $Ca_V1.3$ breeding pairs on a mixed C57BL6:FVB background (Platzer et al., 2000). Tail or ear tissue samples were genotyped using "Cacna1d-1 WT" and neomycin probes in real-time PCR reactions at a commercial vendor (Transnetyx, Cordova, TN). All mice were group housed from birth on a standard 12:12 h light–dark cycle (for day timepoints) or a reverse 12:12 h light–dark cycle (night timepoints). All procedures involving mice were conducted in accordance with the University of Maryland School of Medicine Animal Care and Use Guidelines and approved by the Institutional Animal Care and Use Committee.

Acute SCN Slice Preparation

Mice were sacrificed at zeitgeber time (ZT) 1–3 h for day, or ZT 14–16 h for night, experiments. Brains were rapidly removed and placed into ice-cold sucrose-substituted saline containing (in mM): 1.2 $MgSO_4$, 26 $NaHCO_3$, 1.25 Na_2HPO_4, 3.5 KCl, 3.8 $MgCl_2$, 10 glucose and 200 sucrose. Coronal slices (300 μm) were cut using a VT1000S vibratome (Leica Microsystems, Wetzlar, Germany) at 3–4°C. Slices containing SCN were incubated 1–2 h at 25°C in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 1.2 $MgSO_4$, 26 $NaHCO_3$, 1.25 Na_2HPO_4, 3.5 KCl, 2.5 $CaCl_2$ and 10 D-glucose (300–305 mOsm/kg). Slices were transferred to the recording chamber and perfused *via* gravity flow at 1–2 ml min^{-1} with oxygenated ACSF.

Electrophysiological Recordings

Recordings were performed at the peak (ZT 4–8 h) and nadir (ZT 17–21 h) of the circadian rhythm in spontaneous action potential firing, corresponding to the "day" and "night" timepoints, respectively. Neurons within the center of the SCN were identified in whole-cell current-clamp mode by spontaneous action potential firing or firing following injection of 5–20 pA of current for silent neurons.

Macroscopic BK and Ca^{2+} currents were recorded in whole-cell voltage-clamp mode at 25°C as described previously (Whitt et al., 2018; McNally et al., 2020). For BK currents, electrodes (4–7 MΩ) were filled with intracellular solution (in mM): 123 K-methanesulfonate, 9 NaCl, 0.9 EGTA, 9 HEPES, 14 Tris-phosphocreatine, 2 Mg-ATP, 0.3 Tris-GTP, and 2 Na_2-ATP, pH 7.3 (310–315 mOsm/kg). BAPTA (5 mM) was substituted for EGTA (0.9 mM) in some internal solutions as specified in figure legends. The bath ACSF was composed of (in mM): 125 NaCl, 1.2 $MgSO_4$, 26 $NaHCO_3$, 1.25 Na_2HPO_4, 3.5 KCl, 2.5 $CaCl_2$ and 10 D-glucose (300–305 mOsm/kg). For Ca^{2+} currents, the internal solution was (in mM): 115 cesium gluconate, 10 tetraethylammonium chloride, 10 HEPES, 0.5 EGTA, 2 $MgCl_2$, 20 sodium phosphocreatine, 2 Na_2ATP, and 0.3 Na_3 GTP (pH 7.3, 310–315 mOsm/kg). The bath ACSF was composed of (in mM): 68 NaCl, 3.5 KCl, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 10 D-glucose, 60 tetraethylammonium chloride, and 3 CsCl (300–305 mOsm/kg).

Total voltage-activated BK and Ca^{2+} currents were recorded in 1 μM tetrodotoxin (TTX) before and after focal perfusion (4-min wash-on) of the selective inhibitors paxilline (BK channels) or nimodipine (L-type Ca^{2+}channels), respectively. A minimum 10–15-min wash-out period of focally and bath perfused ACSF was performed before recording from the next cell. BK and L-type Ca^{2+} currents were isolated by subtracting currents elicited in the presence of their respective inhibitors, Nimodipine and Paxilline respectively, from total baseline currents. Three currents were averaged per cell and normalized to cell capacitance (range of 5–10 pF). R_a was <25 MΩ with less than ± 5% change (on average ∼15 MΩ). R_s was compensated at 60%. BK currents were elicited from a holding potential of −90 mV, stepping from −110 to 90 mV for 150 ms in 20-mV increments. Ca^{2+} currents were elicited from a holding potential of −90 mV, stepping from −90 to 50 mV for 150 ms in 10-mV increments. Only cells with a total current size of >100 pA were used in experiments. Voltage values were adjusted for the liquid junction potential (9 mV). Currents were *post hoc* filtered at 1 kHz.

Pharmacology

Drugs used in these experiments were: L-type Ca^{2+} channel inhibitor nimodipine (Nim, 10 μM, Alomone Labs, Jerusalem, Israel, #N-150), Ryanodine Receptor inhibitor dantroline (Dan, 10 μM, Sigma, #D9175), BK current inhibitor Paxilline (Pax, 10 μM, Alomone Labs, Jerusalem, Israel, #P-450) and Sodium channel inhibitor tetrodotoxin (TTX, 1 μM, Alomone Labs, Jerusalem, Israel, #T-550). All drugs were dissolved in DMSO, except TTX, which was dissolved in water. Drugs were focally perfused to the bath at a flow rate of 1 ml min^{-1} by a computer-controlled pressurized perfusion system (ValveLink 8.2; Automate Scientific, Berkeley, CA, USA) at the concentrations indicated from 1,000× stocks.

Membrane Potential Oscillations

In whole-cell current-clamp mode, membrane potential oscillations were recorded in TTX (1 μM) using the same solutions as BK current recordings (McNally et al., 2020). Cells with resting membrane potentials between −30 and 65 mV were

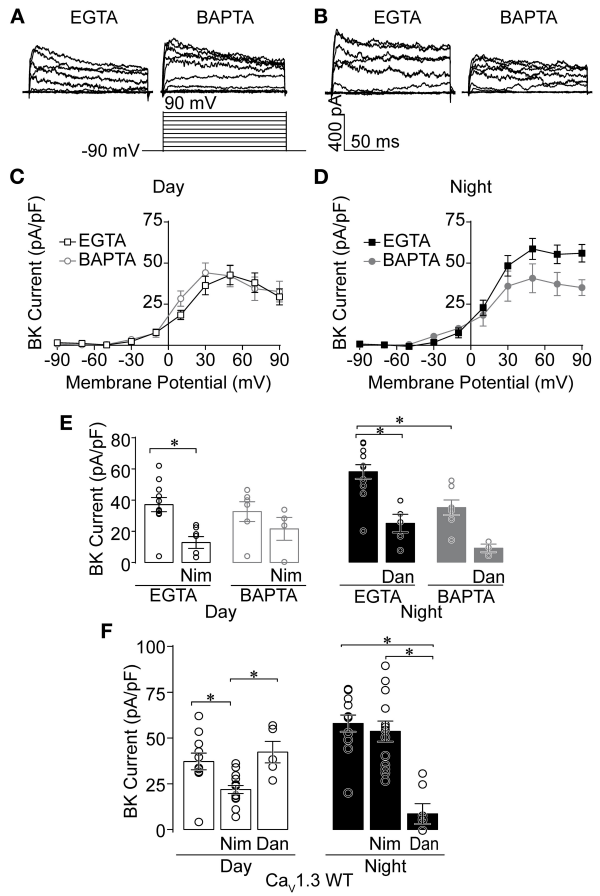

FIGURE 1 | Effects of Ca^{2+} chelators and Ca^{2+} channel inhibitors on BK currents recorded from SCN neurons during the day and night. Paxilline-sensitive macroscopic BK currents were recorded from C57BL6 WT **(A–E)** and $Ca_V1.3$ WT **(F)** SCNs. All intracellular solutions in this study were made with 0.9 mM EGTA, except where 5 mM BAPTA was substituted **(A–E)**. Currents were elicited from a holding potential of −90 mV by 150-ms voltage steps from −110 to +90 mV in +20-mV increments. **(A,B)** Representative BK currents from −90 to +90 mV are shown from day **(A)** and night **(B)** SCN neurons. **(C,D)** Current-voltage plot comparing BK current density recorded in either control EGTA or BAPTA during the day **(C)** and night **(D)**. **(E)** Summary of BK current density at +90 mV recorded with control EGTA or BAPTA with Ca^{2+} channel inhibitors 10 μM nimodipine (Nim) during the day, or 10 μM dantrolene (Dan) at night. In EGTA, Nim decreased BK currents compared to controls ($P = 0.0008$), with no significant difference in BAPTA. At night, BK currents were decreased with BAPTA compared to control EGTA conditions ($P < 0.0001$). Then Dan decreased BK currents in control EGTA ($P = 0.001$), but BAPTA was not significant. **(F)** Ca^{2+} channel inhibitor sensitivity in $Ca_V1.3$ WT SCN neurons. Summary of BK current density at +90 mV recorded with EGTA in control (no drug) conditions, or in the presence of Nim or Dan in day and night. $Ca_V1.3$ WT BK currents were decreased in Nim during the day compared to controls ($P = 0.01$), but not Dan. At night, $Ca_V1.3$ WT BK currents were decreased in Dan compared to control ($P = 0.008$) and Nim ($P < 0.0001$), but Nim and control were not different. *$P < 0.05$, One-way ANOVA with Bonferroni's *post hoc* test between all conditions within day or night. N's represent individual cells recorded from C57BL6 WT (EGTA, BAPTA) day: control (11 neurons, three slices; six neurons, two slices); Nim (seven neurons, two slices; four neurons, one slice) and night: control (12 neurons, three slices; seven neurons, two slices); Dan (five neurons, two slices; three neurons, one slice). $Ca_V1.3$ WT (day, night): control (11 neurons, six slices; 12 neurons, three slices), Nim (14 neurons, four slices; 13 neurons, four slices), and Dan (five neurons, two slices; seven neurons, two slices). Data are mean ± SEM.

included in the dataset. Data were acquired in 10-s sweeps at the cell resting membrane potential (spontaneous oscillations) and during a series of holding potentials stepping from −60 to 0 mV, in 10 mV increments (voltage-dependent oscillations). Oscillations were defined as at least a 5-mV change in membrane potential, with a frequency of 0.2 Hz. Representative traces in figures were at 2 kHz.

Data Analysis and Statistics

BK and Ca^{2+} current-voltage plots were constructed from peak current level at each voltage step. Data are reported as group mean ± SEM. Numbers reported in figure legends are the number of neurons recorded, with 1–6 neurons per animal (one SCN slice per animal). Data for each condition was derived from a minimum of two animals. Statistical significance was determined at $P < 0.05$ using Prism v7 (GraphPad Software, Inc) using unpaired Welch's *t* tests, one-way ANOVA with Bonferroni's *post hoc* test, and Fisher's exact test for categorical data as indicated in results and figure legends. Representative current traces in figures were filtered at 500 Hz.

RESULTS

Effect of Ca^{2+} Buffering on Day and Night BK Current Activation

L-type Ca^{2+} current is larger during the day compared to night in SCN neurons (Pennartz et al., 2002; Whitt et al., 2018; McNally et al., 2020). In conjunction with the diurnal modulation of LTCC current, BK current is predominantly dependent upon LTCCs during the day. However, LTCCs do not significantly contribute to BK current activation at night, suggesting the coupling between BK channels and their Ca^{2+} channel sources could differ between day and night (Whitt et al., 2018). To examine this idea, C57BL6 WT BK currents were recorded in two Ca^{2+} buffering conditions. As a control, BK currents were first recorded under standard whole-cell voltage-clamp conditions with 0.9 mM EGTA in the internal solution, conditions which permit the endogenous Ca^{2+} sources to contribute to BK current activation (Fakler and Adelman, 2008; Whitt et al., 2018; Plante et al., 2021). These BK currents recorded with 0.9 mM EGTA were then compared to BK currents recorded with internal solution containing 5 mM BAPTA, a Ca^{2+} chelator with fast kinetics that disrupts the functional coupling of BK channels located >10–20 nm from their Ca^{2+} source (Berkefeld et al., 2006; Fakler and Adelman, 2008; Cox, 2014). If the coupling between BK channels and their Ca^{2+} channel sources differs between day and night, BAPTA would be expected to reduce BK current below levels recorded in the EGTA control conditions.

Daytime BK currents were not reduced in BAPTA compared to EGTA (**Figures 1A,C,E**), suggesting tight functional coupling and close spatial localization between BK channels and their daytime Ca^{2+} source. However, in contrast to daytime, at night BK current was reduced ∼37% in BAPTA (**Figures 1B,D,E**). This suggests that some BK channels do not remain closely localized with their Ca^{2+} source at night. This change in BAPTA sensitivity was correlated with a change in the Ca^{2+} source that activates BK channels (Whitt et al., 2018). During the day, LTCCs are responsible for the majority of BK current activation. At night BK activation relies primarily on release of Ca^{2+} from intracellular stores through RyRs. This differential activation can be probed with inhibitors for these Ca^{2+} sources.

Consistent with previous studies, daytime BK current was sensitive to inhibition by nimodipine, a selective inhibitor of LTCCs, in control EGTA (**Figure 1E**) (Whitt et al., 2018). In the presence of BAPTA, the reduction in BK current was not statistically significant (**Figure 1E**). At night, BK current in control EGTA was sensitive to inhibition by dantrolene, a selective inhibitor of RyR Ca^{2+} release from intracellular stores. In BAPTA, a similar result to daytime recordings was observed, in that this condition also lacked significance (**Figure 1E**). Thus, taken together, the inhibitor sensitivity in BAPTA still leaves open whether co-localized BK-Ca^{2+} channel complexes contain either LTCCs during the day or RyRs at night. During the day, part of the lack of a definitive effect could be due to the potential for multiple Ca_V1 isoforms ($Ca_V1.2$ and $Ca_V1.3$) to contribute to the Ca^{2+} current in SCN neurons (Nahm et al., 2005; Huang et al., 2012; Kim et al., 2015; Cheng et al., 2018).

Given the influence of $Ca_V1.3$ channels on firing rate and membrane oscillations during the day (Huang et al., 2012), and BK's ability to partner with $Ca_V1.3$ without requiring other proteins in heterologous cells (Vivas et al., 2017; Plante et al., 2021), we first examined the specific contribution of $Ca_V1.3$ to BK current activation by recording from $Ca_V1.3$ KO SCNs (Platzer et al., 2000). $Ca_V1.3$ KO mice were obtained on a C57BL6:FVB background, and $Ca_V1.3$ WT littermates on this mixed strain background were used as controls. First, the changeover in Ca^{2+} source was verified in this strain from recordings in $Ca_V1.3$ WT SCNs. As with inbred C57BL6 SCN neurons (EGTA recordings, **Figure 1E**) (Whitt et al., 2018), $Ca_V1.3$ WT daytime BK currents were sensitive to inhibition by nimodipine, while dantrolene had a negligible effect (**Figure 1F**). At night, BK currents became nimodipine-insensitive, but dantrolene-sensitive (**Figure 1F**). This demonstrates that daily changeover in BK channel activation by its Ca^{2+} source occurs on the mixed strain background harboring the $Ca_V1.3$ transgene.

Ca^{2+} Current in $Ca_V1.3$ KO SCNs

Next, the Ca^{2+} currents were characterized in $Ca_V1.3$ WT and KO SCNs to determine if loss of $Ca_V1.3$ channels results in decreased Ca^{2+} current (**Figure 2**). Ca^{2+} currents (**Figures 2A,E**) were recorded using the voltage protocol shown in **Figure 2E**. In daytime $Ca_V1.3$ WT SCN neurons, the peak nimodipine-sensitive current is 42% of the total current. At night, this decreases to 16% (**Figures 2B,C**). This differential contribution generated a day vs. night difference in the nimodipine-sensitive LTCC current (**Figure 2D**) that is consistent with previous studies on other strain backgrounds (Pennartz et al., 2002; Whitt et al., 2018; McNally et al., 2020).

In the absence of $Ca_V1.3$ channels, there was no change in the total Ca^{2+} current in the $Ca_V1.3$ KO compared to WT (at −10 mV, $P = 0.59$ during the day and $P = 0.76$ at night, respectively; unpaired *t*-test) (**Figures 2F,G**). However, the peak nimodipine-sensitive current was reduced during the day in $Ca_V1.3$ KO cells compared to WT (**Figure 2I**), comprising

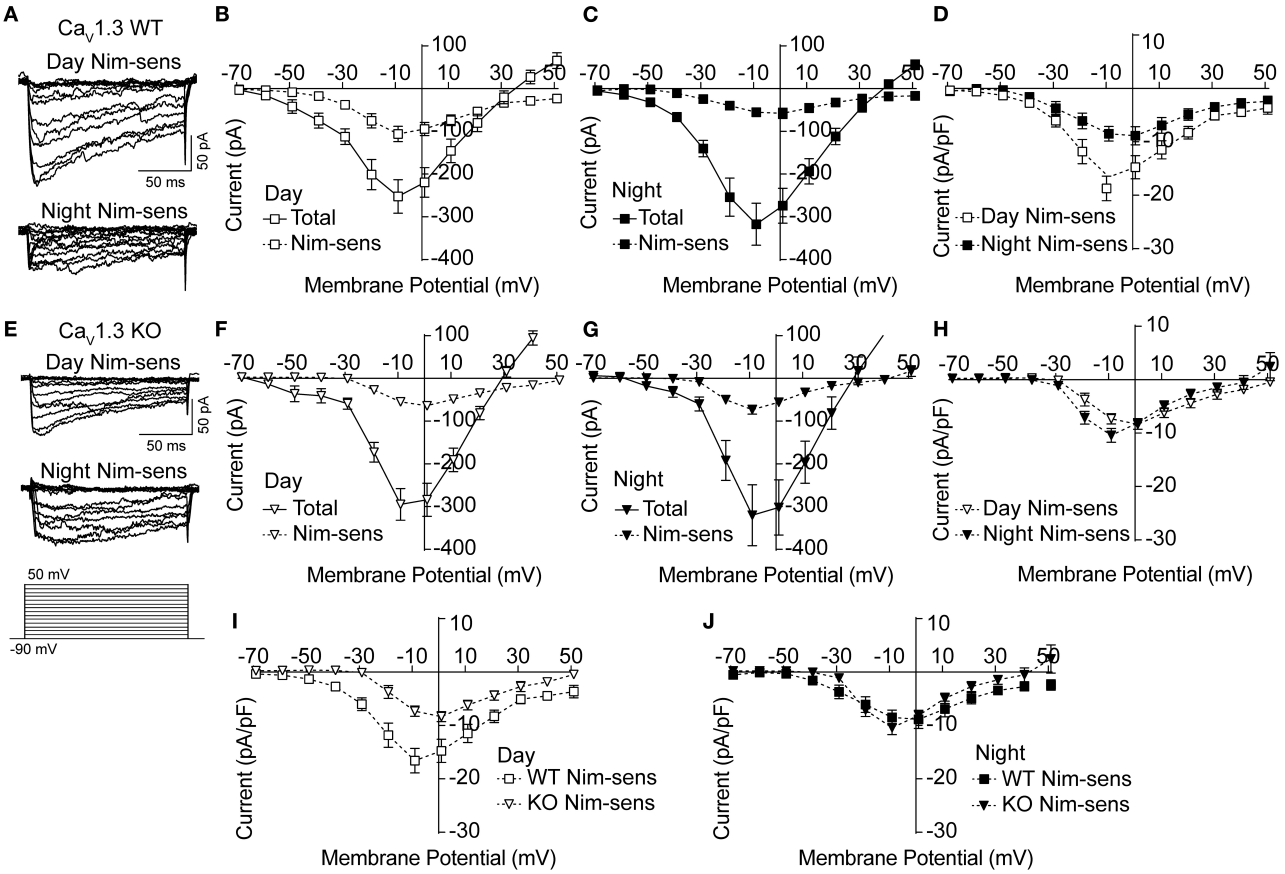

FIGURE 2 | Nimodipine-sensitive Ca^{2+} currents from $Ca_V1.3$ WT and $Ca_V1.3$ KO SCN during the day and night. Macroscopic Ca^{2+} currents were elicited from a holding potential of −90 mV by 150-ms voltage steps from −90 to +50 mV in +10-mV increments. Nimodipine-sensitive LTCC currents were isolated by subtracting currents obtained in 10 μM nimodipine from the total cell current. **(A,E)** Representative nimodipine-sensitive (Nim-sens) current traces from $Ca_V1.3$ WT **(A)** and $Ca_V1.3$ KO **(E)** neurons during the day (top current traces) and night (bottom). **(B,C)** Current-voltage plot for $Ca_V1.3$ WT Ca^{2+} currents before nimodipine (total) and the nimodipine-sensitive (Nim-sens) current from day **(B)** and night **(C)** neurons. **(D)** Comparison of $Ca_V1.3$ WT nimodipine-sensitive normalized current density between day and night. $Ca_V1.3$ WT nimodipine-sensitive currents were larger during the day (at −10 mV) compared to night (at 0 mV) ($P = 0.01$). **(E)** Representative nimodipine-sensitive currents from $Ca_V1.3$ KO neurons during the day (top current traces) and night (bottom). **(F,G)** Current-voltage plot of $Ca_V1.3$ KO total and nimodipine-sensitive currents from day **(F)** and night **(G)** neurons. **(H)** Comparison of $Ca_V1.3$ KO nimodipine-sensitive normalized current density between day and night. $Ca_V1.3$ KO nimodipine-sensitive currents were not different between day (at 0 mV) and night (at −10 mV) ($P = 0.2$). **(I,J)** Comparisons of nimodipine-sensitive current densities from $Ca_V1.3$ WT and $Ca_V1.3$ KO SCN during the day **(I)** and night **(J)**. $Ca_V1.3$ KO nimodipine-sensitive currents were smaller than $Ca_V1.3$ WT currents during the day ($P = 0.009$) but not at night ($P = 0.5$). $P < 0.05$, unpaired Welch's t tests. N's represent individual cells recorded from $Ca_V1.3$ WT (eight neurons, three slices day; 10 neurons, four slices night) and $Ca_V1.3$ KO (12 neurons, two slices day; nine neurons, one slice night). Data are mean ± SEM.

only 23% of the total Ca^{2+} current (**Figures 2E,F**). In addition, the peak of the daytime current-voltage relationship for the nimodipine-sensitive current also shifts from −10 mV ($Ca_V1.3$ WT) to 0 mV ($Ca_V1.3$ KO), consistent with loss of the low voltage activating $Ca_V1.3$ channels (**Figure 2I**). Together these changes in the voltage-dependence and current magnitude identify that $Ca_V1.3$ channels contribute to the daytime LTCC current in SCN neurons.

At night, the contribution of the nimodipine-sensitive current to the total Ca^{2+} current in $Ca_V1.3$ KO neurons was similar to daytime contribution (22%, **Figures 2G,H**). Unlike $Ca_V1.3$ WT, the relative nimodipine-sensitive current to the total current was not smaller at night. Moreover, there was no shift toward depolarizing potentials of the peak voltage of the nimodipine-sensitive current-voltage relationship compared to $Ca_V1.3$ WT (**Figure 2J**). Taking into account the lack of change in both the total and nimodipine-sensitive currents in $Ca_V1.3$ KO neurons, the results raise the possibility that the loss of $Ca_V1.3$ channels might be homeostatically compensated by other LTCCs such as Cav1.2. Thus, the nighttime data are less conclusive and leave open the question of whether $Ca_V1.3$ contributes to the night LTCC Ca^{2+} current in SCN neurons.

$Ca_V1.3$ KO SCNs thus exhibited a different day vs. night profile for LTCC current magnitude. The daytime change in nimodipine-sensitive current magnitude eliminated the diurnal

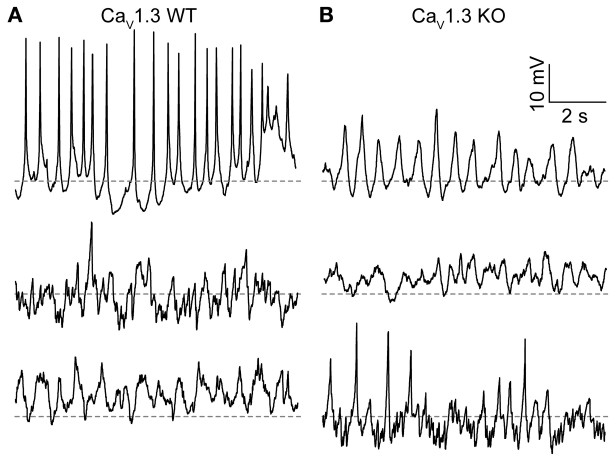

FIGURE 3 | Membrane potential oscillations in $Ca_V1.3$ WT and $Ca_V1.3$ KO SCN neurons. **(A,B)** Representative current-clamp recordings of membrane potential oscillations from $Ca_V1.3$ WT **(A)** and $Ca_V1.3$ KO **(B)** neurons recorded during the day. Recordings were made in 1 μM TTX using the same ACSF and 0.9 mM control EGTA intracellular solution used to record BK currents. Dotted lines indicate −40 mV.

variation in LTCC current in $Ca_V1.3$ KO neurons. Whereas WT neurons had a larger LTCC current during the day (**Figure 2D**), $Ca_V1.3$ KO neurons showed an abrogation of this diurnal difference (**Figure 2H**). This observation primarily resulted from the decreased daytime nimodipine-sensitive current in $Ca_V1.3$ KO compared to WT, which was reduced by about half. The net effect was a loss of the diurnal variation in LTCC current in $Ca_V1.3$ KO SCNs.

Another property of SCN neurons involving LTCCs is the generation of spontaneous subthreshold membrane potential oscillations. During the day, the frequency of these oscillations is higher, and they are eliminated by nimodipine, demonstrating their dependence on LTCC function (Pennartz et al., 2002; Jackson et al., 2004; Huang et al., 2012; McNally et al., 2020). $Ca_V1.3$ channels have been proposed to underlie these oscillations due to their activation at subthreshold membrane potentials. Alteration of Ca^{2+}-dependent inactivation of $Ca_V1.3$ channels is correlated with a decrease in the membrane potential oscillation frequency (Huang et al., 2012). In this study, membrane potential oscillations were revealed in $Ca_V1.3$ WT neurons during the day by application of TTX. SCN neurons exhibit a wide range of oscillatory behavior (**Figure 3**) (Pennartz et al., 2002; Jackson et al., 2004; McNally et al., 2020).

Just over half (56%) of $Ca_V1.3$ WT neurons exhibited membrane potential oscillations. Fifteen neurons had spontaneous oscillations, and three neurons had oscillations manifesting with voltage steps from −60 to 0 mV (out of 32 neurons from three slices; **Figure 3A**). The average resting membrane potential was −44.4 ± 1.8 mV (n = 47). $Ca_V1.3$ KO neurons also exhibited oscillatory membrane behavior (**Figure 3B**). Fifty seven percent of $Ca_V1.3$ KO neurons exhibited oscillations (six neurons with spontaneous and two neurons with oscillations from voltage-steps, out of 14 neurons total from one slice). The average $Ca_V1.3$ KO neuron resting membrane potential was −39.8 ± 2.2 mV (n = 14), which was not different from WT (P = 0.06, unpaired t-test). These results demonstrate that $Ca_V1.3$ is not the sole Ca^{2+} channel required to produce spontaneous membrane potential oscillations or regulate resting Ca^{2+}-dependent K^+ conductances in SCN neurons.

BK Current in $Ca_V1.3$ KO SCN Neurons

Because $Ca_V1.3$ makes a clear contribution to the daytime nimodipine-sensitive current (**Figure 2**), corresponding to the time of day when BK current is more sensitive to nimodipine inhibition (**Figure 1F**), we investigated the role of this LTCC channel subtype in BK current activation (**Figure 4**). BK current levels were first quantified at +90 mV, where the largest diurnal difference is routinely quantified (Montgomery and Meredith, 2012; Whitt et al., 2016). We verified that BK current is larger at night in $Ca_V1.3$ WT SCN neurons on the mixed strain background (**Figure 4A**), consistent with previous studies on inbred mouse backgrounds (Pitts et al., 2006; Montgomery and Meredith, 2012; Montgomery et al., 2013). Most $Ca_V1.3$ WT neurons had a BK current, both during the day and at night (**Figure 4B**). In $Ca_V1.3$ KO neurons, the day vs. night difference in BK current level also persists in the absence of $Ca_V1.3$ channels (**Figure 4A**). Although the proportion of $Ca_V1.3$ KO neurons exhibiting a BK current was lower at 64% during the day, and was similar proportionally to $Ca_V1.3$ WT neurons with nimodipine applied, this difference compared to WT was not significant (**Figure 4B**). At night, all $Ca_V1.3$ KO neurons had a BK current. These data show that $Ca_V1.3$ is not absolutely required to activate BK currents in most neurons, or for the overall circadian difference in BK current magnitude in the SCN.

In whole-cell recordings, multiple Ca^{2+} channel subtypes can contribute to BK current activation. Although $Ca_V1.3$ KO neurons with BK currents do not require $Ca_V1.3$ channels, these channels may still contribute to BK current activation in combination with other LTCC subtypes (such as $Ca_V1.2$). This possibility was addressed by examining the current-voltage relationships for $Ca_V1.3$ WT and KO BK currents. In this comparison for daytime SCN neurons, there was little difference in BK current levels across the voltage range, except at the highest voltage (**Figure 4C**). Although this reduction could suggest contribution from $Ca_V1.3$ channels to BK current activation, the peak Ca^{2+} influx due to $Ca_V1.3$ channels occurs around −20 to −10 mV. For BK channels activated directly by $Ca_V1.3$ Ca^{2+} currents, the largest relative BK current activation is thus observed between −10 and 0 mV (Vivas et al., 2017; Plante et al., 2021). Lack of a difference in BK current observed at these voltages suggests that $Ca_V1.3$ channels may not make a notable contribution to BK current activation in SCN neurons during the day.

When BK channels are coupled to the other neuronal L-type Ca^{2+} channel subtype, $Ca_V1.2$, the BK current activation profile is shifted to higher voltages (+30 mV) (Berkefeld et al.,

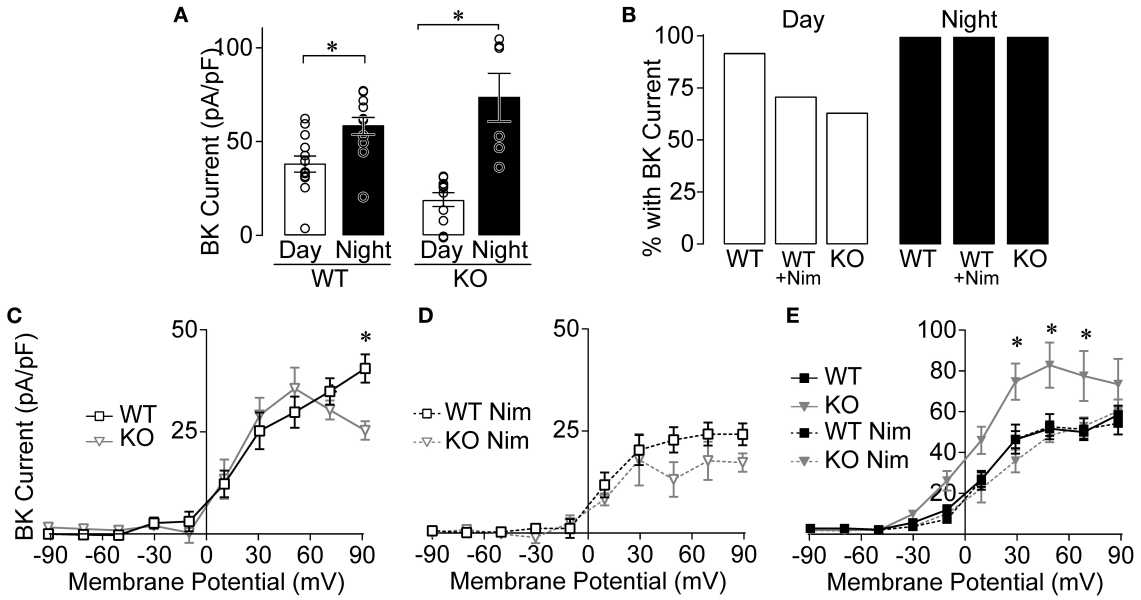

FIGURE 4 | BK currents from $Ca_V1.3$ WT and $Ca_V1.3$ KO SCN during the day and night. Paxilline-sensitive macroscopic BK currents were recorded as in **Figure 1**. **(A)** Summary of BK current densities at +90 mV recorded from $Ca_V1.3$ WT and $Ca_V1.3$ KO neurons during the day and night. BK currents were increased at night for both $Ca_V1.3$ WT ($P = 0.004$) and $Ca_V1.3$ KO ($P = 0.007$) compared to the day. *$P < 0.05$, unpaired Welch's *t* tests. **(B)** Percentage of SCN neurons with BK current in $Ca_V1.3$ WT and $Ca_V1.3$ KO neurons recorded in control conditions or in 10 μM nimodipine (Nim) during the day and night. The number of neurons exhibiting BK currents was not different between $Ca_V1.3$ WT and $Ca_V1.3$ KO ($P = 0.14$, Fischer's exact test). N's: $Ca_V1.3$ WT, 12/13 (cell with BK current/total number recorded); $Ca_V1.3$ WT Nim, 10/14; $Ca_V1.3$ KO, 7/11 and night: $Ca_V1.3$ WT, 12/12; $Ca_V1.3$ WT Nim, 13/13; $Ca_V1.3$ KO, 6/6. **(C,D)** Current-voltage plot for normalized BK current densities from $Ca_V1.3$ WT and $Ca_V1.3$ KO neurons in control conditions **(C)** and after application of Nim **(D)** during the day. $Ca_V1.3$ WT BK currents were increased at +90 mV compared to $Ca_V1.3$ KO in control conditions ($P = 0.03$) but not in Nim ($P = 0.6$). *$P < 0.05$, Repeated measures ANOVA with Bonferroni's *post hoc* test between WT and KO across −10 to +90 mV. **(E)** Current-voltage plot for BK current densities from $Ca_V1.3$ WT and $Ca_V1.3$ KO at night, in control and after application of Nim. $Ca_V1.3$ KO BK currents were increased at +30 ($P = 0.007$), +50 ($P = 0.002$) and +70 mV ($P = 0.01$) compared to $Ca_V1.3$ WT in control conditions at night. N's represent individual cells recorded in (day, night): $Ca_V1.3$ WT (13 neurons, six slices; 12 neurons, three slices), $Ca_V1.3$ WT Nim (14 neurons, four slices; 13 neurons, four slices), and $Ca_V1.3$ KO (11 neurons, four slices; six neurons, two slices), and $Ca_V1.3$ KO Nim (three neurons, one slice; three neurons, two slices). Data are mean ± SEM.

2006; Plante et al., 2021). In the $Ca_V1.3$ KO, the contribution of $Ca_V1.2$ channels to BK current activation can be indirectly inferred by applying nimodipine. Inhibiting the remaining LTCC current in $Ca_V1.3$ KO neurons reduced BK current levels (at +50 mV, $P = 0.006$, two-way repeated measures ANOVA with Bonferroni's *post hoc* test) (**Figure 4C vs. D**), suggesting that it can be attributed to $Ca_V1.2$ channels. However, in the presence of nimodipine, BK current levels were not significantly different between $Ca_V1.3$ WT and KO neurons (**Figure 4D**). The reduction in BK current to similar levels with nimodipine in both genotypes indirectly suggests that there is little compensatory upregulation of $Ca_V1.2$ that affects BK current during the day.

At night, there was a paradoxical increase in BK current in $Ca_V1.3$ KO neurons compared to $Ca_V1.3$ WT (**Figure 4E**). However, in this case, the increase likely comes from compensatory upregulation of $Ca_V1.2$, since nimodipine reduces BK current back to WT levels. This result precludes formulating conclusions about the contribution of $Ca_V1.3$ to BK current activation in nighttime neurons in this study. Yet because nimodipine normally does not significantly decrease the nighttime BK current level in WT neurons in this (**Figure 1F**) and prior studies (Whitt et al., 2018), it is reasonable to conclude that there is no major role for $Ca_V1.3$ channels in BK current activation at night.

DISCUSSION

$Ca_V1.3$ has been proposed to play a role in circadian excitability based on its expression and functional modulation in SCN neurons (Huang et al., 2012; Kim et al., 2015; Cheng et al., 2018). Here we show that $Ca_V1.3$ channels contribute to the daytime LTCC current in SCN neurons. Their contribution may account for up to half of the LTCC current during the day, providing part of the basis for the diurnal difference in Ca^{2+} current levels. In contrast, it is not clear whether $Ca_V1.3$ contributes to daytime membrane potential oscillations or contributes to the nighttime LTCC current. Spontaneous Ca^{2+} oscillations are dependent on the function of LTCCs (Pennartz et al., 2002; Jackson et al., 2004; Huang et al., 2012; McNally et al., 2020). Although $Ca_V1.3$ has been proposed to be important for generating these oscillations

(Huang et al., 2012; Comunanza et al., 2014), the presence of membrane oscillations in $Ca_V1.3$ KO neurons posits that other Ca^{2+} channels are also competent to generate them. Moreover, at night where there is no reduction of the nimodipine-sensitive Ca^{2+} current in $Ca_V1.3$ KO cells and no change in the total Ca^{2+} current, it suggests that $Ca_V1.3$ does not make a significant contribution to the nighttime LTCC current in SCN neurons. However, the data do not completely rule out the possibility of a compensatory upregulation of another LTCC channel subtype, such as $Ca_V1.2$, that is sufficient to maintain the nighttime Ca^{2+} current in $Ca_V1.3$ KO SCNs at a similar level to that observed for $Ca_V1.3$ WT. This compensation is further suggested by the increase in BK current observed in $Ca_V1.3$ KO neurons at night. In other tissues, compensation has been shown to occur *via* upregulation of $Ca_V1.2$ in the absence of $Ca_V1.3$ function (Namkung et al., 2001; Zhang et al., 2005; Marcantoni et al., 2010; Jurkovičová-Tarabová et al., 2012; Poetschke et al., 2015).

BK channels are Ca^{2+}-activated, and LTCCs are a major source of Ca^{2+} required for their activation during the day in SCN neurons (Whitt et al., 2018). Several factors motivated the specific investigation of $Ca_V1.3$ channels in BK current activation. In several types of excitable cells, in heterologous cells, and across diverse animal species, BK and $Ca_V1.3$ channels have been shown to functionally couple (Grunnet and Kaufmann, 2004; Berkefeld et al., 2006; Marcantoni et al., 2010; Bellono et al., 2017; Vivas et al., 2017; Plante et al., 2021). In this study, we found that while the contribution of $Ca_V1.3$ channels to the daytime LTCC current is significant, the effect on BK current is more limited. First, the day vs. night difference in BK current levels that is critical for maintaining proper circadian rhythm in SCN activity is still expressed in the $Ca_V1.3$ KO. This stands in contrast to the LTCC current recorded from $Ca_V1.3$ KO neurons, which does not show a diurnal difference in levels. Second, many SCN neurons still possess detectable BK currents and retain mostly normal BK current activation in the $Ca_V1.3$ KO. Lastly, in prior behavioral studies, daily patterns of locomotor activity were shown to be essentially normal in $Ca_V1.3$ KO mice on a regular light:dark cycle (Busquet et al., 2010). If $Ca_V1.3$ were a fundamental source of Ca^{2+} for BK channel activation, some disruption of locomotor activity might be expected, along the lines of the alterations in behavioral rhythms observed due to loss of BK channel function (Meredith et al., 2006). Taken together, these data suggest that $Ca_V1.3$ channels are not the predominant LTCCs contributing to the net steady-state BK current levels under these recording conditions.

Yet the contribution of $Ca_V1.3$ channels could be more subtle or underestimated. BK–$Ca_V1.3$ channel complexes expressed in heterologous cells were sensitive to low concentrations of BAPTA (0.1 mM) (Vivas et al., 2017), while the SCN BK currents in this study were resistant to higher concentrations of BAPTA (5 mM). This raises the possibility that the predominant BK—LTCC complex in SCN neurons is more tightly coupled than BK–$Ca_V1.3$ channel complexes, making their contribution harder to assess. There were also some minor changes in BK currents from daytime SCN neurons, such as a trend toward fewer neurons with detectable BK currents and a drop-off in the BK current at the peak voltage tested compared to WT. The lack of significance in this data could result from the variability in the Ca^{2+} currents recorded between SCN neurons (Whitt et al., 2018; McNally et al., 2020). Moreover, it is possible that the major contribution for $Ca_V1.3$ channels could be in the smaller population of neurons that had no BK currents in $Ca_V1.3$ KO neurons. Alternatively, the changes in BK current observed at +90 mV in the $Ca_V1.3$ KO could reflect an indirect role for the channel, such as in the gating of RyR-mediated Ca^{2+} release. A small portion of daytime SCN BK current has been previously reported to be sensitive to dantrolene (Whitt et al., 2018), but the pathway mediating the opening of RyRs is not currently understood in SCN neurons (Harvey et al., 2020). Lastly, $Ca_V1.3$ is less sensitive nimodipine than $Ca_V1.2$ (Xu and Lipscombe, 2001), raising the possibility that a small contribution to BK current could be lost within the variability of Ca^{2+} currents from cell to cell.

The data in this study indirectly suggest $Ca_V1.2$ as a more significant LTCC for providing the Ca^{2+} influx that activates BK channels in the daytime SCN. BK current activation is preserved to a large extent when $Ca_V1.3$ channels are absent, and a portion of that current is sensitive to nimodipine. BK channels are also well-described to couple to $Ca_V1.2$ channels in a variety of cell types, including in neurons and heterologous cells (Berkefeld et al., 2006, 2010; Berkefeld and Fakler, 2008; Hou et al., 2016; Plante et al., 2021). Besides $Ca_V1.3$, $Ca_V1.2$ is likely the relevant LTCC subtype to consider in SCN neurons, since $Ca_V1.1$ is restricted to skeletal muscle and $Ca_V1.4$ to retina (Lipscombe et al., 2004; Lee et al., 2015).

A factor complicating some interpretations in this study include the issue of LTCC compensation in the $Ca_V1.3$ KO. Substantia nigra and lateral superior olive neurons, as well as adrenal chromaffin cells, exhibit upregulation of other Ca^{2+} currents in $Ca_V1.3$ KOs (Marcantoni et al., 2010; Jurkovičová-Tarabová et al., 2012; Poetschke et al., 2015). A distinct $Ca_V1.3$ KO mouse line also exhibited compensatory upregulation of $Ca_V1.2$ channels in heart tissues and pancreatic β-cells (Namkung et al., 2001; Zhang et al., 2005), suggesting $Ca_V1.3$ and $Ca_V1.2$ have a connected expression relationship. In this study, the increased BK current at night in $Ca_V1.3$ KO neurons comes from LTCC compensation, since it is nimodipine sensitive. However, the total and nimodipine-sensitive Ca^{2+} currents did not increase compared to WT, as expected if there were compensation. This observation leaves open the question of whether LTCC compensation actually obscured a decrease in the $Ca_V1.3$ KO LTCC current at night. While this issue makes it difficult to assess the role for $Ca_V1.3$ in the nighttime Ca^{2+} current, it is unlikely to affect the conclusion that it does not play a role in BK current activation at night. Interestingly, the increase in BK current at night suggests that when extra LTCC channels are made, they can aberrantly couple to BK channels at the wrong time of day. Since it is not yet known how BK channels change their functional Ca^{2+} channel associations over the circadian cycle in SCN (Whitt et al., 2018; Harvey et al., 2020), the $Ca_V1.3$ KO

model may provide a new context to test mechanisms for BK–Ca_V coupling.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore.

AUTHOR CONTRIBUTIONS

BM performed the experiments. BM, AP, and AM analyzed the data. AM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Calcium-Activated Chloride Channels in Myometrial and Vascular Smooth Muscle

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In smooth muscle tissues, calcium-activated chloride channels (CaCC) provide the major anionic channel. Opening of these channels leads to chloride efflux and depolarization of the myocyte membrane. In this way, activation of the channels by a rise of intracellular [Ca^{2+}], from a variety of sources, produces increased excitability and can initiate action potentials and contraction or increased tone. We now have a good mechanistic understanding of how the channels are activated and regulated, due to identification of TMEM16A (ANO1) as the molecular entity of the channel, but key questions remain. In reviewing these channels and comparing two distinct smooth muscles, myometrial and vascular, we expose the differences that occur in their activation mechanisms, properties, and control. We find that the myometrium only expresses "classical," Ca^{2+}-activated, and voltage sensitive channels, whereas both tonic and phasic blood vessels express classical, and non-classical, cGMP-regulated CaCC, which are voltage insensitive. This translates to more complex activation and regulation in vascular smooth muscles, irrespective of whether they are tonic or phasic. We therefore tentatively conclude that although these channels are expressed and functionally important in all smooth muscles, they are probably not part of the mechanisms governing phasic activity. Recent knockdown studies have produced unexpected functional results, e.g. no effects on labour and delivery, and tone increasing in some but decreasing in other vascular beds, strongly suggesting that there is still much to be explored concerning CaCC in smooth muscle.

Keywords: calcium-activated chloride channels, vascular smooth muscle, myometrial smooth muscle, TMEM16A, Anoctamin 1, excitability, calcium, scramblases

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INTRODUCTION

Chloride (Cl^-), its control, transport and contribution to fluid and volume control and excitability, is of long-standing interest to physiologists. It is abundant extracellularly in all cell types, at ~100–110 mM, but of note, in smooth muscles, its concentration intracellularly is unusually high at 30–50 mM, due to the activities of Cl^-/HCO_3^- exchanger and $Na^+K^+Cl^-$ co-transporters. Consequently, when chloride channels open, chloride effluxes and the myocytes depolarize. In smooth muscles, there are volume-sensitive, bestrophins and CFTR chloride channels, but the most important, and the subject of this review, are Ca^{2+}-activated chloride channels (CaCC). Unlike epithelial cells, in smooth muscle, CaCC are the major anion channel, but surprisingly

much about CaCC remains enigmatic. Molecular and electrophysiological studies report CaCC properties that differ between tissues. By comparing two smooth muscles with different properties and regulatory mechanisms, myometrium and vascular, we will probe consistencies and differences in CaCCs. We will relate these findings to the functional roles and activation mechanisms of CaCCs. We start by summarising the background and current understanding of CaCCs, followed by an overview of the channels in smooth muscle, before moving on to the detailed review of them in myometrium and vascular smooth muscle (VSM).

CaCC AND THE TMEM16 FAMILY

Several reviews of CaCC composition, structure and regulation have been published and can be consulted for further details of the orientating overview we present here (Pedemonte and Galietta, 2014; Falzone et al., 2018; Kalienkova et al., 2021). The molecular identity of CaCC was established in 2008 (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). TMEM16 were an orphan family of membrane proteins with 10 transmembrane domains (Brunner et al., 2014; Dang et al., 2017; Paulino et al., 2017a,b). This protein family was also called anoctamins, as they *were thought* to be anion-selective (An) and have eight (Oct) transmembrane domains. The topology and structure of CaCC are still being researched, but we know now, based on X-ray and cryo-EM data, that there are 10, not eight, transmembrane domains (Brunner et al., 2014; Paulino et al., 2017a). There are 10 members of the TMEM16 family and all are Ca^{2+}-activated. TMEM16A and TMEM16B (ANO1 and 2) are the only pure anion channels, i.e. CaCCs, in the family (Suzuki et al., 2013). They form dimers (Fallah et al., 2011), with each monomer activated by Ca^{2+}, thus forming a double-barrelled channel (Jeng et al., 2016; Lim et al., 2016; Paulino et al., 2017a,b). Most, if not all these other members of the TMEM16 family function predominantly as phospholipid scramblases, which are important for maintaining bilayer symmetry and function. Although still controversial, it appears that the scramblases can also be non-selective ion channels conducting cations, and in some cases anions (Yang et al., 2012; Grubb et al., 2013; Shimizu et al., 2013; Kim et al., 2018; Lin et al., 2018). Interestingly, with regard to this, there has been suggestions about whether CaCCs permit a degree (~15–20%) of cation flux [see discussions in Picollo et al. (2015); Falzone et al. (2018)]. Liposome studies of the pure CaCCs, however, show no dissipation of a KCl gradient supporting anion selectivity, as does the positive shift in reversal potential when SCN^- is substituted for Cl^- in the extracellular medium (Ferrera et al., 2011; Paulino et al., 2017a). It has been shown that single point mutations of the pore will greatly decrease anion selectivity and increase cationic (Yang et al., 2008, 2012; Peters et al., 2018). To explain this, it has been suggested that the CaCC channel has voltage-dependent conformational changes which may allow it to conduct cations (Peters et al., 2018). The details of the pharmacological and biophysical properties of native CaCCs vary depending on the tissues under study, possibly due to splice variations, heterodimers or even association of different subunits. Splicing leads to multiple isoforms and can have significant effects on channel function, such as changing Ca^{2+} sensitivity (Ferrera et al., 2009). Recently allosteric modulation of splice variants of CaCC by phosphatidylinositol 4,5-bisphosphate (PIP_2) and CaMKII has been reported, adding to the complexity of channel regulation (Ko et al., 2020).

CALCIUM-ACTIVATED CHLORIDE CHANNELS ARE PRESENT IN SMOOTH MUSCLES

Before the molecular identification of CaCC (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008), the presence of a Ca^{2+}-activated Cl^- current (I_{ClCa}) had been identified in SMCs, and functional studies conducted. Electrophysiological studies demonstrated slow (relative to L-type Ca^{2+} currents), voltage-dependent (at submaximal [Ca^{2+}]), Ca^{2+}-activated current, with outward rectification and corresponding functional studies showed depolarization and contraction [see (Large and Wang, 1996) for an excellent review of this early literature and (Janssen and Sims, 1992; Akbarali and Giles, 1993; Greenwood et al., 1995)]. A variety of not-so-specific CaCC inhibitors, including niflumic acid (NFA), 9-AC (9-Anthracenecarboxylic acid) and DIDS, (4,4'-Diisothiocyano-2,2′-stilbenedisulfonic acid), blocked the current, produced hyperpolarisation and relaxed smooth muscles (Large and Wang, 1996). L-type Ca^{2+} channels (LTCC) represent the major pathway for the increase in Ca^{2+} needed for contraction in smooth muscles, but CaCCs can provide a positive feedback mechanism in the myocytes, as they are activated by the Ca^{2+} entry, and help to maintain depolarization as Cl^- effluxes through their pore.

The channels and currents have been found in all smooth muscle tissues, indicating an important functional role [Urethra (Drumm et al., 2021); bladder (Kajioka et al., 2004; Bijos et al., 2014); ureter (Iqbal et al., 2012; Hunziker et al., 2020); airway (Kotlikoff and Wang, 1998; Gallos et al., 2013); GI tract (Sanders et al., 2012); and oesophagus (Saha et al., 1992; Akbarali and Giles, 1993; Zhang and Paterson, 2002)]. There are, however, some key areas of uncertainty. With particular relevance to smooth muscles, (1) is the source of activating Ca^{2+} coming from the extracellular entry of Ca^{2+} (L-type and possibly TRP channels) or intracellular stores, i.e. the sarcoplasmic reticulum (SR) or mitochondria?, (2) is channel activation directly by Ca^{2+} or indirectly *via* a Ca^{2+}-activated intermediary, e.g. calmodulin-activated kinases, and (3) are expression and functional effects in some smooth muscle tissues due to interstitial cells of Cajal (ICC), not myocytes,

Abbreviations: AP, action potential; SMC, smooth muscle cell; VSM, vascular smooth muscle; CaCC, Ca^{2+}-activated chloride channel; PIP_2, phosphatidylinositol 4,5-bisphosphate; LTCC, L-type Ca^{2+} channel; MLCK, myosin light chain kinase; PLC, phospholipase C; DAG, diacylglycerol; TMEM16A, ANO, anoctamin; SR, sarcoplasmic reticulum; STIC, spontaneous transient inward current; STOC, spontaneous transient outward current; CaMKII, Ca^{2+}-calmodulin kinase II.

expressing the CaCC and passing the depolarization *via* gap junctions to the myocytes?

By comparing two types of smooth muscle, one spontaneous and phasic, and one tonic, we asked whether the answers to these three questions differed by type, but also if there was support for the suggestion that the contribution of CaCC to excitability also differed.

CALCIUM-ACTIVATED CHLORIDE CHANNELS IN MYOMETRIUM

The myometrium is a spontaneously active (myogenic) smooth muscle. The processes leading to cell activation and contraction are complex and involve the activity of several ion channels to promote a change in membrane potential (V_m). The resting V_m of pregnant myocytes is around −60 mV which shifts to −40 mV towards term in rodents and humans (Parkington et al., 1999) in line with myometrial transition from quiescence during pregnancy to an actively contracting organ in labour. As is the case in other SMCs, membrane depolarization results in activation of L-type, voltage-gated Ca^{2+} channels (LTCCs) to provide calcium entry and action potential (AP) generation. The accompanying rise in $[Ca^{2+}]_i$ gives rise to contraction, and oscillations in V_m give rise to rhythmic contractions (**Figure 1A**).

The identity of the channel/s (and ions) responsible for the initial depolarisation to reach the threshold needed for LTCC activation is unknown. However, CaCCs have been implicated in carrying this current (Wray et al., 2015; Wray and Arrowsmith, 2021).

The presence of Cl^- currents in the myometrium was first described by Parkington and Coleman in single channel recordings in intact tissue strips of guinea-pig myometrium (Coleman and Parkington, 1987). A Cl^- current induced by oxytocin was also observed by Arnaudeau in rat myometrial cells following short-term culture (Arnaudeau et al., 1994). Later, our group, using freshly isolated rat myometrial cells, showed that this channel was activated by calcium entering *via* LTCC (Jones et al., 2004). The current was present in one-third of cells as a slowly deactivating tail current which was observed upon repolarisation following stepwise depolarisation. A tail current is the current remaining after the initial depolarizing stimulus has been removed and is an indicator of the timing of channel closures. That a tail current was observed following repolarisation when the LTCC are closed and L-type Ca^{2+} current would be inactive, suggested other channel species were present, open and carrying this late inward current. The current's reversal potential was found to be close to that of Cl^- and was sensitive to $[Cl^-]$. Moreover, it was sensitive to Ca^{2+} but not Ba^{2+} and was enhanced by the LTCC agonist, BayK8644 (Jones et al., 2004).

In addition to Ca^{2+} entry, other sources of activating Ca^{2+} could include that from the SR, either *via* local Ca^{2+} sparks from the spontaneous opening of Ryanodine receptors (RyR) or from a more global rise in Ca^{2+} *via* agonist-mediated IP_3 release. However, calcium sparks do not occur in myometrium (Burdyga et al., 2007) and the RyRs are non-functional (Dabertrand et al., 2007). IP_3 receptors are present and oxytocin is associated with activation of CaCCs (Arnaudeau et al., 1994). In cultured mouse myometrial cells, an inward current was recorded, which was blocked by CaCC inhibitors (Bernstein et al., 2014). The inhibitors also reduced agonist-mediated increases in Ca^{2+} and the authors suggested that SR release can also assist channel opening in myometrium (Bernstein et al., 2014). But whether SR Ca^{2+} release is a requirement for channel activation was not determined. As Ca^{2+} entry in the absence of an agonist was shown to activate CaCCs, Jones et al. concluded that L-type Ca^{2+} entry was the source of activating Ca^{2+}. A similar logic would also hold for other intracellular organelles, such as mitochondria, but this has not been directly investigated.

Whether functional CaCC or other TMEM16 family members are present intracellularly, e.g. on SR or mitochondria is a controversial area but such expression has been reported in human myometrium (Pedemonte and Galietta, 2014; Danielsson et al., 2018). Whether there is a functionally important role for these channels within the SR or if their presence is simply due to protein processing and trafficking is not known.

Following the identification of TMEM16A and B (ANO1/2) and the development of TMEM16 A and B-selective inhibitors, their function as the putative CaCCs in myometrium has been studied. Both are expressed in human and rodent myometrium (Bernstein et al., 2014; Danielsson et al., 2015). Pharmacological inhibition of TMEM16A and B eliminated inward currents in patched mouse myometrial cells (Bernstein et al., 2014), produced hyperpolarisation in immortalised human myometrial cells [measured indirectly using a fluorescent potentiometric indicator and induced relaxation in human myometrial strips precontracted with oxytocin (Danielsson et al., 2018; Hyuga et al., 2018)]. siRNA knockdown of TMEM16A in primary or human myometrial cells also resulted in an attenuation of the oxytocin-induced increase in filamentous to globular actin ratio which is a marker of actin polymerisation and indicator of cell contraction (Danielsson et al., 2018). The findings from this group, albeit somewhat indirect and not on fresh myocytes, add to the suggestions of TMEM16A contributing to myometrial excitability.

CaCC Function in Myometrium

That CaCCs are both voltage-gated and activated by increases in $[Ca^{2+}]_i$, makes them suitable candidates for participating in excitation-contraction coupling and AP generation in myometrium. The activation of CaCC will depolarize or maintain depolarization of the myometrial membrane, increase excitability and the open probability of LTCC, and AP generation. In this way, CaCCs in the myometrium are likely to contribute to both spontaneous and oxytocin-stimulated contractions with the latter thought to also involve IP_3-mediated release of Ca^{2+} from the SR (**Figure 1A**).

The frequency, amplitude and duration of contractions have long been associated with the frequency and duration of AP firing (Burdyga et al., 2007, 2009). In rat myometrial tissues, CaCCs have also been implicated in stabilising the plateau

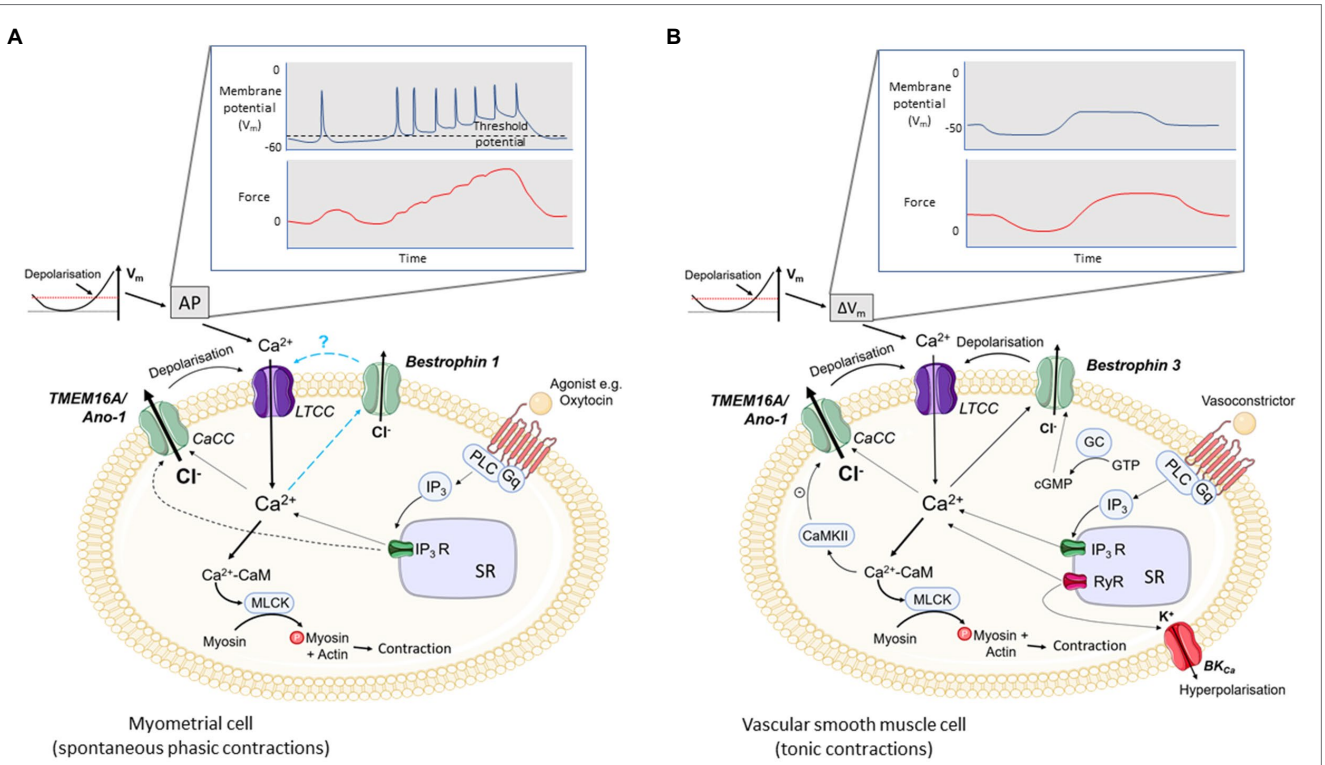

FIGURE 1 | Excitation-contraction coupling in uterine and vascular smooth muscle cells (VSMCs) and the potential roles for CaCCs. **(A)** In spontaneously active smooth muscles, e.g. myometrium, depolarisation of the membrane potential (V_m) to threshold initiates an action potential (AP) and phasic contractions. **(B)** In most vascular smooth muscles which are not spontaneously active, slow, tonic changes in V_m give rise to tonic contractions. In both, depolarisation leads to opening of voltage-gated calcium channels (L-Type, LTCC) and calcium entry. Calcium binds to calmodulin and the Ca^{2+}-CaM complex activates myosin light chain kinase, MLCK. MLCK phosphorylates myosin promoting actin and myosin cross-bridge formation and contraction. Agonists, such as oxytocin in the myometrium **(A)** or vasoconstrictors in VSMCs **(B)**, binding to their receptor activate PLC which in turn hydrolyses PIP_2 to IP_3 and DAG (not shown). IP_3 binds to its receptor, IP_3R, on the SR and facilitates Ca^{2+} efflux into the cytoplasm. The rise in $[Ca^{2+}]_i$ is thought to activate calcium-activated chloride channels (CaCCs; TMEM16A/ANO-1 in myometrium and TMEM16A/ANO-1 and Bestrophin 3 in VSMCs), producing a chloride efflux, depolarisation of the cell membrane and opening of LTCCs. In myometrium, activation of CaCCs may be responsible for the initial depolarisation required to activate LTCCs. Ca^{2+} entry *via* LTCCs may also facilitate the opening of CaCCs which further increases the open probability of LTCC opening. The role of Bestrophins in excitation-contraction coupling in myometrium is not known. Additionally, in VSMCs, spontaneous calcium release through RyRs (Ca^{2+} sparks) on the SR can also contribute to CaCC opening (STICs) and depolarisation. Ca^{2+} sparks also activate BK_{Ca} leading to K^+ efflux, STOCs and hyperpolarisation. Bestrophin channels are positively regulated by cGMP and TMEM16A/ANO1 channels are inhibited by CaMKII. Ca^{2+} entry *via* store – operated Ca^{2+} entry and *via* TRPC6 channels can also stimulate CaCC current and depolarisation (not shown).

phase of the AP and being responsible for prolonging the duration of contraction (Young and Bemis, 2009). A role for CaCCs in establishing or contributing to cell excitability is further supported by the reduced frequency of contraction following the application of several CaCC inhibitors (Yarar et al., 2001; Kaya et al., 2002; Jones et al., 2004; Bernstein et al., 2014; Danielsson et al., 2018; Hyuga et al., 2018). As mentioned previously, some CaCC blockers are non-specific, and off-target actions may synergise with their inhibition of CaCC to produce myometrial relaxation (tocolysis).

Interestingly, I_{ClCa} was only present in around one-third of freshly isolated rat myometrial cells (Jones et al., 2004), and only 5% of cultured murine cells showed auto-rhythmicity (Bernstein et al., 2014). This suggests that those cells expressing CaCC could be the pacemakers or electrogenic cells in the myometrium, equivalent to the ICC cells in gastric smooth muscle. Modelling of excitation-contraction coupling events by integrating transcriptomic data with electrophysiological data also supports a role for CaCCs in generating spontaneous depolarisations (Atia et al., 2016). Tantalising as these data are, direct evidence that CaCC are a major contributor to pacemaking and spontaneous activity in myometrium is lacking. Furthermore, TMEM16A expression was found to be downregulated (15-fold) in late gestation (non-labouring) pregnant human myometrium compared to non-pregnant (Danielsson et al., 2018). This reduced expression may reflect that the uterus is in a state of quiescence required to maintain pregnancy. Alternatively, TMEM16A channels may not be as important as suggested; SMC-specific deletion of TMEM16A in pregnant mice did not alter calcium signalling, uterine contraction or change the length of gestation (Qu et al., 2019), which casts doubt on the importance of the channel in myometrial physiology and its role in pacemaking for spontaneous, or as a depolarising channel for agonist-induced contractions. However, the extent of the reduction in TMEM16A in these conditional knockout mice is not clear.

Other channels known to display Ca^{2+} sensitivity and conduct Cl^- include Bestrophins (BEST 1, 2 and 3). Unlike TMEM16A/ANO1, however, they are not voltage sensitive. In expression systems, BEST-1 has been shown to modulate the LTCC current (Reichhart et al., 2010) by interacting with the channel's β-subunits and regulating the number of pore forming subunits (Milenkovic et al., 2011). Its combined role as a Ca^{2+}-dependent anion channel and regulator of LTCCs is thought to provide a feedback mechanism to control Ca^{2+}-dependent Cl^- transport (Milenkovic et al., 2011). BEST-3 is a CaCC, which displays cGMP dependence [see later, Matchkov et al. (2008)]. BEST-1 is expressed in non-pregnant rat myometrium (Mijuskovic et al., 2015) and inhibition of bestrophins using DIDS has been implicated in mediating the relaxatory effect of hydrogen sulphide in myometrium (Mijuskovic et al., 2015). Their role in excitation-contraction coupling in myometrium, however, has not been studied.

Questions that remain unanswered include whether there is upregulation of TMEM16A or B in labour in humans to facilitate a pro-contractile drive, or similarly, whether expression and/or function of the channel is altered in preterm labour, and whether its inhibitors relax the labouring uterus. This is particularly important if they are to be used as targets for tocolysis – a major goal of the research in this area. Of note, both intra and extracellular protons have been shown to regulate TMEM16A activity (Cruz-Rangel et al., 2017): raising extracellular protons and subsequent channel protonation increase TMEM16A activation without changing their Ca^{2+} sensitivity. During contractions, the myometrium undergoes transient acidification (Larcombe-Mcdouall et al., 1999), which we have shown increases myometrial activity (Pierce et al., 2003; Alotaibi et al., 2015). Could CaCCs therefore, contribute to the stimulation seen under extracellular acidic conditions in labour?

SUMMARY

In myometrium, TMEM16A CaCC are expressed. We conclude that (1) L-type Ca^{2+} entry is the major source of activating calcium, (2) that this activation is direct, and (3) their expression is on myocytes, but only a subset of those present. More direct studies on labouring and non-labouring myometrium are urgently needed.

CALCIUM-ACTIVATED CHLORIDE CURRENTS IN VSM

Most VSM exists in a state of partial contraction (myogenic tone) from which it can dilate or constrict according to physiological requirements. There are some exceptions, such as portal vein, which exhibit spontaneous phasic contractions. Here, we will concentrate primarily on tonically contracting vessels as a comparison to the spontaneously active myometrial SM. However, as important work has been carried out in the phasic portal vein (which is arterial to the liver), and data on myometrium are limited, where relevant we will discuss data on both types of VSM and compare to the myometrium.

Ca^{2+}-activated Cl^- currents (I_{ClCa}) were first reported in VSM cells isolated from guinea-pig pulmonary artery (Byrne and Large, 1987). Since then, I_{ClCa} has been identified in many different tonic vessels (e.g. aorta, coronary, pulmonary, cerebral and mesenteric arteries) from multiple species (Lamb et al., 1994; Large and Wang, 1996; Davis et al., 2010, 2013; Manoury et al., 2010; Thomas-Gatewood et al., 2011; Matchkov et al., 2013; Dam et al., 2014; Cil et al., 2021). The currents have also been shown in spontaneously active vessels, e.g. in rabbit portal vein (Byrne and Large, 1988) and guinea-pig mesenteric vein (Van Helden, 1991).

The tail current amplitude varies between vessels and species: I_{ClCa} is large in conduit vessels (tail current density 40 pA/pF) and very large in the pericytes of the microvasculature (130 pA/pF), but small (5–10 pA/pF) or absent in small arteries of the mouse (Heinze et al., 2014). However, other studies have identified these currents in rat, rabbit and human small arteries [5–10 pA/pF in rabbit pulmonary and coronary arteries (Greenwood et al., 2001) and 20 pA/pF in rat cerebral arteries (Thomas-Gatewood et al., 2011)], although current density was not shown in all (Klockner and Isenberg, 1991; Thomas-Gatewood et al., 2011; Dam et al., 2014). In myometrium, the amplitude of the tail current was 162 ± 48 pA in rat myocytes, equivalent to a current density of approx. 1.5 pA/pF (Jones et al., 2004), which is small compared to the current in the SM of conduit vessels and microvessels. A similarly small peak current density of 5 pA/pF was recorded in murine portal vein (Ohshiro et al., 2014). In spontaneously active venous tissues, spontaneous depolarizations are observed that have been linked to I_{ClCa} (Van Helden, 1991). ICC-like cells have been identified in portal vein (Povstyan et al., 2003; Huang et al., 2010), but it is not clear whether they act as pacemaker cells, as SMC also generate spontaneous depolarizations. Interestingly, it was found that 40% of portal vein myocytes express an I_{ClCa} compared to 90% of myocytes from the tonic pulmonary and coronary arteries (Greenwood et al., 2001), an expression difference perhaps linked to the differential nature of a tonic vessel and spontaneously active vessel with pacemaker activity.

CaCC Identity in VSM

TMEM16A/ANO1 has been identified as the 'classical' CaCC in VSM and TMEM16A mRNA and protein are widely expressed (Davis et al., 2010; Manoury et al., 2010; Thomas-Gatewood et al., 2011). Inhibition of CaCC by traditional non-selective blockers [e.g. NFA (Criddle et al., 1996, 1997; Kamouchi et al., 1997; Sun et al., 2012)] or more recent selective inhibitors [e.g. $T16A_{inh}$-A01 and TM_{inh}-23 (Davis et al., 2010, 2013; Cil et al., 2021)] leads to vasorelaxation of tonic VSM. siRNA knockdown of TMEM16A also reduces arterial constriction (Bulley et al., 2012; Dam et al., 2014; Heinze et al., 2014; Jensen et al., 2018).

Multiple splice variants of TMEM16A have been identified in murine thoracic aorta, carotid artery and portal vein (Davis et al., 2010; Ohshiro et al., 2014). Splice variants can exhibit

differing Ca^{2+} sensitivities and voltage-dependence (Ferrera et al., 2009). In portal vein, Ohshiro and co-workers (Ohshiro et al., 2014) have demonstrated the ability of splice variants to homo and hetero-dimerize. Varied co-expression of splice variants and homo/heterodimerization of channels provides great scope for diversity of physiological functioning of CaCC across different vascular beds.

Many VSMs also co-express a second Ca^{2+}-dependent Cl^- conductance that is distinct from the classical I_{ClCa}, in that it requires cGMP for activation, is not voltage-dependent and is resistant to inhibition by NFA (Matchkov et al., 2005). Both channels co-exist, but relative distribution varies along the vascular tree (Matchkov et al., 2005). This $I_{Cl(cGMP,Ca)}$ current is encoded by bestrophin 3, rather than the TMEM16A/ANO1 gene, since knockdown of the bestrophin gene leads to disappearance of the cGMP-dependent chloride current, but not the classical I_{ClCa} current (Matchkov et al., 2008; Broegger et al., 2011). $I_{Cl(cGMP,Ca)}$ does not mediate agonist-stimulated contraction but instead plays a role in regulation of tissue perfusion by mediating tone oscillations in VSM (Boedtkjer et al., 2008; Broegger et al., 2011). Both cGMP-dependent and classical I_{ClCa} currents are expressed in spontaneously active portal vein (Matchkov et al., 2005). This differs from myometrium, where only classical CaCC are expressed and BEST-3 has not been reported, but BEST-1 has (Mijuskovic et al., 2015). Thus, $I_{ClCaGMP}$ is a feature of VSM – irrespective of it being phasic or tonic.

CaCC Function in VSM

Similar to myometrium and other SM, CaCC play a role in excitation-contraction coupling in VSM cells (Large and Wang, 1996). The Ca^{2+} required to activate CaCC in tonic VSM can come from multiple sources including extracellular and agonist-(e.g. vasoconstrictor) mediated SR release (**Figure 1B**). Compared to myometrium, in VSM, the mechanisms that have been shown to activate CaCC are more varied. Depolarising currents that activate Ca^{2+} entry *via* LTCC trigger I_{ClCa}, either directly [coronary artery (Lamb et al., 1994), renal artery (Gordienko et al., 1994)] or by stimulating Ca^{2+}-induced Ca^{2+} release *via* RyR (Lamb et al., 1994). Agonist-mediated release of Ca^{2+} from the SR [pulmonary artery (Helliwell et al., 1994; Yuan, 1997)] is another important route by which I_{ClCa} is activated. Ca^{2+} entry *via* store-operated Ca^{2+} entry (SOCE) [pulmonary artery (Forrest et al., 2010; Angermann et al., 2012)] and *via* TRPC6 channels [cerebral artery (Wang et al., 2016)] can also stimulate CaCC current and depolarisation.

In VSM, the spontaneous release of Ca^{2+} from the SR *via* RyRs (Ca^{2+} sparks) is associated with stimulation of Ca^{2+}-activated channels on the sarcolemma. When large-conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}) are activated in this way, spontaneous transient outward currents (STOCs) are generated, and hyperpolarisation and vasodilation ensue (Nelson et al., 1995). When CaCC are activated, spontaneous transient inward currents (STICs) occur and cause depolarisation and vasoconstriction, thus playing a role in determining vasomotor tone. The balance between these two pathways varies from vessel to vessel and will determine the overall contractility of the VSM [cerebral artery (Zhao et al., 2017) and renal arterioles (Yip et al., 2018)]. Ca^{2+} sparks and associated CaCC-mediated STICs have been identified in both tonic and phasically contracting VSMs (Wang et al., 1992; Large and Wang, 1996; Yip et al., 2018).

Diverse sources of Ca^{2+} activate I_{ClCa} in the spontaneously active portal vein preparation [L-type Ca^{2+} entry, agonist-induced SR Ca^{2+} release and spontaneous release of Ca^{2+} from the SR (sparks) and reverse mode sodium-calcium exchange (NCX)] (Wang et al., 1992; Leblanc and Leung, 1995; Greenwood and Large, 1996; Burt, 2003; Saleh and Greenwood, 2005). In contrast, few mechanisms of I_{ClCa} activation have been identified in the spontaneously active myometrial smooth muscle (**Figure 1A**). As mentioned above, in the myometrium, Ca^{2+} sparks are not found and RyRs are non-functional. Nor have the role of NCX, SOCE, TPRC6 been determined in relation to CaCC activation.

In addition to mediating the response to contractile agonists, CaCC are involved the vascular myogenic response. CaCC antagonists or TMEM16A knockdown hyperpolarises and dilates vessels (Nelson et al., 1997; Bulley et al., 2012; Yip et al., 2018) and reducing extracellular Cl^- augments myogenic tone (Doughty and Langton, 2001). CaCC are also implicated in the generation of vasomotion (Boedtkjer et al., 2008; Dam et al., 2014) and spontaneous contraction of portal vein (Wang et al., 1992).

Vascular smooth muscle-specific disruption of TMEM16A/ANO1 in mice abolished I_{ClCa} in VSM of the aorta, carotid artery and small arterioles of the brain and retina and resulted in lowering of blood pressure (Heinze et al., 2014). This hypotensive effect is likely mediated *via* small diameter arterioles, where many CaCC are expressed and the vasocontractility they mediate will affect peripheral resistance. In the spontaneously hypertensive rat, TMEM16A/ANO1 is overexpressed in aorta, carotid, mesenteric and hind limb arteries (Wang et al., 2015). siRNA knockdown or pharmacological inhibition of these channels prevented hypertension development in this model (Wang et al., 2015). In addition, TMEM16A expression and activity are significantly upregulated in various pulmonary hypertension models (Forrest et al., 2012; Sun et al., 2012; Papp et al., 2019). However, in an alternative model of hypertension, the 2 kidney 2 clip model, TMEM16A expression and I_{ClCa} are reduced and CaCC activity is negatively correlated with blood pressure and medial cross-sectional area of the basilar artery (Wang et al., 2012), suggesting that downregulation of CaCC is associated with the cerebrovascular remodelling that occurs during hypertension.

In a recent study of heterozygous TMEM16A/ANO1 knockout mice, a 50% decrease in I_{ClCa} reduced aortic contractility as expected, but paradoxical increases in tail/saphenous artery contractility were observed (Matchkov et al., 2020). Clearly the *in vivo* functional role of CaCC is complicated and requires further examination.

Regulation of Classical CaCC in VSM

TMEM16A/ANO1 channels are gated by Ca^{2+}, but sustained Ca^{2+}-activation induces desensitisation. When TMEM16A is expressed in HEK293T cells, PIP_2 is required for channel

activation and guards against Ca^{2+}-induced inactivation (Arreola and Hartzell, 2019; Le et al., 2019). This means that agonist-induced IP_3 production on the one hand activates CaCC, but simultaneously PIP_2 hydrolysis reduces the availability of PIP_2 and reduces channel opening. Similar findings were reported in detrusor SMCs (Yarotskyy et al., 2019); however, the only study to examine the role of PIP_2 in VSM found that PIP_2 tonically inhibits CaCC (Pritchard et al., 2014). Further investigations are required to determine whether PIP_2 is inhibitory in all VSM and why the mechanism differs between cell types.

Phosphorylation by Ca^{2+}-calmodulin-dependent kinase II (CaMKII) attenuates activation of CaCC in a variety of VSMs (Greenwood et al., 2001; Wiwchar et al., 2009). This represents another important negative feedback mechanism whereby vasoconstricting agonists can limit the depolarising influence of CaCC. Calmodulin mediates the Ca^{2+}-dependent inactivation of TMEM16A (Yang et al., 2014; Yang and Colecraft, 2016) but not its activation. Since phosphorylation inactivates CaCC, phosphatases, such as calcineurin, PP1 and PP2, can enhance channel function (Ledoux et al., 2003; Ayon et al., 2009).

Although TMEM16A expression is relatively evenly spread across the VSM sarcolemma (Davis et al., 2010), it is suggested that TMEM16A are to some extent localised to lipid microdomains, as the amplitude and pharmacology of I_{ClCa} are significantly altered by cholesterol depletion with methyl-β-cyclodextrin (Sones et al., 2010), although this has only been studied in mouse portal vein thus far. There is an intriguing overlap in pharmacology between BK_{Ca} channels and CaCC (Greenwood and Leblanc, 2007), which may be partly related to their co-localisation in lipid rafts given a loss of shared pharmacology after cholesterol depletion (Sones et al., 2010), although it is likely more complicated than simple co-localisation.

As mentioned earlier, changes in pH affect channel activity (Cruz-Rangel et al., 2017) and in smooth muscles, this may lead to changes in cell excitability. In pulmonary artery myocytes, chronic hypoxia, which will also change pH, strongly increases TMEM16A expression and CaCC currents, which may be the cause of the enhanced serotonin-mediated vasoreactivity associated with pulmonary hypertension (Sun et al., 2012).

Comparison of Tonic and Phasic Smooth Muscles

We have described CaCC expression, function and regulation in two very different smooth muscles: the myometrium and tonic VSMs, with some comparisons made to spontaneously active VSM. Returning to the questions we posed at this start of this review, in relation to the source of CaCC-activating Ca^{2+}, there appears to be genuine, rather than experimental differences between smooth muscles, e.g. in uterus, L-type Ca^{2+} entry is the activator, whereas in blood vessels, SR Ca^{2+} release through RyRs (and other alternative sources, e.g. SOCE) is also important. In myometrium, any SR Ca^{2+} activating the CaCCs will be from agonist-mediated release through IP_3Rs. Other smooth muscles not discussed in this review also show these differences [e.g. urethra (Drumm et al., 2018)].

Whether channel activation is directly by Ca^{2+} or *via* an intermediary, e.g. CaM kinase II, has been a controversial area. Evidence to support direct binding of Ca^{2+} activating the channel has come from expression of human TMEM16A in liposomes (Terashima et al., 2013), in which all the properties of CaCC, including Ca^{2+} sensitivity, were recapitulated in the absence of calmodulin. The review Yang and Colecraft (2016) concluded that 'there is now overwhelming evidence that Ca^{2+}-dependent activation occurs through CaCC binding directly to the channel, and does not require CaM'. In fact, phosphorylation by CaMKII attenuates activation of CaCC in many VSMs (Greenwood et al., 2001; Wiwchar et al., 2009) and to date, such regulation has not been studied in myometrium.

In phasic smooth muscles, a major question is around the identity of the cells expressing and conducting the CaCC current. In the GI tract, CaCC channel expression and pacemaking activity are exclusively in the ICC cells and in urethra, both myocytes and ICCs express the channel (Sanders, 2019). ICC-like cells have been identified in human uterus (Duquette et al., 2005), but their role in uterine contractile activity is still unclear. A third of rat myometrial SM cells express an I_{ClCa} (Jones et al. Pusch, 2004): but are these then the myometrial pacemaker cells? As mentioned above, ICC-like cells have also been identified in portal vein (Povstyan et al., 2003; Huang et al., 2010), but since both the ICC and SMC generate spontaneous rhythmic inward currents, it is not clear exactly where the pacemaker activity lies. It is interesting however that I_{ClCa} is confined to 40% of portal vein myocytes (Greenwood et al., 2001), a similar number to myometrium, whereas the majority of cells in tonic vessels expresses I_{ClCa}.

Most VSM express both the classical I_{ClCa} (mediated by TMEM16A) and a cGMP-dependent $I_{Cl(cGMP,Ca)}$ (mediated by BEST-3). So far myometrium has also been shown to express the classical I_{ClCa} (TMEM16A) and although BEST-1 is expressed, BEST-3 and $I_{Cl(cGMP,Ca)}$ have not yet been identified.

Although the data are far from exhaustive or always in agreement, we consider that CaCCs cannot explain the differences in electrical activity and hence contraction, between different smooth muscles.

CONCLUSION AND FUTURE DIRECTIONS

Calcium-activated chloride channels are an important aspect of SMC physiology, particularly their contribution to excitation and regulation of contraction. Targeting them pharmacologically to modulate their activity is an attractive goal and may aid treatment of several SMC disorders including preterm labour and hypertension. The identification of TMEM16A as the channel-forming protein has enabled deeper insight into their role and expression in several SMCs and in different species, which has brought us somewhat closer to achieving this aim. However, as we have discussed, differences in their role (e.g. whether CaCCs participate in initial depolarisations and AP generation),

their activation (e.g. from L-type entry as seen in uterus, or SR Ca^{2+} release, or both as seen in VSMs) and their regulation exist between different SMs and between species, so we cannot simply extrapolate or generalise findings. The non-specificity of classical CaCC inhibitors was problematic for many years, but the recent development of more selective inhibitors should aid the ongoing elucidation of the role of CaCC in smooth muscles. The role of TMEM16A splice variants and their effect on function needs further elucidating and appears not to have been examined in the myometrium. That TMEM16A mutations are associated with a range of pathologies is also interesting and may point towards future pharmacogenetic profiling or personalised medicine approaches.

The localisation of CaCCs to intracellular membranes including the SR is interesting given the repertoire of Ca^{2+} release channels present and begs more questions into their role there. Could SR TMEM16A interfere with the function of these Ca^{2+}-release channels? Could they provide a counter current to SR Ca^{2+} release and/or determine the rate of cytosolic Ca^{2+} increase? Further work is also needed to determine their involvement and interaction with other signalling proteins and channels within membrane microdomains, as well as understanding more about how endogenous modulators or changes to the extracellular milieu can regulate their function.

AUTHOR CONTRIBUTIONS

SW, CP, and SA contributed equally to the writing of the manuscript and have approved the submitted version.

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REVIEW
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BK Channel Gating Mechanisms: Progresses Toward a Better Understanding of Variants Linked Neurological Diseases

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The large conductance Ca^{2+}-activated potassium (BK) channel is activated by both membrane potential depolarization and intracellular Ca^{2+} with distinct mechanisms. Neural physiology is sensitive to the function of BK channels, which is shown by the discoveries of neurological disorders that are associated with BK channel mutations. This article reviews the molecular mechanisms of BK channel activation in response to voltage and Ca^{2+} binding, including the recent progress since the publication of the atomistic structure of the whole BK channel protein, and the neurological disorders associated with BK channel mutations. These results demonstrate the unique mechanisms of BK channel activation and that these mechanisms are important factors in linking BK channel mutations to neurological disorders.

Keywords: BK channel, voltage, calcium, activation, neurological disorders, loss of function mutation, gain of function mutation

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INTRODUCTION

BK channels are activated by membrane depolarization and intracellular Ca^{2+} binding. Due to its large single channel conductance of 100–300 pS (Latorre et al., 1989), which gives rise to its name as the big K^+ (BK) channel, the opening of the channel effectively repolarizes the membrane and stops Ca^{2+} from entering the cell due to deactivation of voltage gated Ca^{2+} channels (Lancaster and Nicoll, 1987; Storm, 1987). Therefore, BK channels are important in controlling cellular excitation and Ca^{2+} homeostasis. In 2005, a mutation of the BK channel was found to associate with epilepsy and movement disorder in human patients (Du et al., 2005). The mutation in the *KCNMA1* gene that encodes the Slo1 α-subunit of BK channels causes a missense change, D434G. This mutation alters Ca^{2+} dependent activation of the channel, resulting in an enhanced Ca^{2+} sensitivity (Du et al., 2005; Díez-Sampedro et al., 2006; Lee and Cui, 2009; Yang et al., 2010). With the progress in human genetics, more *KCNMA1* variants that link to neurological disorders have been identified. Some of the mutations in BK channels due to these variants have been functionally characterized, and the results show that these mutations alter voltage and Ca^{2+} dependent activation to different effects (Bailey et al., 2019; Miller et al., 2021). These results demonstrate that the changes in voltage and Ca^{2+} dependent activation of BK channels are important factors linking the *KCNMA1* variants to neurological disorders.

Voltage and Ca^{2+} dependent activation of BK channels has been studied intensively since the discovery of BK channels in early 1980's (Marty, 1981; Pallotta et al., 1981). During the course

of these studies, the DNA sequence of Slo1 was identified (Atkinson et al., 1991; Adelman et al., 1992; Butler et al., 1993) and the atomistic structures of the channel protein were solved. The first atomistic structure data came from MthK, a K^+ channel that lacks the voltage sensor but has a cytosolic structure resembling that of BK channels (Jiang et al., 2002a,b). The cytosolic structure of BK channels was subsequently solved using X-ray crystallography (Wu et al., 2010; Yuan et al., 2010; Yuan et al., 2012), and recently the structure of the whole BK channel with and without the association of the modulatory β4 subunit was solved using cryo-EM (Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019). Each of these structural discoveries has revealed a new dimension in the organization of the molecular components important for voltage and Ca^{2+} dependent activation, allowed the use of additional approaches to explore the mechanisms, and as a result, led to a leap of understanding. In this article I will first review the studies prior to the publication of cryo-EM structures of BK channels, which have defined the important frameworks for understanding BK channel activation. The cryo-EM structures of the whole BK channel have revealed the interactions among structural domains with and without Ca^{2+} binding (Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019) that are important for understanding the couplings of Ca^{2+} and voltage sensors to the opening of the pore. These structures also help reveal novel mechanisms of BK channel activation that differ from canonical mechanisms that were known in other K^+ channels. Finally, the changes in voltage and Ca^{2+} dependent activation with some variants linked to neurological diseases will be described, and the relationship between the change of molecular mechanisms and clinical presentations will be discussed.

Established Frameworks for Understanding BK Channel Activation

Ion channel activation involves three major molecular processes: sensor activation, sensor-pore coupling, and pore opening. Sensors in channel proteins change conformation upon the stimulation of signals, such as changes in the membrane potential and ligand binding. The conformational change of sensors is propagated to the pore *via* interactions between sensors and the pore, known as sensor-pore coupling. Finally, the pore opens to allow ionic flow across the membrane. BK channel activation depends on both membrane potential and intracellular Ca^{2+}. The studies of BK channel activation have revealed the following frameworks for understanding how the two stimuli open the same pore.

First, Ca^{2+} and voltage activate the channel with distinct mechanisms. In early studies the relation between Ca^{2+} and voltage in activating BK channels was an important question. The identification of Slo1 gene *KCNMA1* and the availability of cDNA of Slo1 for functional expression of BK channels in exogenous cells (Atkinson et al., 1991; Adelman et al., 1992; Butler et al., 1993) allowed the studies to distinguish the distinct Ca^{2+} and voltage dependent activation mechanisms (Cui et al., 1997). When the intracellular Ca^{2+} concentration was kept low (≤0.5 nM) BK channels opened in response to membrane depolarization with a rate that exceeded the diffusion limit for Ca^{2+} to bind, suggesting that the channel can open by voltage without Ca^{2+} binding (Cui et al., 1997; **Figures 1A,B**). On the other hand, when the voltage sensor was kept at the resting state by negative membrane potentials (≤−140 mV) (Horrigan et al., 1999; Cui and Aldrich, 2000) the open probability of BK channels increased by 4 orders of magnitude when Ca^{2+} concentration was elevated to 100 μM (Horrigan and Aldrich, 2002; Yang et al., 2010; **Figures 1C,D**), suggesting that the channel can open by Ca^{2+} binding without voltage. Subsequent studies identified the voltage sensor and Ca^{2+} binding sites in different structural domains of the channel. While the residues responsible for voltage sensing are located in the transmembrane segments (Diaz et al., 1998; Cui and Aldrich, 2000; Ma and Horrigan, 2005; Pantazis et al., 2010a; Zhang et al., 2014), the Ca^{2+} binding sites are found to reside in the cytosolic domain (Schreiber and Salkoff, 1997; Shi et al., 2002; Xia et al., 2002; Bao et al., 2004; Yusifov et al., 2008; Wu et al., 2010; Yuan et al., 2010; Zhang et al., 2010; Javaherian et al., 2011; Yuan et al., 2012; Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019). Thus, voltage and Ca^{2+} activate the channel by perturbing different structure domains that harbor the respective sensors and can open the channel independent of each other.

Second, Ca^{2+} and voltage activate the channel with allosteric mechanisms. In the extreme experimental conditions with low Ca^{2+} concentrations (≤0.5 nM) and negative membrane potentials (≤−140 mV) BK channels were observed to open with a small but measurable open probability ($\sim 10^{-7}$) (Horrigan et al., 1999; Cui and Aldrich, 2000; **Figure 1C**). In these experimental conditions the channel opening was not controlled by Ca^{2+} binding or voltage sensor activation, but was an intrinsic spontaneous event. The open probability of BK channels increases with Ca^{2+} binding or voltage sensor activation, since at the open conformation the channel has a higher Ca^{2+} affinity and a facilitated voltage sensor activation (McManus and Magleby, 1991; Cox et al., 1997; Horrigan et al., 1999; Horrigan and Aldrich, 2002). Each BK channel contains eight high-affinity Ca^{2+} binding sites (Schreiber and Salkoff, 1997; Shi et al., 2002; Xia et al., 2002; Bao et al., 2004; Wu et al., 2010; Yuan et al., 2010, 2012; Zhang et al., 2010; Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019) and four voltage sensors (Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019; **Figure 2**), and the open probability of the closed-open transitions increases with each Ca^{2+} binding and voltage sensor activation. This mechanism of Ca^{2+} and voltage dependent activation can be quantitatively described by a model that accounts for BK channel activation with the changes of Ca^{2+} concentration and voltage at a broad range that includes and extends beyond physiological conditions (Horrigan and Aldrich, 2002; **Figure 2A**). This mechanism is in contrast to Shaker K^+ channels, for which the opening was tightly controlled by voltage sensor activation even when the open probability was at 10^{-7} (Islas and Sigworth, 1999). Thus, the mechanism of coupling between sensor activation and pore opening in Shaker K^+ channels is thought to be obligatory, while in BK channels is allosteric. The allosteric mechanism makes it possible for BK channels to sense both Ca^{2+} binding and voltage sensor activation for opening. In a channel with the pore tightly

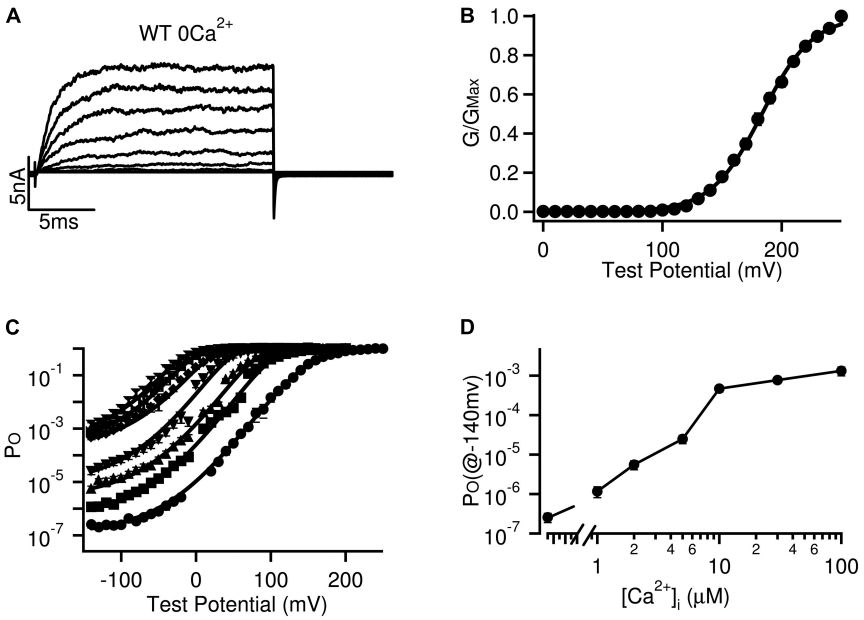

FIGURE 1 | Independent voltage and Ca^{2+} activation of BK channels. **(A)** Current traces activated by voltage pulses in low Ca^{2+} concentration ($\leq$0.5 nM) without binding to Ca^{2+}. **(B)** Steady state conductance of the channels (measured at the end of the current traces in panel **(A)**) at different voltages. The GV relation reflects voltage dependence of open probability of the channels. **(C)** Open probability of BK channels at different voltage and Ca^{2+} concentrations. At negative voltages ($V < -100$ mV) channels open in response to Ca^{2+} concentration changes without dependence on voltage sensor activation. **(D)** Dependence of open probability at -140 mV on Ca^{2+} concentration. The same results have been published previously (Yang et al., 2010).

controlled by the voltage sensor, such as Shaker, Ca^{2+} binding would not have been able to open the channel without voltage sensor activation.

Third, interactions among structural domains are important for Ca^{2+} and voltage dependent activation of BK channels (**Figure 2B**). The primary sequence of Slo1 channels, in comparison with voltage gated K^+ (Kv) channels, indicated that the channel included three structural domains, the transmembrane pore-gate domain (PGD), the transmembrane voltage sensor domain (VSD), and the cytosolic domain (CTD) (Cui, 2010; Lee and Cui, 2010). The structure of MthK (Jiang et al., 2002a,b) and subsequently the structures of the BK CTD (Wu et al., 2010; Yuan et al., 2010; Yuan et al., 2012) showed that the CTD contains two RCK (Regulator of K^+ Conductance) domains in each Slo1 subunit, and the eight RCK domains of the four subunits form a ring-like structure called the gating ring. Based on the sequence homology with Kv channels, the structures of the VSD, which contains the transmembrane segments S1–S4, and PGD, which contains S5–S6, were modeled after the structure of Kv1.2 (Long et al., 2005a; Lee and Cui, 2009). Two Ca^{2+} binding sites, the Ca^{2+} bowl that is primarily formed by the RCK2 residues (Schreiber and Salkoff, 1997; Bao et al., 2004; Yusifov et al., 2008; Wu et al., 2010; Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019) and the site that is located in RCK1 (Shi et al., 2002; Xia et al., 2002; Wu et al., 2010; Zhang et al., 2010; Yuan et al., 2012; Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019), were found in the CTD of each Slo1. Residues in the VSD that are important for voltage sensing were identified (Ma and Horrigan, 2005; Zhang et al., 2014). Thus, the VSD and CTD harbor the voltage and Ca^{2+} sensors and propagate the stimuli to open the pore in PGD *via* interactions among these structure domains.

Domain-Domain Interactions for Sensor-Pore Couplings in BK Channel Activation

Prior to the publication of the cryo-EM structures of the whole BK channel (Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019; **Figures 2B,C**) the coupling of Ca^{2+} binding in the CTD to the opening of the PGD had been proposed to involve two types of interactions among the structure domains. A study Niu et al. (2004) showed that voltage and Ca^{2+} dependent activation of BK channels was changed by altering the covalent link between the PGD and the CTD *via* a peptide of 15 residues, known as the C-linker. The changes in activation depended on the changes in C-linker length by addition or deletion of amino acids in a relationship as if the interaction between the PGD and CTD were like pulling a spring for channel activation. Thus, it was proposed that Ca^{2+} may activate the channel by causing a conformational change in the CTD that directly tugs the pore to open *via* the C-linker. On the other hand, the CTD was proposed to also interact with the VSD *via* non-covalent interactions, and such interactions may indirectly open the pore *via* VSD-PGD interactions (Lee and Cui, 2009). This proposal derived from the finding that the residues from both the VSD and CTD form a Mg^{2+} binding site that modulates voltage dependent activation

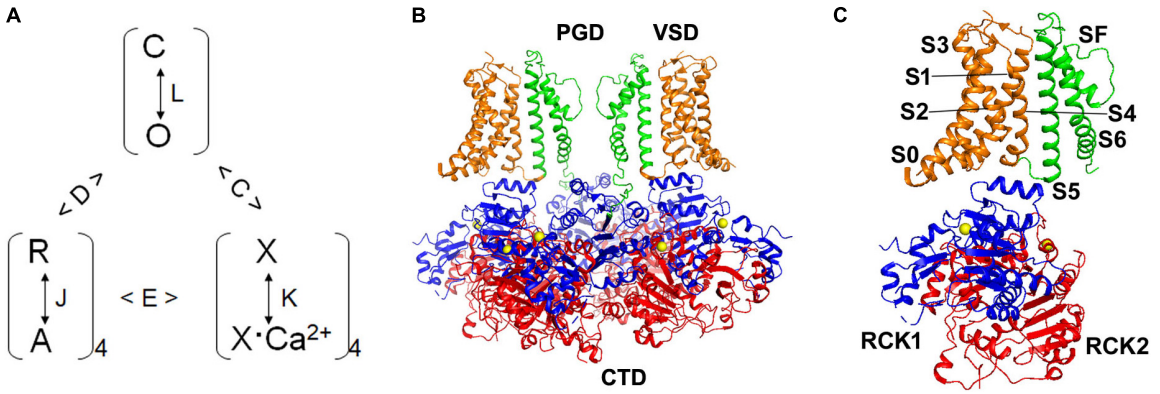

FIGURE 2 | Allosteric activation mechanism and structure of BK channels. **(A)** The allosteric model of BK channel activation. The channel undergoes an intrinsic closed-open transition (C and O), which is regulated by voltage sensor activation (R to A) and Ca^{2+} binding (X to X:Ca^{2+}) with allosteric mechanisms. i.e., with each voltage sensor activation the channel open state is favored by an allosteric factor D; and with each Ca^{2+} binding to the channel the channel open state is favored by an allosteric factor C. L, J, and K are equilibrium constants for the respective transitions. Although voltage and Ca^{2+} can activate the channel independently, voltage sensor activation and Ca^{2+} binding affect each other with an allosteric factor E. In this model only 4 Ca^{2+} binding sites are assumed. Similar models containing 8 Ca^{2+} binding sites can also fit the electrophysiology data (Savalli et al., 2012). **(B)** BK channel structure with four Slo1 subunits (PDB entry: 6V38). Only two subunits are shown for the membrane spanning part of the channel. VSD: voltage sensing domain, PGD: pore-gate domain, CTD: cytosolic domain. **(C)** A single Slo1 subunit (PDB entry: 6V38). S0–S6: transmembrane segments, SF: selectivity filter, the two Ca^{2+} bound to the channel are shown in yellow.

(Shi and Cui, 2001; Hu et al., 2003; Zeng et al., 2005; Yang et al., 2007; Yang et al., 2008), and thus VSD and CTD are located closely. This mechanism was consistent with an earlier finding that the part of the CTD that is located close to the VSD is important in determining the different Ca^{2+} sensitivities between different BK channel homologs (Krishnamoorthy et al., 2005), and subsequent studies showed that alterations of the VSD-CTD interactions indeed affected Ca^{2+} dependent activation (Yang et al., 2013; Geng et al., 2020).

For voltage dependent activation, it was found that a negatively charged residue E219 in the VSD, which contributed to voltage sensing, interacted with charged residues E321 and E324 in the PGD to mediate the coupling of voltage sensor activation to channel opening (Zhang et al., 2014). The interaction, however, was also found to be involved in the coupling between Ca^{2+} bindings and pore opening. Similar to Ca^{2+} dependent activation that involves interactions among all CTD, VSD, and PGD, voltage dependent activation also involves all three structure domains. A comparison between the voltage dependent activation of the wild type BK channel with that of a truncated BK channel with CTD deletion (Budelli et al., 2013) showed that the CTD was important in the coupling between VSD activation and pore opening (Zhang et al., 2017). The CTD undergoes conformational changes during voltage dependent activation as detected by fluorescence signals (Miranda et al., 2018). Ca^{2+} binding to the CTD also alters voltage dependence of channel activation (Horrigan and Aldrich, 2002; Sweet and Cox, 2008; Savalli et al., 2012; Lorenzo-Ceballos et al., 2019). These results suggest that the interactions among all three structure domains are involved in voltage and Ca^{2+} dependent activation.

The CTD-VSD non-covalent interactions were more clearly shown by the cryo-EM structures of the whole BK channels (Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019; **Figures 2B,C**). The CTD gating ring is located closely to the membrane spanning domains of the channel, with an extensive interface between the N-terminus of the CTD with the cytosolic part of the VSD (585 $Å^2$ per subunit in the Ca^{2+} structure) (Hite et al., 2017). Comparing the structure with Ca^{2+}/Mg^{2+} bound to the metal-free structure the N-lobes of the RCK1 domain, which face the VSD, tilted in a rigid body fashion away from the pore axis. In the Ca^{2+}/Mg^{2+} bound structure the cytosolic part of the VSD also showed a corresponding outward displacement, and the movements of both the RCK1 and VSD alter the interface between these domains. This Ca^{2+} dependent change in the interactions between CTD and VSD suggests that these interactions are part of the coupling mechanism for Ca^{2+} binding to open the channel, and is consistent with the studies to show that mutations in the VSD-CTD interface alter Ca^{2+} sensitivity (Yang et al., 2013; Geng et al., 2020). The C-linker in the cryo-EM structures is partly α-helical and partly extended and nearly identical in both the structures with and without Ca^{2+}/Mg^{2+} bound, but undergoes a large positional displacement laterally as a rigid unit. Recent studies showed that the C-linker can interact with the membrane directly to alter BK channel activation (Tian et al., 2019; Yazdani et al., 2020b). These results suggest that the C-linker may not affect channel activation simply as a passive link between the CTD and PGD, but directly interact with the membrane and other parts of the channel protein such as the gating ring to mediate Ca^{2+} and voltage dependent activation.

In the tetrameric structure of the Kv1.2 channel the VSD of each subunit is adjacent to the PGD of its neighboring subunit, showing a domain-swapped configuration (Long et al., 2005b). The BK channel, however, does not show such a domain-swapping. Instead, the VSD and PGD of the Slo1 subunit interact within each subunit (Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019; **Figure 2C**). This difference as revealed by the cryo-EM structure of BK channels suggests that the coupling between VSD activation to pore opening differs from

the canonical electromechanical coupling mechanism for the domain-swapped Kv channels. In domain swapped Kv channels the peptide linking the VSD and the PGD of a subunit, the S4–S5 linker, interacts with the cytosolic part of the transmembrane helix S6 (Long et al., 2005b). S6 lines the inner pore, and the helices from four subunits cross at the cytosolic side of the membrane to form the activation gate that controls ionic flow during the opening and closing of these Kv channels (Liu et al., 1997; Long et al., 2005a,b). The interactions between the S4–S5 linker and S6 has been shown to mediate electromechanical coupling, which open the activation gate in response to VSD movements (Lu et al., 2001; Long et al., 2005b; Hou et al., 2020; Cowgill and Chanda, 2021). However, in BK channels, the S4–S5 linker of each subunit is short and not located close to S6, but interacts with the N-terminus of RCK1 (Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019; **Figure 2C**). The VSD seems to be in close contact with the PGD within the same Slo1 subunit only with the interface between S4 and S5 helices (**Figures 2B,C**). The contact between S4 and S5 helices is extensive, which suggests that the interactions between the two helices are important for mediating the coupling between VSD activation and pore opening.

The extensive interactions between the S4 and S5 would also restrict the motion of S4, which may be responsible for the unique VSD movements during BK channel activation. In domain swapped Kv channels the charged residues that sense voltage for channel activation, known as gating charges, are primarily found in the S4 transmembrane helix (Aggarwal and MacKinnon, 1996; Mannuzzu et al., 1996; Seoh et al., 1996). S4 moves across the membrane with a large distance upon membrane potential depolarization, resulting in a transfer of total 12–16 equivalent gating charges across the electric filed in the membrane (Zagotta et al., 1994a,b; Aggarwal and MacKinnon, 1996; Mannuzzu et al., 1996; Seoh et al., 1996; Bezanilla, 2000; Gandhi and Isacoff, 2002). However, in BK channels gating charges are found in S2 (D153 and R167), S3 (D186), and S4 (R213, E219) segments (Ma and Horrigan, 2005; Pantazis et al., 2010a; Zhang et al., 2014). During voltage dependent activation of BK channels a total 2.32 equivalent gating charges are moved across the electric filed in the membrane (Horrigan and Aldrich, 1999; Ma and Horrigan, 2005), much smaller as compared to those in domain swapped Kv channels. These results suggest that the VSD may undergo small movements that involve many transmembrane segments during voltage dependent activation (Ma and Horrigan, 2005; Pantazis et al., 2010a,b; Pantazis and Olcese, 2012; Pantazis et al., 2018). Furthermore, the pore opening in BK channels also retrospectively control VSD activation, such that VSD activation increases with multiple time courses, and some of which are in parallel with channel opening (Horrigan and Aldrich, 1999; Savalli et al., 2006), consistent with the idea that the S4–S5 interactions may restrict VSD movements during BK channel activation.

Hydrophobic Gate in BK Channels

Cryo-EM structures of BK channels reveal that the S6 helices from the four Slo1 subunits do not cross at the cytosolic side, either with or without Ca^{2+}/Mg^{2+} bound (**Figure 2B**). If the metal-free structure represents the closed state of the channel, while the Ca^{2+}/Mg^{2+} bound structure represents an open state (Hite et al., 2017), the cytosolic side of the pore is thus wide open at both the open and closed states of the channel. This result is consistent with the suggestion from the studies of BK channel block by quaternary ammonium (QA) molecules (Li and Aldrich, 2004; Wilkens and Aldrich, 2006; Tang et al., 2009) and other smaller molecules (Zhou et al., 2011; Zhou et al., 2015) that the channels do not have an intracellular gate so that the blockers can enter the pore even at the closed state. The study of a peptide blocker suggests that the cytosolic part of the pore may undergo a conformational change to reduce the pore size during BK channel closing (Li and Aldrich, 2006), but the change is not sufficient to close the pore to restrict the entrance of K^+ ions or QA blockers. These results indicate that the activation gate of BK channels may be located above the cytosolic side, possibly at the selectivity filter, which is the narrowest part of the pore (**Figures 2B,C**) and with a conserved function among K^+ channels to select K^+ ion over other ions for permeation (Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019).

Recently, an alternative mechanism for the activation gate was proposed. The structures of the metal free and Ca^{2+}/Mg^{2+} bound BK channels suggested that the inner pore of the channel underwent a conformational change in the absence of metal binding, which mainly involved an amphipathic segment of S6, $V_{319}PEIIE_{324}$, to expose hydrophobic residues V319 and I323 to the pore inner surface (Hite et al., 2017; Tao et al., 2017; Tao and MacKinnon, 2019). As a result of these changes the inner pore beneath the selectivity filter becomes narrower, more elongated and hydrophobic. A molecular dynamics simulation study found that these changes promote dewetting transitions that completely deplete the inner pore of liquid water, giving rise to a vapor barrier to block the ion flow (Jia et al., 2018). Importantly, the dry pore remains physically open with an average diameter of ∼6 Å, allowing QA blockers to access the deep-pore region to block the channel even in the closed state. Such a hydrophobic gate depends on the hydrophobicity of the surface residues and the geometry of the inner pore (Aryal et al., 2015; Yazdani et al., 2020a). A functional study showed that the mutations in the BK channel inner pore A316D and A316V made the channel constitutively open and harder to open, respectively (Chen et al., 2014). Consistent with these results molecular dynamics simulations found that A316D reduced hydrophobicity of the pore and prevented dewetting transitions, while A316V enhanced hydrophobicity of the pore and made dewetting transitions faster with less water molecules remaining in the pore (Jia et al., 2018).

At present the hydrophobic gating mechanism is primarily based on molecular dynamic simulations, which can explain the experimental results of QA blockers on BK channel and some mutations. There has not been a direct experimental validation of this mechanism, partly due to the dilemma that once the vapor barrier forms the channel is closed and devoid of any functional detection. Comparing to the hydrophobic gate, the alternative mechanism, the selectivity filter acting as the activation gate, is not clearly defined with computational or experimental evidence. Where is the activation gate of BK channels and how it operates

are not only part of the fundamental mechanism of voltage and Ca^{2+} dependent activation but also important for understanding BK channel modulation by compounds that act in the pore of BK channels, such as the QA blockers, paxilline (Zhou et al., 2020), and NS11021 (Rockman et al., 2020; Schewe et al., 2019). Some of these modulators have been excellent tools for the studies of the roles of BK channels in cellular and tissue function (Kaczorowski and Garcia, 2016; Cui, 2020), and may serve as the lead for therapies for BK variants linked neurological diseases as described in the following sections.

Slo1 Mutation D434G Enhances Ca^{2+} Sensitivity and Is Associated With Epilepsy and Paroxysmal Non-kinesigenic Dyskinesia

In a study of a family with inherited generalized epilepsy and coexistent paroxysmal dyskinesia (GEPD), a heterozygous A-G transition in exon 10 of *KCNMA1* was identified, which results in the D434G mutation in the BK channel subunit Slo1 (Du et al., 2005; **Figure 3A**). The inheritance followed an autosomal dominant pattern, and sixteen members of the family were affected. Among these individuals nine had early childhood onset of absence epilepsy, showing the characteristic episodes of loss of awareness, with vacant staring and unresponsiveness, and electroencephalography (EEG) showing synchronous spike-and-wave discharges (SWDs). Three of these individuals also developed generalized tonic-clonic seizures. The seizures in some of these individuals were responsive to anti-epilepsy medicine valproate, lamotrigine, or clonazepam. Among the sixteen affected individuals twelve had paroxysmal dyskinesia including five with both seizures and paroxysmal dyskinesia. These individuals were described to have involuntary dystonic or choreiform movements of the mouth, tongue, and extremities. The episodes were not induced by sudden movements, but induced by alcohol, fatigue, and stress, so that the paroxysmal dyskinesia was classified as paroxysmal non-kinesigenic dyskinesia (PNKD). It was noted that the episodes of PNKD developed around the same age as the onset of the seizures, but during the episodes the individual had preserved consciousness.

Comparison of the currents of the D434G BK channels with the wild type (WT) BK currents showed that the D434G currents were increased with a faster activation time course at the same voltage and intracellular Ca^{2+} concentrations $\geq$1 μM (Du et al., 2005; Díez-Sampedro et al., 2006; Lee and Cui, 2009; Wang et al., 2009; Yang et al., 2010; Moldenhauer et al., 2020), which is the cytosolic Ca^{2+} concentration at neuronal excitations (**Figures 3B,C**). These results suggest that more currents flow through the D434G BK channels during an action potential. At these Ca^{2+} concentrations, the voltage dependence of channel conductance (GV relation) of D434G BK channels was shifted to more negative voltages (**Figure 3C**), indicating that channel activation was increased. The increased channel activation was actually primarily because of an increased Ca^{2+} sensitivity without changes in voltage dependent activation, because the mutation increased the apparent affinity for Ca^{2+} in channel activation (Díez-Sampedro et al., 2006; Wang et al., 2009; Yang et al., 2010), while at low Ca^{2+} concentrations (∼0.5 nM) with few Ca^{2+} binding to the channel the D434G mutation did not alter GV relation (Díez-Sampedro et al., 2006; Yang et al., 2010). The residue D434 is located close to the Ca^{2+} binding site in RCK1 (**Figure 3A**). The mutation D434G neutralizes a negative charge close to the Ca^{2+} binding site and makes the protein structure more flexible around the site, which may be responsible for the mutation to specifically enhance the affinity of Ca^{2+} for the RCK1 site and strengthen the coupling between Ca^{2+} binding to the site with pore opening (Yang et al., 2010).

Recently a knock-in mouse model carrying BK D434G mutation was characterized (Dong et al., 2021). The BK D434G mutation mice manifested symptoms resembling the human patients affected by the BK D434G mutation. Simultaneous video-EEG recordings showed that the BK-D434G mice had frequent episodes of spontaneous, generalized SWDs, and during which behavioral arrests, characteristic of absence seizures. These mice were also more susceptible to the induction of generalized seizures by injection of low dose of pentylenetetrazole (PTZ), a convulsant, than the WT mice, consistent with that some BK-D434G patients developed generalized tonic-clonic seizures (Du et al., 2005). Consistent with the human patients, the mutant animals exhibited severe locomotive defects in a battery of motor tests when there was no absence seizure. However, due to the limitations of mouse models, it is difficult to clearly tell if the animals also had PNKD. Both the heterozygous BK-D434G mutation ($BK^{DG/WT}$) and the homozygous mutation ($BK^{DG/DG}$) mice exhibited these symptoms, but the incidents were more frequent or severe in $BK^{DG/DG}$ mice. All these symptoms could be suppressed by the treatment with a BK channel inhibitor, paxilline (PAX). These results support the idea that the D434G mutation in BK channels causes a gain of function of the channel that results in epilepsy and movement disorders.

Acute brain slice recordings from the BK D434G mutation mice further showed that the mutation caused hyperexcitability in both the cortical pyramidal neurons and the cerebellar Purkinje cells (Dong et al., 2021), which play essential roles in the pathogenesis of absence seizures (Crunelli et al., 2020) and contribute to the motor defects (Sausbier et al., 2004), respectively. These neurons from the $BK^{DG/WT}$ mice exhibited a significantly increased action potential frequency compared with the $BK^{WT/WT}$ mice. The action potentials in $BK^{DG/WT}$ neurons showed a much faster repolarization with a shortened duration and augmented after-hyperpolarization amplitude (AHP). These results are consistent with that the BK-D434G channels have a higher Ca^{2+} sensitivity (Díez-Sampedro et al., 2006; Wang et al., 2009; Yang et al., 2010), allowing the channels to activate more during an action potential following membrane depolarization and the opening of voltage gated Ca^{2+} channels. The enhanced BK currents more efficiently hyperpolarize the membrane, enabling a faster recovery of the voltage-gated sodium channels from inactivation and potentially facilitating the activation of the hyperpolarization-activated cation (HCN) channels (Lancaster and Nicoll, 1987; Storm, 1987; Shah, 2014), which allow the neurons fire at a higher frequency. The application of BK channel

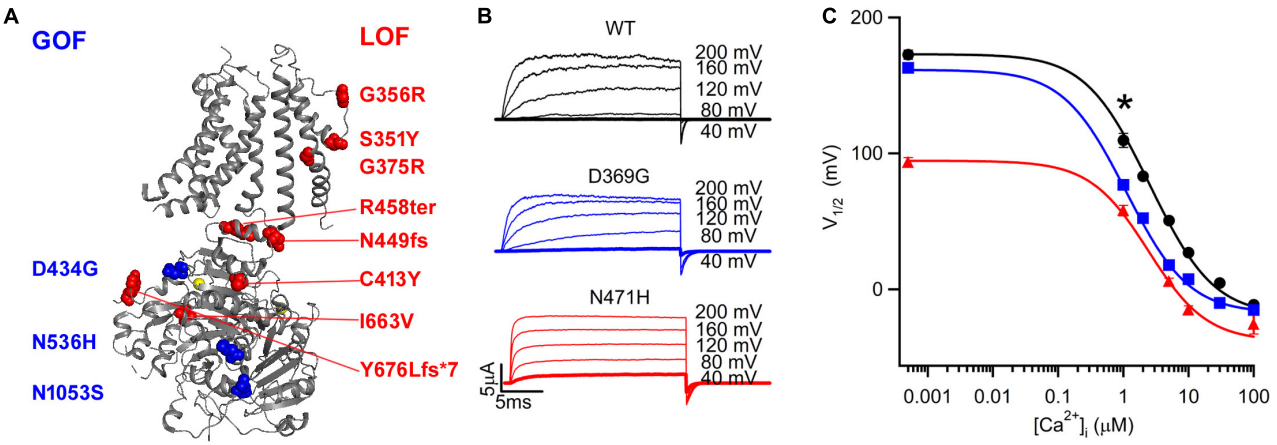

FIGURE 3 | BK mutations alter activation and link to neurological diseases. **(A)** The GOF (blue) and LOF (red) mutations mapped onto Slo1 structure. Yellow circles: Ca^{2+} ions bound to the channel. **(B)** The GOF mutation D434G and N536H in human Slo1 corresponds to D369G and N471H in mouse Slo1, respectively. Both mutations enhance currents at the same voltage and Ca^{2+} concentration. The current trace at +40 mV, which is most relevant physiological membrane potential, is highlighted in a thicker line. **(C)** Ca^{2+} dependence of the $V_{1/2}$ of GV relations (the voltage where GV relation is half maximum, which is used to measure the voltage range in which the channel activates). The result shows that with the D369G and N471H mutations GV shifts to more negative voltages at all Ca^{2+} concentrations. The * shows that 1 μM Ca^{2+} concentration is most physiologically relevant. The same results have been published previously (Yang et al., 2010; Zhang et al., 2020).

inhibitor PAX widened the action potential, reduced AHP and decreased firing frequency.

The seizures in patients affected by BK D434G were responsive to general anti-epilepsy medicine (Du et al., 2005), and so were in the BK D434G mutation mice (Dong et al., 2021). Both ethosuximide and valproate suppressed generalized SWDs (Dong et al., 2021). It was shown that the anti-epilepsy medicine acetazolamide had no direct effect on BK channels (Moldenhauer et al., 2020), suggesting that BK channels were not the target of these general anti-epilepsy medicines. It is reported that 30% of absence epilepsy patients are pharmaco-resistant with the first-line anti-absence medicines (Crunelli et al., 2020). The results of BK channel inhibitor PAX (Dong et al., 2021) suggest that BK channels as a new target for anti-absence therapy may be developed to treat those pharmaco-resistant patients. However, the effects of PAX in suppressing generalized SWDs in BK D434G mutation mice only lasted for ∼30 min, which indicates that novel BK channel inhibitors with appropriate efficacy, specificity and pharmacokinetics need to be developed. The development of such novel drugs will benefit from the understanding of molecular mechanisms of BK channel gating.

Neurological Diseases Associated With Other BK Channel Gain of Function Mutations

The second disease associated *KCNMA1* variant was identified in 2015, which resulted in the N1053S mutation in Slo1 (Zhang et al., 2015; **Figure 3A**). Subsequently more patients with the same Slo1 mutation were identified [also called N995S (Li et al., 2018), or N999S (Heim et al., 2020)]. These mutations were all *de novo* in unrelated patients. Some of these patients suffered movement disorders, diagnosed with PNKD (Zhang et al., 2015) or cataplexy (Heim et al., 2020), while some only suffered absence epilepsy or mixed with myoclonic seizures (Li et al., 2018). Only one patient suffered both symptoms (Heim et al., 2020). These patients also showed various degrees of developmental delay or intellectual disability. Whether symptoms respond to anti-epileptic medicines also varied among these patients.

As the symptoms of BK-N1053S patients showed some resemblance to those of BK-D434G patients, N1053S mutation is also a gain of function (GOF) mutation. Similar to D434G, the N1053S mutation increased BK currents and the activation kinetics at physiological intracellular Ca^{2+} concentrations with all depolarizing voltages (Li et al., 2018; Moldenhauer et al., 2020), suggesting that the mutation in neurons would shorten action potentials, increase after hyperpolarization and enhance spike firing frequencies (Li et al., 2018). However, the molecular mechanism of N1053S in causing functional changes differs from that of D434G. While G434G primarily enhances Ca^{2+} sensitivity, N1053S does not seem to alter Ca^{2+} dependent activation. The G-V relation of the N1053S BK channels shifts to negative voltages with a similar amount at different Ca^{2+} concentrations, and the deletion of Ca^{2+} binding sites does not prevent the G-V shift of the N1053S BK channels (Li et al., 2018).

Recently another *de novo* GOF *KCNMA1* variant that resulted in a Slo1 N536H mutation (**Figure 3A**) was identified, with which the patient suffered frequent dystonic/atonic spells (Zhang et al., 2020). She also had disorders of autism spectrum, attention deficit hyperactivity, and intellectual disability. No obvious seizure was observed in the patients. The symptoms were not responsive to antiepileptic medicines, but dextroamphetamine, a central nervous system stimulant, completely controlled her dystonic episodes from >100 per day to none. N536H is also a GOF mutation that increases BK currents at 1 μM Ca^{2+} concentration by shifting G-V relation to negative voltages (**Figures 3B,C**). Similar to N1053S, but unlike G434G, N536H does not alter Ca^{2+} dependent activation of the BK channel.

The findings so far suggest that the BK channel GOF mutations are associated with absence epilepsy with possible development of myoclonic seizures, movement disorders, or both symptoms. However, it needs to be emphasized that these symptoms vary from patient to patient. The patients may also have cerebellar atrophy, development delays, autism spectrum and intellectual disability to various degrees. These different symptoms derive from increased BK currents either due to increased Ca^{2+} sensitivity or shift of G-V relation with an intact Ca^{2+} dependent activation. The larger BK currents may result in hyperactivity in different neuron types to cause various symptoms. Future studies are needed to dissect how the GOF BK channel mutations with increased Ca^{2+} sensitivity or shift of G-V relation with an intact Ca^{2+} dependent activation lead to different neurological symptoms.

Neurological Diseases Associated With BK Channel Loss of Function Mutations

A report in Tabarki et al. (2016) described two sisters harboring a homozygous *KCNMA1* variant that resulted in the frameshift mutation T676Lfs*7, which changed T676 to L and caused a frame shift after 7 residues downstream (**Figure 3A**). The patients suffered development delays, severe cerebellar atrophy and seizures of myoclonic type, which progressed to tonic seizures with one of the siblings. The homozygous frameshift mutation presumably abolished the BK channel function although no functional studies were conducted. Several other loss of function (LOF) *de novo* BK channel mutations that either completely destroyed BK channel function or reduce BK channel currents were subsequently identified in unrelated patients. The mutations that destroyed BK channel function included G375R, S351Y, G356R, N449fs, I663V (Liang et al., 2019) and the truncation mutation R458ter (Yesil et al., 2018). Other mutations, including C413Y and P805L, decrease BK currents either by reduced protein expression or a shift of the G-V relation to the positive voltages (Liang et al., 2019; **Figure 3A**). These patients suffered neurological diseases that vary in symptoms and severity, most had development delay, intellectual disability, movements disorders (such as ataxia and axial hypotonia), and cerebellar atrophy. Some of the patients also had seizures.

These results reveal that the normal neurological functions are sensitive to BK channel function. Either a GOF or LOF BK channel mutation could tip the balance of neural function and lead to neurological diseases. While the symptoms of both GOF and LOF BK channel mutations show some overlap, such as development delay, intellectual disability, movement disorder, and epilepsy, the LOF BK channel mutations result in more severe cerebellar atrophy. The patients with BK G375R mutation even had visceral and cardiac malformations, connective tissue symptoms, and dysmorphic features, suggesting that BK channel mutations can impact organs beyond the nervous system (Liang et al., 2019). In a recent study a BK LOF mutation, G354S (**Figure 3A**), was identified in association with cerebellar degeneration, ataxia, developmental delay and intellectual disability in a young girl (Du et al., 2020). The mutation caused a shift of G-V relation of BK channel activation to more negative voltages. However, due to its location in the selectivity filter of the channel the G354S mutation reduced single channel conductance and increased Na^{+} permeability, thereby decreasing the macroscopic currents and making the currents less effective in repolarizing the membrane potential. Viral transfection of the G354S BK in mouse brain induced ataxia in the animals. Transfection of the G354S BK into dividing PC12 cells, the mutant channel suppressed outgrowth of neurites by reducing the neurite length. The mutant BK channel was also toxic to mitochondria, with the cells expressing the G354S BK showing reduced mitochondria content, disrupted mitochondria superstructure, altered mitochondria dynamics to increase fragmented fission forms, and decreased mitochondria membrane potential. NS1619, a BK channel activator, protected the cells expressing G354S BK from the reduced neurite outgrowth, cell death, and changes in mitochondria, suggesting that the recovery of BK channel function can be a therapy for the neurological diseases associated with BK channel LOF mutations.

CONCLUDING REMARKS

In ion channel activation sensors change conformation upon physical or chemical stimulation, which induces pore opening *via* interactions between sensors and the pore known as sensor-pore coupling. BK channel activation has shown unique characteristics in all these molecular steps as compared to canonical activation mechanisms in voltage gated K^{+} (Kv) channels. First, BK channels sense both membrane voltage and intracellular Ca^{2+} with distinct voltage sensor and Ca^{2+} binding sites. For these two stimuli to control the opening of the same pore independently the sensors are coupled with the pore with allosteric mechanisms. Second, the VSD and PGD domains in BK channels are not domain swapped among subunits, which differs from most Kv channels, and thus the VSD-pore coupling mechanism in BK channels is also unique. Third, the activation gate in BK channels is not a physical barrier at the intracellular side of the pore, but may be a vaper barrier as in a hydrophobic gating mechanism.

Mutations of BK channels that either increase BK channel currents (GOF) or decrease currents (LOF) at physiological voltages and Ca^{2+} concentrations are associated with neurological disorders. The changes in BK channel currents alter excitability of various neurons that may induce the symptoms. The LOF BK mutations may also directly disrupt mitochondria functions and cause cell death in brain and other organs. In the future, it is critical to translate the comprehensive understanding of BK channel structure and function to uncover the neurological mechanisms of BK channelopathy and design precision therapies to treat these patients. A key question is how the mutations of BK channels that either increase BK channel currents (GOF) or decrease currents (LOF) at physiological voltages and Ca^{2+} concentrations are associated with such a wide spectrum of neurological disorders. Animal models carrying different BK channel mutations and induced pluripotent stem cells (iPSCs) directly derived from patients are invaluable tools to address this question.

On the other hand, the change of voltage and Ca^{2+} dependent activation by the disease-associating mutations also provide

unique insights for further understanding of BK molecular mechanisms. For instance, while we can reason that G375R (Liang et al., 2019; **Figure 3A**) may disrupt the opening of the activation gate since this is part of the diglycine hinge for the BK channel activation gate (Magidovich and Yifrach, 2004) and G356R may destroy the selectivity filter (Tao and MacKinnon, 2019; **Figure 3A**), we have no clue how the mutations in the cytosolic domain, such as N1053S (Zhang et al., 2015), N536H (Zhang et al., 2020), C413Y, or I663V (Liang et al., 2019; **Figure 3A**) may affect voltage dependent activation or the intrinsic gate opening in BK channels. Further studies of these mutations may provide insights to how the CTD interacts with VSD and PGD to control BK channel activation.

The studies of human genetics on BK channelopathy, biophysical characterizations, and the animal models carrying either GOF or LOF BK channel mutations indicate that BK channels can be a promising drug target for treating associated neurological diseases (Du et al., 2020; Dong et al., 2021). The studies of molecular mechanisms of BK channel activation and neurological diseases associated with aberrant BK channel function are valuable in directing the diagnoses of such diseases and the development of BK channel specific modulators for the therapy.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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The Large-Conductance, Calcium-Activated Potassium Channel: A Big Key Regulator of Cell Physiology

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Large-conductance Ca^{2+}-activated K^+ channels facilitate the efflux of K^+ ions from a variety of cells and tissues following channel activation. It is now recognized that BK channels undergo a wide range of pre- and post-translational modifications that can dramatically alter their properties and function. This has downstream consequences in affecting cell and tissue excitability, and therefore, function. While finding the "silver bullet" in terms of clinical therapy has remained elusive, ongoing research is providing an impressive range of viable candidate proteins and mechanisms that associate with and modulate BK channel activity, respectively. Here, we provide the hallmarks of BK channel structure and function generally, and discuss important milestones in the efforts to further elucidate the diverse properties of BK channels in its many forms.

Keywords: BK channels, smooth muscle, nervous system, membrane potential, intracellular Ca^{2+}

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INTRODUCTION

Large-conductance Ca^{2+}-activated K^+ or BK [Big Potassium (K^+)] channels, also known as Maxi-K, Slo1 or $K_{Ca}1.1$ channels, are ubiquitously expressed in a broad array of excitable and non-excitable cells including neurons/glial cells (Trimmer, 2015; Hayashi et al., 2016; Latorre et al., 2017), a variety of vascular or nonvascular smooth muscle (Nelson et al., 1995; Nelson and Quayle, 1995; Brenner et al., 2005; Herrera et al., 2005; Hu and Zhang, 2012; Kyle et al., 2013; Krishnamoorthy-Natarajan and Koide, 2016; Dopico et al., 2018), skeletal muscle (Pallotta et al., 1981), neuroendocrine cells (Solaro et al., 1995) and, epithelial cells (Manzanares et al., 2011; Yang et al., 2017). These channels are characterized by exhibiting a high K^+ selectivity, a large single channel conductance of 200–300 pS (~10–20-fold greater that other K^+ channels), and an exquisite ability to be dually activated by two distinct physiological stimuli: membrane depolarization and local increases in intracellular Ca^{2+} (Marty, 1981; Pallotta et al., 1981; Barrett et al., 1982; Latorre et al., 1982, 1989; Marty, 1989). Given their unusual and very impressive large unitary conductance, the stimulation of BK channels leads to a rapid efflux of K^+, which results in membrane hyperpolarization. This capability therefore confers an important physiological mechanism to modulate membrane excitability and intracellular Ca^{2+} homeostasis. Thus, BK channels are key players in a plethora of physiological processes such as smooth muscle contraction (Brayden and Nelson, 1992; Nelson et al., 1995; Pérez et al., 1999; Wellman and Nelson, 2003), neuronal signaling (Robitaille and Charlton, 1992; Hu et al., 2001; Raffaeli et al., 2004; Wang, 2008), hormone secretion (Braun et al., 2008) and audition (Miranda-Rottmann et al., 2010). In the brain,

astrocyte endfeet express functional BK channels with the ability to sense astrocytic Ca^{2+}. This in turn signals to neighboring arteriolar smooth muscle cells by the focal release of K^+ into the perivascular space, thus playing an essential role in neurovascular coupling and the regulation of brain blood flow (Filosa et al., 2006).

In harmony with these vital physiological functions, both malfunctioning and abnormal expression (loss or gain of function) of BK channels can have detrimental consequences on the excitability of neuronal or vascular networks (N'Gouemo, 2014; Contet et al., 2016). Thus, BK channels are now recognized to play a role in numerous pathophysiological conditions including seizures and epilepsy (Du et al., 2005; N'Gouemo, 2011), movement disorders (Du et al., 2005; Imlach et al., 2008), autism and mental retardation (Laumonnier et al., 2006), cerebral ischemia and hypoxia (Gribkoff et al., 2001; Kumar, 2007; Liao et al., 2010; Tao et al., 2015), hypertension (Brenner et al., 2000; Pabbidi and Roman, 2017), obesity (Jiao et al., 2011) and diabetes mellitus (Gutterman and Durand, 2014). In this review, we provide an overview of the basic biophysical features, including structural, functional and pharmacological properties of mammalian BK channels, with a particular focus on their pathological implication as well as their potential as molecular targets for the development of innovative and promising therapeutic strategies in the nervous and cardiovascular systems.

BIOPHYSICAL FEATURES OF LARGE-CONDUCTANCE Ca^{2+}-ACTIVATED K^+ CHANNELS

The Structure of BK Channels

As members of the TM6 voltage-gated ion channel superfamily, BK channels share partial topology with voltage-gated K^+ (K_v) channels and, constitute tetramers of the pore-forming α-subunits or Slo1 proteins, encoded by a single gene, termed *Slo1* or *KCNMA1* in mammals. The *Slo1* gene undergoes extensive alternative splicing (Tseng-Crank et al., 1994; Navaratnam et al., 1997), giving rise to a high degree of functional diversity in BK channels. It was firstly identified in the *Slowpoke* mutant of *Drosophila melanogaster*. This mutant exhibited abnormal locomotor patterns and obvious impaired flight ability due to a deficiency in a Ca^{2+}-activated conductance (Elkins et al., 1986; Atkinson et al., 1991). Each α-subunit, containing about 1,200 amino acids, is comprised of 7 membrane-spanning domains (i.e., S0-S6; ~330 amino acids) (Wallner et al., 1996) with S4 considered as a conserved positively charged domain that acts as a well-defined voltage sensor as seen in K_v channels (Liman et al., 1991; Lopez et al., 1991; Logothetis et al., 1993; Seoh et al., 1996). The pore-gated domain is constituted by S5 and S6 transmembrane segments; it forms the center of the BK channel and acts as a K^+ selective filter. Additionally, the α-subunit contains an extensive (~840 amino acids) C-terminal cytosolic region with four additional hydrophobic segments (S7–S10) containing two non-identical domains (RCK1 and RCK2), serving as regulators for K^+ conductance. Each of the RCK domains contain a high-affinity binding Ca^{2+} site and multiple regulatory domains for a variety of ligands (Jiang et al., 2002; Xia et al., 2002; Sweet and Cox, 2009) or divalent cations including Mg^{2+} (Shi et al., 2002; Xia et al., 2002; Yang et al., 2008; **Figure 1**). Interestingly, the "gating ring" of the tetramer is constituted by these four RCK1-RCK2 arrangements (Tao et al., 2017). While Ca^{2+} ions are powerful promoters of BK channel open probability, other Ca^{2+}-activated K^+ channel species, activated by lower intracellular Ca^{2+} concentrations [i.e., small (SK) or intermediate (IK) K^+ channels], display a completely dissimilar channel gating mechanism. This process requires calmodulin (CaM), a small but highly conserved Ca^{2+}-modulating protein, to bind with Ca^{2+} ions (Fanger et al., 1999; Adelman, 2016). Specifically, four CaM molecules attach to the channel tetramer causing a conformational change of the S4-S5 linker that promotes the opening of the channel pore in a cooperative manner with high Ca^{2+} sensitivity (Xia et al., 1998).

The structural features of the α-subunit confer unique biophysical properties to the channel including ion permeation,

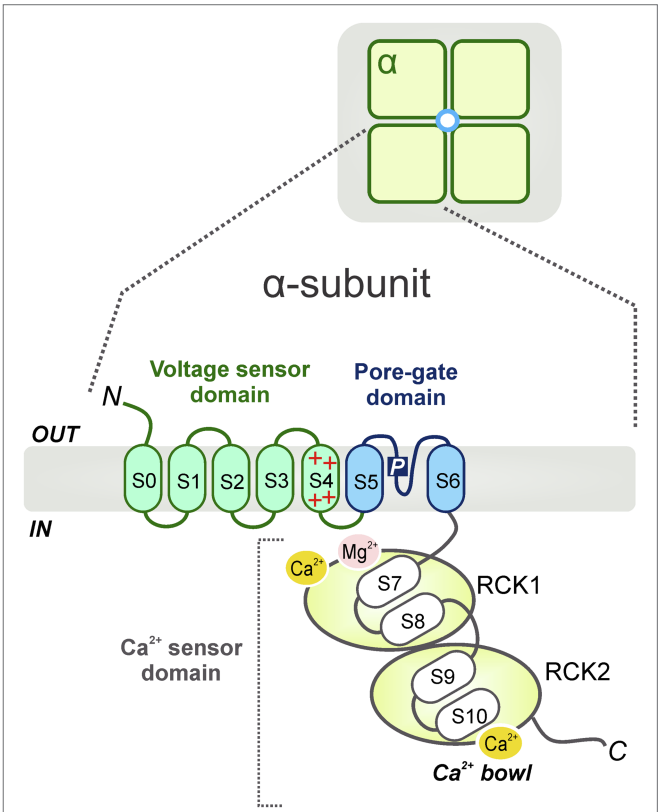

FIGURE 1 | Schematic diagram of the general BK channel structure. BK channels constitute tetramers of the channel pore-forming α-subunits (*top*). Each α (*Slo1*) subunit contains 3 main domains: a voltage sensor domain (VSD, S0-S4), a pore-gate domain (S5-S6) and a C-terminal cytosolic region, which functions as a Ca^{2+} sensor domain (S7-S10) (*bottom*). The Ca^{2+} sensor domain is constituted by two non-identical domains (i.e., RCK1 and RCK2) which contain high-affinity binding Ca^{2+} sites (Ca^{2+} *bowl*) and several modulatory domains for multiple ligands or cations including Mg^{2+}.

gating and regulation by diverse ligands and intracellular molecules and ions. Notably, the pharmacology and functional features of BK channels, such as their sensitivity to voltage and intracellular Ca^{2+}, are prominently impacted by their association with auxiliary and non-pore-forming modulatory β (β_{1-4}) (Brenner et al., 2000; Yan and Aldrich, 2010), γ (γ_{1-4}) (Yan and Aldrich, 2010, 2012), and LINGO1 subunits (Dudem et al., 2020). Most importantly, the co-assembling with diverse auxiliary subunits gives rise to the existence of distinct BK channel phenotypes with varied functionality, thus increasing channel heterogeneity. Specifically, the identity of the regulatory subunits that associate with BK channels dictates important electrophysiological and kinetic features of the channel including the voltage range of activation, inactivation or deactivation, single-channel current rectification characteristics, as well as pharmacological sensitivity to different channel blockers (Gonzalez-Perez and Lingle, 2019). In this aspect, recent literature has centered on BK channel gating modulation by these auxiliary subunits (Contreras et al., 2012; Torres et al., 2014; Zhang and Yan, 2014), with a particular emphasis on the unique role of the β-1 subunit in BK channels in smooth muscle and kidney (Krishnamoorthy-Natarajan and Koide, 2016; Latorre et al., 2017).

The complexity of BK channel function reflects the intricacy of its protein structure. In the last decade, several studies employing electron cryomicroscopy (cryo-EM) and X-ray crystallographic analysis have begun to provide key structural and biophysical insights into the BK channel gating (Wang and Sigworth, 2009; Lee and Cui, 2010; Yuan et al., 2010; Hite et al., 2017). However, a complete knowledge of BK channel structure is needed to provide not only a more informed understanding of its biological role(s), but also refined targeting in the practical search for novel drugs and compounds to treat diverse BK-associated pathologies while mitigating potential side effects. Importantly, more extensive crystallographic analyses of the diverse auxiliary subunits may add new dimensions to BK channel modulation and add potential targeting options to the channel in a more tissue- or cell-specific manner. This section will review the organization and structural basis for gating the BK channel as they are currently understood.

The Voltage Sensor and Activation of BK Channels by Membrane Voltage

One of the most defining hallmarks of BK channels is their mechanism of activation by membrane depolarization and changes in cytosolic Ca^{2+} levels in a synergetic fashion. This idea is supported by various allosteric models revealing that BK channels can open the pore gates and allow K^+ efflux in the absence of voltage sensor activation and Ca^{2+} binding with an intrinsic open probability (P_o) of ~10^{-7}. (Horrigan et al., 1999). These models further demonstrated that in the practical absence of intracellular Ca^{2+} [(Ca^{2+}) < 1 nM], membrane depolarization is sufficient to reduce the free energy necessary to maximally stimulate voltage-dependent macroscopic ionic currents through BK channels (Cui et al., 1997; Horrigan et al., 1999; Horrigan and Aldrich, 1999). In this sense, a depolarization greater than +200 mV was necessary to promote channel opening at ~0.5 nM intracellular Ca^{2+}. This Ca^{2+}-independent activation of the BK channel is reinforced by the fact that the time constant of current stimulation was three orders of magnitude faster than the averaged diffusion-limited time it would take Ca^{2+} to bind the channel during depolarization (Cui et al., 1997). These latter observations combined with their structural similarity to K_v channels, suggest that BK channels possess a virtuously voltage-dependent mechanism of gating conferred by the existence of an intrinsic voltage sensor domain (VSD, S0-S4).

Structurally, the VSD in BK channels resembles the voltage-sensing apparatus of K_v channels but with an additional N-terminal transmembrane segment (S0), which exhibits similar voltage dependence to the positively charged S4 domain [i.e., contains three Arginine [Arg] residues] and is important for β-subunit modulation (Meera et al., 1997). Diverse electrophysiological assays have demonstrated that the S0 helix is remarkably close to the extracellular domains of S3 and S4 (Liu et al., 2008) and functionally modulates the transition between the resting and the fully active state of the VSD upon membrane depolarization (Koval et al., 2007).

In terms of functionality, the voltage sensor of BK channels differs from that of K_v channels in two main aspects. First, the number of voltage-sensing charges (gating charge) of BK channels – measured as the depolarization-induced movement of the VSD – are smaller compared to Kv channels (0.6*e* vs. ~12–13*e* effective gating charges in BK vs. K_v channels), suggesting the requirement of more membrane depolarization to move the VSD of BK channels into the fully activated state. This relatively weak voltage dependence allows BK channels to function throughout a wide range of membrane potentials. Second, while in K_v channels each of the first four most extracellular charges (Arg residues) of S4 is voltage sensing and contributes to the gating currents (Bezanilla, 2008), only one Arginine residue influences the actual BK channel gating. This observation suggests the existence of additional charged residues outside of S4 with essential contributions to the total amount of gating charges (Ma et al., 2006). Specifically, two supplementary voltage-sensing charged residues (accounting for at least 50% of gating currents) have been described in the BK S2 segment, and the S3 domain carries an additional residue, also involved in charge-related movements of the gating current. Given the various remarkable dissimilarities, it is possible that the BK channels perform a similar but unique mode of structural rearrangement of the VSD during channel gating (Ma et al., 2006). Further studies using the voltage-clamp fluorometry technique have tracked the relative motions of the BK channel VSD domain upon membrane depolarization, providing a fundamental structural basis to gain a better understanding of the voltage-sensing operation of BK channels (Pantazis et al., 2010; Pantazis and Olcese, 2012).

The Calcium Sensor and Calcium Sensitivity of BK Channels

Ca^{2+} binding promotes BK channel opening independently of the voltage-sensing apparatus of the channel. This evidence

was elegantly demonstrated by classic electrophysiology studies in which the steady-state open probability of BK channels increased as the intracellular Ca^{2+} concentration was elevated (from <10 nM to 1,000 μM) at a fixed transmembrane voltage (Markwardt and Isenberg, 1992) or at very negative voltages (less than −80 mV) where voltage sensors are largely in resting states (Horrigan and Aldrich, 2002).

Physiologically, BK channel activity can be enhanced by elevating cytosolic Ca^{2+} concentrations, since Ca^{2+} binds with high affinity to the cytoplasmic domain(s). Several divalent cations (including Ca^{2+}) are sensed by the so-called "gating ring," a large tetrameric arrangement made up of two different regulators of conductance of potassium (i.e., RCK1 and RCK2) domains. As stated above, each RCK domain possesses a high-affinity Ca^{2+} site (commonly known as the "Ca^{2+} bowl") where 3 Ca^{2+} ions can bind (i.e., 24 Ca^{2+} ions/channel), leading to a change in the conformation of the gating ring and switching into a conducting state (Pau et al., 2011). In addition, BK channel subunits contain at least a low-affinity site for Ca^{2+} and Mg^{2+} at the interface between the voltage sensor and the RCK1 domain (Zhang et al., 2001). Interestingly, when Mg^{2+} ions bind to this site, a charged residue in the S4 domain is repelled, therefore enabling the active configuration of the VSD, and indirectly promoting BK channel opening (Hu et al., 2003).

In addition to Ca^{2+} and Mg^{2+}, RCK domains can sense other divalent cations such as Cd^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, or Ni^{2+}, with relatively low selectivity to that for Ca^{2+} (Xia et al., 2002: Zeng et al., 2005). Furthermore, an additional low-affinity-Ca^{2+} binding site for the ions with smaller radii (i.e., Mn^{2+}, Co^{2+}, Mg^{2+} and Ni^{2+}) has been noted by Zeng et al. (2005).

The binding of intracellular Ca^{2+} (or other divalent cations) promotes a leftward shift of the steady-state open probability of BK channels, a process which is correlated with a slowing deactivation of the channel (Barrett et al., 1982). Electrophysiological studies combined with Ca^{2+}-dependent kinetic analysis of the BK channel have determined that its Ca^{2+} affinity resides mainly in the low micromolar range (i.e., 1–10 μM) (Cox et al., 1997; Contreras et al., 2013), and can be modulated by a variety of ligands and metabolic states (Xia et al., 2002). The spatial interaction between BK channels and voltage-gated calcium channels (VGCC) appears to be critical for promoting BK channel activation at low membrane voltages. In fact, several studies have shown colocalization or the existence of BK-VGCC macromolecular complexes which mediate rapid and focalized Ca^{2+}-activated K^+ signaling in neurons (Berkefeld et al., 2006).

In vascular smooth muscle, BK channels are associated with elevations in intracellular Ca^{2+} concentrations by behaving as a negative feedback mechanism to oppose the nearly always partially constricted state of resistance arteries (i.e., vascular tone) exhibited under physiological conditions (**Figure 2**). In particular, highly localized intracellular Ca^{2+} transients, known as "Ca^{2+} *sparks,*" are triggered by the concurrent opening of a number of ryanodine-sensitive Ca^{2+} release (RyR) channels in the sarcoplasmic reticulum. This in turn elevates local Ca^{2+} levels (10–100 μM) that activates multiple adjacent BK channels, leading to transient macroscopic currents referred to as spontaneous transient outward currents (i.e., "STOCs") and subsequent membrane hyperpolarization by closing VGCC and decreasing intracellular Ca^{2+} concentrations (Nelson et al., 1995; Bonev et al., 1997; Porter et al., 1998). Notably, simultaneous recordings of Ca^{2+} sparks and cell membrane potential revealed that Ca^{2+} sparks elicited up to ~20 mV hyperpolarization of arterial smooth muscle through the activation of BK channels (Ganitkevich and Isenberg, 1990). Accordingly, blockade of either Ca^{2+} sparks or BK channels depolarized pressurized cerebral arteries causing an increase in intracellular Ca^{2+} levels and subsequent vasoconstriction (Nelson et al., 1995; Knot et al., 1998; Porter et al., 1998). These relevant studies provided solid evidence for Ca^{2+} sparks functioning as essential regulators of the vascular tone through the activation of BK channels.

PHARMACOLOGY OF BK CHANNELS

BK channel activity can be modulated by numerous endogenous mediators, intracellular signaling proteins, peptide toxins, small-molecule blockers, and/or endogenous or synthetic openers (**Figure 3**). This section reviews these regulating molecules and their potential in delineating the physiological and pathophysiological implications for BK channels.

Regulation by Signaling Molecules or Endogenous Mediators

Physiologically, BK channel activity may be regulated *via* a wide variety of intracellular signaling molecules that bind to the cytoplasmic domain of the channel, including Mg^{2+}, which depending on its concentration, can exert opposing effects in the activity of BK channels. While at physiological concentrations (0.5 mM) and in the presence of relatively low Ca^{2+} levels, Mg^{2+} shifts the voltage-dependent opening of BK channels toward more hyperpolarized voltages, higher levels of this divalent cation (1–4 mM) diminish BK channel unitary amplitude in a voltage-dependent manner (Zamoyski et al., 1989; Ferguson, 1991; McLarnon and Sawyer, 1993; Zhang et al., 1995, 2001; Morales et al., 1996; Shi and Cui, 2001; Shi et al., 2002; Xia et al., 2002).

There is also a growing literature on BK channel activity modulation by intracellular protons (Schubert et al., 2001; Schubert and Nelson, 2001; Avdonin et al., 2003; Brelidze and Magleby, 2004; Raingo et al., 2005; Park et al., 2007; Hou et al., 2009). Electrophysiological analysis using native smooth muscle cells from rat tail arteries revealed that while pH fluctuations (ranging 7.0–7.8) were unable to alter single-channel conductance or voltage-dependence of activation, the amplitude of intact BK channel currents were markedly decreased when lowering the pH from 7.2–6.8 (Schubert et al., 2001). In contrast, Dabertrand et al. (2012) elegantly demonstrated that brain acidosis induces the transformation of Ca^{2+} waves into Ca^{2+} sparks, leading to the activation of BK channels and subsequent dilation of brain parenchymal arterioles. The latter effect is due to the inherent ability of protons to bind to Ca^{2+} sensing residues located at the C-terminus of the BK channel. Specifically,

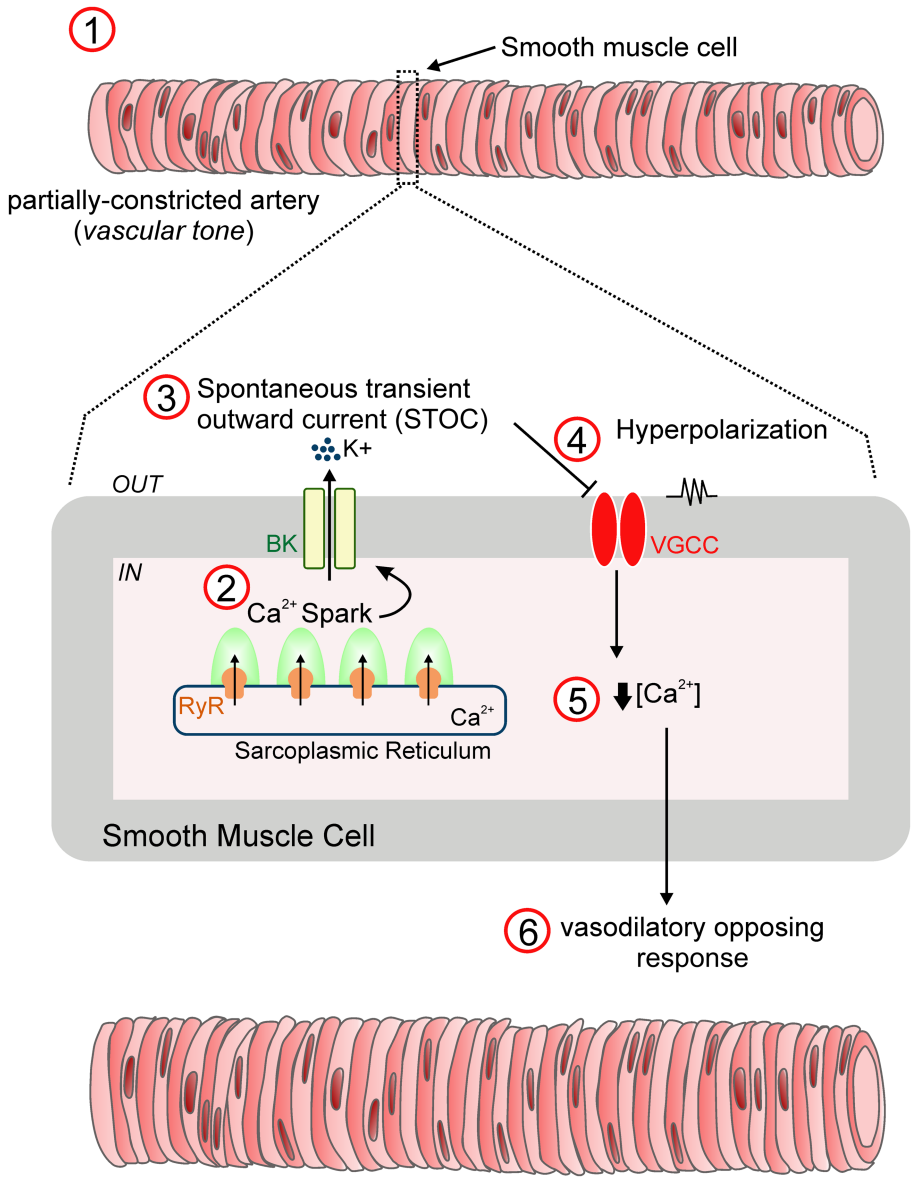

FIGURE 2 | STOC-mediated vasodilation mechanism. In vascular smooth muscle, BK channels are key drivers of negative feedback control *via* the regulation of membrane excitability, an essential mechanism that prevents excessive constriction of resistance arteries (1). Specifically, transient activation of ryanodine receptors (RyR) residing in the sarcoplasmic reticulum leads to the generation of "Ca^{2+} sparks" (2). Single sparks increase the Ca^{2+} concentration in the vicinity of membrane BK channels, provoking their opening and the subsequent development of macroscopic K^+ currents referred to as "Spontaneous transient outward currents (STOCs)" (3). This in turn, contributes to membrane hyperpolarization by reducing the voltage-gated Ca^{2+} channel (VGCC) open probability (4), and a relative reduction in the intracellular Ca^{2+} levels (5). As a result, the resistance artery develops a dilatory response (6), a vital feedback mechanism to optimize arterial tone development (Nelson et al., 1995).

intracellular protons target three residues [i.e., two Histidines (His) and, one Aspartate (Asp)] residing within the RCK1 domain of the BK channel (Avdonin et al., 2003).

Oxidative stress causes contrasting effects on BK channel function. Diverse studies in vascular smooth muscle have shown an increase in BK channel activity by oxidizing agents such as 5'5-dithiobis (2-nitrobenzonic acid, DNTB; Thuringer and Findlay, 1997), nicotinamide adenine dinucleotide (NAD) and glutathione sulfide (Lee et al., 1994), while other redox derivatives including dithiothreitol (DTT), β-mercaptoethanol, NADH or reduced glutathione (GSH) diminished BK currents (Thuringer and Findlay, 1997). Peroxynitrite, an oxidant produced by the near diffusion-controlled reaction between NO and superoxide ion, decreases BK channel open probability in cerebrovascular and coronary smooth muscle, leading to vessel constriction (Brzezinska et al., 2000; Liu et al., 2002). Furthermore, the inhibitory effect of peroxynitrite was reversible and thiol-dependent as the BK current amplitude was rescued by the

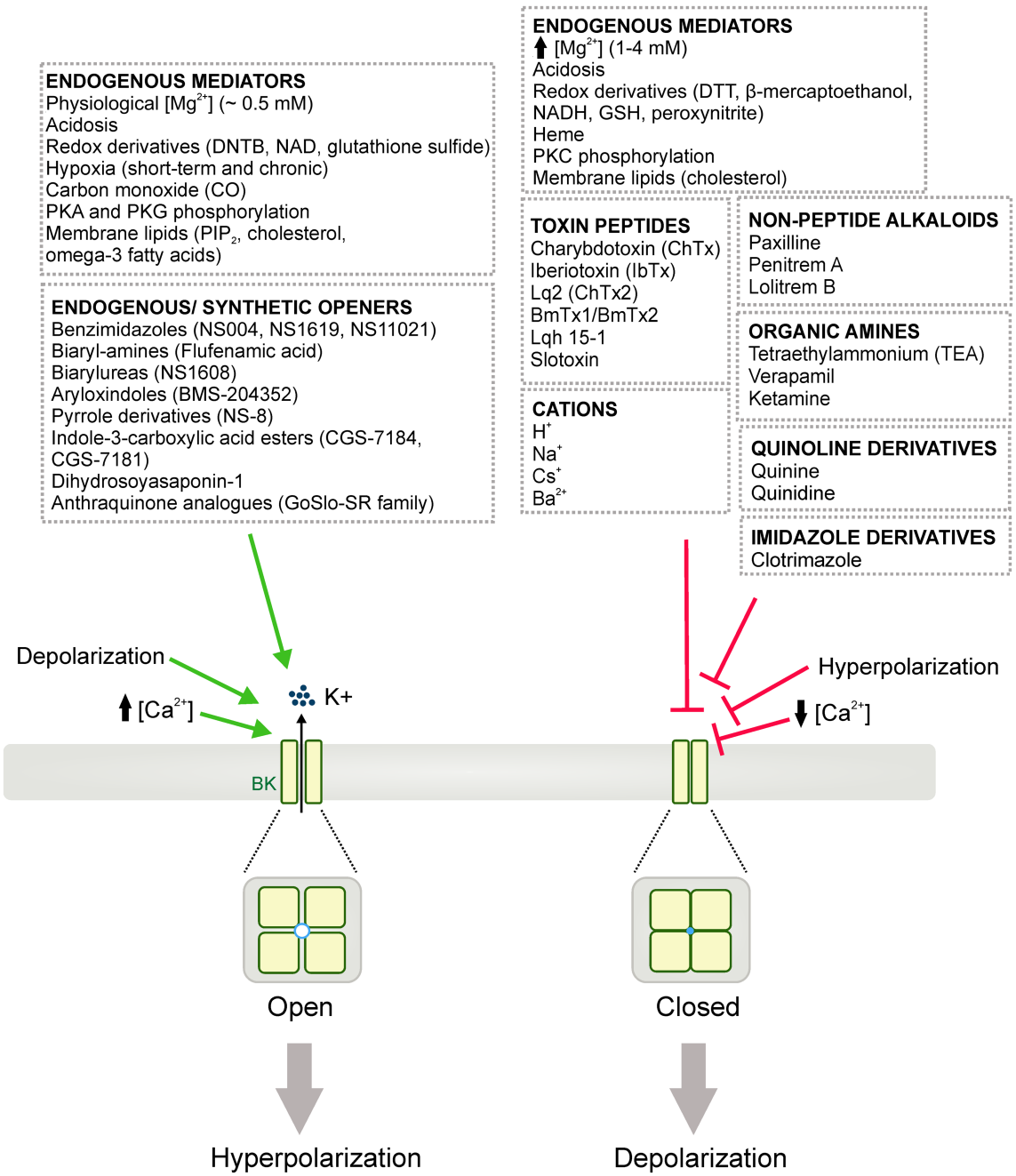

FIGURE 3 | Diagrammatic summary of the pharmacology of BK channels. BK channels can be activated (i.e., opened) or blocked (i.e., closed or inhibited) leading to cell membrane hyperpolarization and depolarization, respectively. Diverse endogenous mediators, redox derivatives and, signaling proteins are able to either potentiate or inhibit BK channel activity. Numerous BK channel inhibitors/blockers have been also reported, including: toxin peptides from scorpion venoms, non-peptide alkaloids, organic amines, quinolone and imidazole derivatives. Additionally, an extensive list of cations including H^+, Na^{2+}, Cs^+ and Ba^{2+} are shown to influence BK channel activity. Similarly, endogenous and synthetic openers have been widely studied as experimental tools and potential therapeutic approaches for different vascular or neurological disorders involving BK channels.

antioxidant GSH. Acute or chronic deprivation of adequate oxygen supply (i.e., hypoxia) also influences the activity of BK channels with downstream effects on vascular tone. During short-term hypoxia, the brain vasculature dilates to increase cerebral blood flow, a mechanism of autoregulation which is thought to be partially mediated by BK channels (Gebremedhin et al., 1994). Similarly, Tao et al. (2015) demonstrated a stimulatory effect of chronic hypoxia on BK channel activity by increasing channel affinity for Ca^{2+} and shifting voltage channel activation to more hyperpolarized membrane potentials.

Heme, an essential cofactor involved in the redox-sensitive reaction of hemoproteins, directly binds to BK channel either in its oxidized or reduced state and drastically inhibits its activity (Tang et al., 2003). Further studies demonstrated that heme can impact the effect of allosteric activators of BK channels by possibly altering the architecture of the channel gating ring and thus, the voltage-sensing apparatus and the intrinsic stability of the open state of the channel (Horrigan et al., 2005). These findings suggest heme can function as a potent brake of BK channel activity, and it represents a clinically relevant agent with a potential cytoprotective role during conditions of "heme stress" or "excess of heme" such as trauma, ischemia or hypoxia (Gribkoff et al., 2001; Doré, 2002; Xu et al., 2002).

Carbon monoxide (CO) induces cerebral artery vasodilation through the activation of smooth muscle BK channels and thus increasing Ca^{2+} spark/STOC coupling. Specifically, CO reverses heme-induced BK channel inhibition by impairing the interaction between heme and its conserved binding domain (Jaggar et al., 2005). More detailed electrophysiology studies using inside-out, excised patch-clamp revealed that CO-induced BK channel activation is independent of the oxidation state of the gating ring of the channel and partly dependent on physiological levels of intracellular Ca^{2+} (Williams et al., 2008).

The action of various kinases including cAMP-dependent protein kinase (PKA), cGMP-dependent (PKG), and PKC directly regulates the apparent Ca^{2+}- and/or voltage-sensitivity of BK channels, and thus, their physiological activity (Schubert and Nelson, 2001). Furthermore, BK channels are shown to contribute to the actions of many endogenous vasodilators [i.e., calcitonin-gene-related peptide (CGRP) or nitric oxide (NO)] that signal *via* adenylyl or guanylyl cyclase resulting in the elevation of cAMP or cGMP intracellular levels (Robertson et al., 1993; Miyoshi and Nakaya, 1995; Peng et al., 1996). In this context, several studies demonstrated the stimulatory influence of PKA and PKG phosphorylation on the kinetics of the BK channel, but not the conductance *per se*, causing a leftward voltage shift and increasing the open probability of the channel (Robertson et al., 1993; Minami et al., 1993a,b). In contrast, PKC phosphorylation directly attenuates BK currents in arterial smooth muscle (Schubert et al., 1999; Taguchi et al., 2000).

The activity of BK channels can be further modulated by the action of several membrane lipids (see Dopico and Bukiya, 2014 for a comprehensive review) including the minor but ubiquitous phospholipid component of cell membranes, phosphatidylinositol 4,5-bisphosphate (PIP_2), the fundamental membrane lipid component cholesterol (Vaithianathan et al., 2008; Dopico et al., 2012; Tang et al., 2014; Tian et al., 2015; Dopico and Bukiya, 2017) and omega-3 fatty acids (Hoshi et al., 2013a,b). PIP_2 directly stimulates vascular smooth muscle BK channels, contributing to vascular tone and blood flow control. Specifically, its negatively charged inositol head group interacts with a conserved motif in the cytoplasmic domain of the channel α subunit. The stimulatory effect of PIP_2 is conferred by the accessory subunits that comprise the channel, potentiated by β_1, which is abundantly expressed in smooth muscle, but not by β_4 subunits. Consequently, pharmacological manipulation of endogenous PIP_2 levels results in endothelium-independent dilation of cerebral resistance arteries, an effect that is blunted by selective BK channel blockers (Vaithianathan et al., 2008). Similarly, omega-3 fatty acids are known to potentiate BK channel activity in coronary artery smooth muscle and cause dilation of isolated coronary arteries (Lai et al., 2009; Wang et al., 2011; Hoshi et al., 2013a,b). Noteworthy, however, cholesterol down-regulates BK channels and its acute depletion using methyl-β-cyclodextrin potentiates channel activity (Dopico and Bukiya, 2014). However, a recent study using native cerebral artery smooth muscle cells revealed that cholesterol enrichment stimulated BK channels, and this effect was driven by increases in cell membrane levels of β_1 subunits (Bukiya et al., 2021). In support to these findings, smooth muscle cells isolated from human coronary atherosclerotic plaque samples exhibited significantly higher channel activity than those obtained from coronary media segments (Wiecha et al., 1997). However, these findings should be interpreted with caution as cholesterol supplementation may directly modify the dynamic physical characteristics of the cell membrane, and consequently the conformation and function of the BK channel.

A long list of additional signaling molecules have been shown to influence BK channel activity including ethanol (Dopico et al., 1998; Bukiya et al., 2014a), paracrine mediators such as NO (Mistry and Garland, 1998) or adiponectin (Baylie et al., 2017); and hormones and circulating agents like angiotensin II (Zhang et al., 2014), leukotrienes (Bukiya et al., 2014b), ghrelin (Mladenov et al., 2008) or cannabinoids (Sade et al., 2006). Furthermore, BK channels are indirectly stimulated by a number of downstream second messengers resulting from the action of endogenous modulators such as adenosine and ATP, prostacyclin or CGRP (Cabell et al., 1994; Strøbaek et al., 1996a; Herzog et al., 2002; Tanaka et al., 2004).

BK Channel Inhibitors and Blockers

Venom from scorpions represent a rich reservoir of bioactive peptides, some of which have robust BK channel inhibitory properties. These toxin peptides display high potency and selectivity, constituting powerful molecular tools for the biophysical characterization of BK channels and the development of BK channel pharmacology. Charybdotoxin (ChTX), a 37-amino-acid peptide obtained from the scorpion *Leiurus quinquestriatus hebraeus* (Gimenez-Gallego et al., 1988), was the first "BK channel blocker" reported. This potent peptide toxin binds electrostatically to the outer face of the BK channel and physically blocks its activity by interfering with K^+ efflux through the ion conduction pathway. Despite its high-affinity, ChTX is known to block other subtypes of K^+ channels including voltage-dependent K^+ channels ($K_v1.2$, $K_v1.3$, and $K_v1.6$) and intermediate-conductance calcium-activated K^+ channels (Judge and Bever, 2006; Panyi et al., 2006), a property that results in its lack of selectivity and thus, prompts the requirement to use more selective BK channel blockers. The 37-amino-acid peptide Iberiotoxin (IbTX), a toxin purified from the African scorpion *Buthus tamulus*, shares extensive sequence homology (i.e., ~70%) with ChTX with an identical peptide backbone configuration, but exhibits more selectivity for BK channels

as it does not inhibit other K^+ channels apparently sensitive to ChTX (Galvez et al., 1990). The high selectivity of this toxin was determined by structural studies indicating that IbTX in fact binds to a different receptor on the external face of the BK channel which is allosterically coupled to the ChTX-binding site (Candia et al., 1992). This therefore, positions IbTX as a valuable pharmacological tool to study the structure and function of BK channels. In addition to ChTX and IbTX, a number of other toxin peptides have been purified from scorpion venom and similarly described as BK channel blockers with diverse and selective pharmacology, including Lq2 (Lucchesi et al., 1989), BmTx1/BmTx2 (Blanc et al., 1998), Lqh 15–1 (also called ChTx2; Marshall et al., 1994), and slotoxin (Garcia-Valdes et al., 2001). While all these peptide toxins are useful tools for experimentation, they do not display true potential as therapeutics given their inherent pharmaceutical disadvantages (e.g., rapid degradation, poor blood–brain permeability, ineffective orally active formulation) and poor reversibility.

A distinct group of highly selective and potent BK channel blockers include a series of non-peptide alkaloid molecules such as the fungal tremorgenic indole-diterpenes paxilline, penitrem A and lolitrem B, and the organic amines tetraethylammonium (TEA), verapamil and ketamine. While these alkaloids are capable of inhibiting BK channels in a highly specific fashion, their respective structures and mechanisms of action differ considerably (Kaczorowski et al., 1996; Nardi et al., 2003). Among them, tremorgenic mycotoxins, which are known to elicit a neurotoxic disorder in cattle called "ryegrass staggers" syndrome, are the most potent and selective non-peptide blockers of BK channels to date (Norris et al., 1980). In particular, paxilline has been the most extensively used in experimentation due to its apparent high-specificity and reversibility of action. This largely rigid molecule potently blocks BK channels at low nanomolar concentrations ([nM]) by interacting with binding sites residing on the α-subunit – distinct but allosterically coupled to those associated with ChTX (DeFarias et al., 1996; Sanchez and McManus, 1996). Additionally, a recent study from Zhou et al. (2020), identified a novel and highly specific site involved with paxilline-mediated inhibition that may represent a useful tool to further elucidate BK channel function as well as to design new modulators with promising clinical applications.

Quaternary amines such as TEA and its analogues belong to the group of organic amines able to block BK channels in a voltage-dependent manner but also a wide variety of other voltage-gated K^+ channels, lacking therefore of high selectivity and applicability. In contrast to the toxin peptides – which only binds to the outer face of the BK channel – TEA blocks BK channels through either the internal or external side of the membrane, implying a complex mechanism of action. However, it exhibits different affinities depending on its site of action (external vs. internal). Specifically, BK channels are more sensitive to external TEA, and this particularity is attributed to a phenylalanine ring located near the mouth of the channel pore (Heginbotham and MacKinnon, 1992). This well-defined binding site is selective for TEA and it seems to act as a filter to differentiate the diverse TEA analogs by size. Given its ability to block BK channels, TEA has been suggested as a possible treatment to improve the persistent hypotension associated with septic shock. However, a study using a BK channel α subunit knockout mouse line demonstrated that BK channels are not a potential therapeutic target for sepsis-induced hypotension, suggesting therefore that the pressor effect of TEA may be attributed to other potassium channel species (O'Brien et al., 2011).

BK channel activity is also sensitive to other organic amines including verapamil and ketamine, quinoline derivatives such as quinine and quinidine, and imidazole derivatives (clotrimazole). The antihypertensive and antiarrhythmic agent verapamil and its analogues, are potent L-type Ca^{2+} channel blockers known to cause vasodilation and a decrease in arterial blood pressure. Verapamil is able to block BK channels (with an efficacy comparable to that reported for Ca^{2+} channels) by binding to a residue within the channel pore. The intravenous general anesthetic, ketamine is also reported to indirectly inhibit BK channels, and this effect is attenuated by increases in intracellular Ca^{2+} levels, suggesting that both ketamine and Ca^{2+} compete for the same binding site on the channel protein (Denson and Eaton, 1994). Among the quinoline derivatives, quinine and quinidine inhibit K^+ efflux through BK channels, a blockade characterized by fast flickering of the channel between the open and closed states with a consequent reduction in open channel amplitude (Wong, 1989; Mancilla and Rojas, 1990). In addition, the imidazole antimycotic P450-inhibitor clotrimazole is also capable of diminishing the open probability of BK channels without affecting single-channel conductance (Wu et al., 1999).

Furthermore, a number of cations including H^+, Na^+, Cs^+ and Ba^{2+} are also known to bind to the K^+-conduction pathway and block single-channel (i.e., unitary) currents through BK channels, thus constituting vital experimental tools in characterizing their multi-ion pore conduction mechanism.

Direct blockade of BK channels may offer therapeutic benefit in certain pathologies. However, the use of the abovementioned peptide or non-peptide BK channels blockers in the clinical setting has been extremely limited due to their poor pharmaceutical features. Thus, a demand for more targeted and selective drugs still exists for meaningful pharmacotherapy strategies and improved patient outcomes. It is clear that only through dedicated research and development initiatives, we can expect a novel BK channel inhibitor compound to satisfy strict criteria for specific channel targeting and clinically acceptable pharmacokinetics/pharmacodynamics properties in humans.

BK Channel Activators and Openers

Several synthetic and endogenous BK channels openers have been investigated at whole-cell and single channel levels using the patch-clamp technique in a diverse array of native vascular, non-vascular tissues from different animal species, and culture cell models. These small-molecule BK openers include the synthetic benzimidazoles NS004 and NS1619 (McKay et al., 1994; Lee et al., 1995a), the biaryl-amine flufenamic acid (Ottolia and Toro, 1994), the biarylurea

NS1608 (Strøbaek et al., 1996b), the aryloxindol BMS-204352 (Gribkoff et al., 2001), the pyrrole derivative NS-8 (Tanaka et al., 2003), the indole-3-carboxylic acid esters CGS-7184 and CGS-7181 (Hu et al., 1997), and the natural modulator dihydrosoyasaponin-1 (Gribkoff et al., 1996). Among them, NS1619 has been widely studied as a potential therapeutic treatment for various conditions involving vascular and non-vascular smooth muscle such as shock-induced vascular hyporeactivity (Hu et al., 2014), pulmonary hypertension (Revermann et al., 2014), bladder hyperactivity (La Fuente et al., 2014), and erectile dysfunction (Gonzalez-Corrochano et al., 2013). However, the therapeutic perspective of NS1619 is limited given its relatively low potency and selectivity. A more selective BK channel opener, NS11021, has been reported to protect the heart against ischemia–reperfusion injury (Bentzen et al., 2009), enhance erectile responses in rodents (Kun et al., 2009) and reduce excitability and contractility of detrusor smooth muscle in the urinary bladder (Layne et al., 2010). Finally, the GoSlo-SR family constitute a group of anthraquinone analogues with higher potency than NS11021, which has been suggested as a useful starting template for the design of more tissue-specific BK openers (Roy et al., 2012).

While these BK channel openers would offer some limited clinical applications for conditions of neuronal and muscular hyperexcitability, they have not borne meaningful fruit for the pharmaceutical industry as therapeutic treatments. High on the list of potential reasons for difficulty to utilize such agents is that they may induce epilepsy and/or paroxysmal movement disorder(s). It appears that the abnormally increased BK channel activity may paradoxically lead to an enhancement in excitability in certain cases by triggering rapid depolarization of action potentials and therefore, contributing these pathological conditions (Du et al., 2005). Nonetheless, Cheney et al. (2001) demonstrated that the fluoro-oxindole BK channel opener BMS-204352 might be selectively beneficial for the treatment of experimental traumatic brain injury, in this case induced by lateral fluid percussion, as its administration significantly improved neurologic motor deficits and prevented the extent of regional cerebral edema at ~1–2 weeks post-injury. This pharmacological agent was subsequently suggested as a promising therapeutic strategy for ischemic stroke as it was able to diminish neuronal excitability and excitatory transmitter release in a rodent model of stroke (Jensen, 2002).

BK CHANNEL DIVERSITY

Post-transcriptional Modifications

The pore-forming α-subunits of the mammalian BK channel are encoded by only one gene (*Slo1*; *KCNMA1*) which displays extensive alternative splicing of pre-messenger RNA. The powerful regulatory strategy of alternative splicing allows a large number of phenotypic splice variants to be generated from a single gene with high degree of diversity, particularly with respect to their physiological roles, tissue distribution, and biophysical features such as apparent sensitivity to calcium/voltage, unit conductance, activation/deactivation voltage range, and phosphorylation susceptibility by endogenous protein kinases or other intracellular signaling pathways (Tian et al., 2001; Chen et al., 2005; McCartney et al., 2005; Fodor and Aldrich, 2009). Alternative splicing also acts as a regulator of BK channel trafficking by finely tuning their cell surface expression according to certain physiological needs (Zarei et al., 2004; Singh et al., 2012). A variety of sites of alternative splicing within the α-subunits have been identified, and the intracellular C-terminal domain comprises the majority of them (Shipston, 2001). Using transcript scanning, Chen et al. (2005) analyzed the biophysical profile of five distinct splice variants resulting from alternative splicing at a single site – the mammalian site of splicing C2 residing in the C-terminal domain – and described the high variability among them in terms of functionality and biophysical properties. Thus, this widespread phenomenon represents a powerful mechanism to increase BK channel molecular heterogeneity and determine cellular excitability in a given tissue.

Post-translational Modifications

BK channel activity is robustly regulated by an eclectic array of major post-translational processes including phosphorylation, palmitoylation, glycosylation and ubiquitination (for an extensive review see Shipston and Tian, 2016). For instance, BK channels are potently and reversibly controlled by PKA-mediated phosphorylation in neurons and smooth muscle cells (Lee et al., 1995b; Zhou et al., 2000). Several studies have identified various putative PKA-mediated phosphorylation C-terminal motifs including $RQPS_{899}$ and the stress regulated exon (STREX), with remarkable properties contributing to promote either BK channel activation and inhibition, respectively (Tian et al., 2004). Interestingly, the cytosolic C-terminal of the STREX insert can also undergo palmitoylation of a conserved cysteine-rich domain, providing a conditional gate for BK channel regulation by PKA phosphorylation (Tian et al., 2008). Additional cysteine-enriched sites for palmitoylation have been identified – independent of and outside the STREX insert – within the intracellular linker between the S0 and S1 transmembrane domains with key roles in controlling BK channel cell-surface expression (Jeffries et al., 2010). This complex cross-talk between palmitoylation and phosphorylation explains the dramatic functional diversity of the BK channel among different cell types and tissues. N-linked glycosylation has been also reported to control BK channel stability, trafficking, and function. While direct evidence in the α subunits is sparse, β subunits have shown to be more susceptible to be N-glycosylated at two residues (Asn 53 and Asn 90 in the human β-4 subunit) in the large extracellular loop (Wallner et al., 1996; Jin et al., 2002). Finally, multiple sites in the C-terminal domain of the α subunits may be exposed to subsequent polyubiquitination which in turn results in BK channel accumulation in the endoplasmic reticulum. Accordingly, transgenic mouse models lacking the ubiquitination molecular machinery exhibit increased levels of BK channels at the cell surface and develop neuronal hyperexcitability and spontaneous epileptic seizures. These

findings effectively suggest that this post-translational mechanism is critical to prevent this neurological disorder through BK channels (Liu et al., 2014).

Association With Auxiliary Subunits

The association of the BK channel α-subunit with tissue-specific auxiliary subunits generates considerable functional channel diversity in several tissues and cell types of large mammals. Two main families of auxiliary proteins have been extensively characterized thus far, the regulatory β and γ subunits.

Among the β subunits, four different subtypes have been cloned (i.e., β1- β4) which share a similar architecture consisting of two transmembrane domains (i.e., TM1 and TM2) linked by a 100-amino acid extracellular loop, and short intracellular C- and N-terminals. Although to a different extent, each β subunits is generally able to impact the Ca^{2+} sensitivity, voltage dependence, and gating mechanisms of the BK channels they interact with, and thus, influence the cell membrane excitability in a tissue-specific manner (Brenner et al., 2000, 2005). Importantly, these auxiliary subunits may also alter the sensitivity of BK channels to regulatory molecules including hormones and lipids (King et al., 2006; Hoshi et al., 2013a; Martín et al., 2014). In regard to general tissue distribution, β-1 subunits are mainly found in vascular smooth muscle, urinary bladder and some areas of the brain, β-2 are highly expressed in chromaffin cells of the adrenal gland, pancreas, kidney and hippocampal neurons, β-3 is predominant in chromaffin cells, kidney, heart, liver and lung, and β-4 is almost exclusively expressed in the brain although it may be also found in smooth muscle (Contreras et al., 2013).

The family of auxiliary γ-subunits is equally composed of 4 distinct members (1γ- γ4), encoded by four different genes. The γ-subunit is made up of a single transmembrane segment, a large extracellular domain containing leucine-rich repeat proteins, and a short intracellular C-terminal domain. Specifically, these leucine-rich repeat proteins are critical in modifying the BK channel activation profile (Yan and Aldrich, 2010, 2012). More recently, a novel regulatory subunit termed LINGO1 has been discovered and constitutes the subject of ongoing studies. This protein, which shares a number of structural characteristics with $\gamma_{1\text{-}4}$ subunits and has been associated with motor disorders and tremor such as Parkinson's disease and essential tremor, was found to be in close association with BK channels and reduced BK channel activity in culture models and human cerebellar tissues (Dudem et al., 2020).

CONCLUDING REMARKS

Many landmark studies have contributed to the understanding of BK channel structure and function. The reporting of the BK channel crystal structure had initially raised hopes about therapeutic potential, but difficulties generating a safe, reliable and specific pharmaceutical compound for therapy have been particularly problematic. The continued work to fully characterize the crystal structure(s) of the various BK channel accessory subunits may offer promise to provide alternative targets to modulate channel function and improve therapies. While such drugs and interventions may be some years away from clinical practice, efforts to study BK channel function using "laboratory-based" research compounds still provide important tools to further understand the various roles of BK channels in the context of cellular-, tissue-, and organ-specific studies.

AUTHOR CONTRIBUTIONS

MS and BDK wrote the manuscript, and designed the figures. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Coronary Large Conductance Ca^{2+}-Activated K^+ Channel Dysfunction in Diabetes Mellitus

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Diabetes mellitus (DM) is an independent risk of macrovascular and microvascular complications, while cardiovascular diseases remain a leading cause of death in both men and women with diabetes. Large conductance Ca^{2+}-activated K^+ (BK) channels are abundantly expressed in arteries and are the key ionic determinant of vascular tone and organ perfusion. It is well established that the downregulation of vascular BK channel function with reduced BK channel protein expression and altered intrinsic BK channel biophysical properties is associated with diabetic vasculopathy. Recent efforts also showed that diabetes-associated changes in signaling pathways and transcriptional factors contribute to the downregulation of BK channel expression. This manuscript will review our current understandings on the molecular, physiological, and biophysical mechanisms that underlie coronary BK channelopathy in diabetes mellitus.

Keywords: BK channel, diabetes mellitus, coronary arteries, blood vessels, regulation

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INTRODUCTION

Diabetes mellitus (DM) has become a global epidemic. The incidence and the prevalence of DM have steadily increased over the past few decades. According to the WHO report in 2021, over 422 million people worldwide have DM with a prevalence of 8.6%, causing 1.6 million deaths annually.[1] Type 1 diabetes mellitus (T1DM) accounts for 5–10% of the total cases of DM and is caused by autoimmune-mediated destruction of pancreatic β-cells, leading to hyperglycemia and insulin dependence (Bluestone et al., 2010; Op De Beeck and Eizirik, 2016). Type 2 diabetes mellitus (T2DM) represents 90–95% of the total cases of DM and is caused by insulin resistance with hyperinsulinemia, hyperglycemia, and hyperlipidemia in most patients (Pandey et al., 2015; Halim and Halim, 2019).

Both T1DM and T2DM are intimately related to micro-vascular and macro-vascular diseases, including ischemic heart disease, cerebrovascular disease, and peripheral vascular disease, resulting in myocardial infarction, stroke, retinopathy, nephropathy, and neuropathy with organ and tissue damages in 70% of diabetic patients (Kurisu et al., 2003; Yeung et al., 2012; Beckman and Creager, 2016; Sorop et al., 2016). The clinical consequences of diabetic vascular complication are devastating. DM is the leading cause of end stage renal disease, new cases of blindness,

[1]https://www.who.int/health-topics/diabetes#tab=tab_1

and non-traumatic lower extremity amputation, imposing global direct health expenditure of $ 760 in 2019 with a projected $ 825 billion by 2030 and $ 845 billion by 2045 (Williams et al., 2020). Hence, it is critically important to understand the mechanisms of vascular dysregulation in DM so that better diagnostic and therapeutic approaches can be developed to treat diabetic vascular complications more effectively.

Ionic mechanisms play a central role in the regulation of vascular reactivity. Vascular large conductance Ca^{2+}-activated K^+ (BK) channels are major determinants of such regulation. BK channels are densely populated in vascular smooth muscle cells (SMCs), particularly in small resistance arteries, and provide tight regulation of vascular tone and tissue perfusion. It is well established that vascular BK channel expression and function are abnormal in DM. Diabetic patients are known to have worse cardiovascular events and outcome, with higher risks of ischemic heart disease and myocardial infarction (Kurisu et al., 2003; Yeung et al., 2012; Sorop et al., 2016). In this review, we will focus on recent findings in the coronary arterial SMCs, highlighting the diabetes-mediated changes in channel expression, function, and intrinsic properties, as well as the molecular mechanisms associated with these changes.

STRUCTURE AND FUNCTION OF VASCULAR BK CHANNELS

Cardiac perfusion is regulated by vasoactive agents released by the endothelium from mechanical sensing of luminal shear stress, including endothelium-derived relaxation factors (EDRF) and endothelium-derived hyperpolarizing factors (EDHF), the pharmacologic action of neuroendocrine factors, and the response of coronary arteriolar SMCs to intralumenal pressure (Goodwill et al., 2017). Functional vascular BK channels are composed of the pore-forming α-subunits (BK-α) and the accessory β1-subunits (BK-β1) and/or γ1-subunits (BK-γ1; **Figure 1**; Knaus et al., 1994; Yan and Aldrich, 2012). Four BK-α and four BK-β1 assemble to form a functional BK channel. The stoichiometry and interaction between BK-α and BK-γ1 are currently unclear. BK-α is expressed ubiquitously on the cell surface and in mitochondrial membranes of excitable and non-excitable cells, while BK-β1 is distributed in the cell membranes of excitable cells. BK-γ1 is mainly found in the cell membrane of non-excitable cells (Singh et al., 2013; Li et al., 2016). BK-α (encoded by the *KCNMA1* gene) contains the structure of six transmembrane domains (S1–S6) of voltage-gated K^+ channels in which S1–S4 constitute the voltage-sensing domain (VSD) and the S5-P loop-S6 form the ion permeation domain, containing the conserved K^+ selectivity filter (TVGYG; Ma et al., 2006; Cui et al., 2009). In addition, the BK channel has a unique S0 segment unit in the extracellular N-terminus and a large C-terminal domain (CTD). The CTD has four cytosolic domains (S7–S10) with two regulators of K^+ conductance domains (RCK1 and RCK2) that contain two high-affinity Ca^{2+} binding sites (Wu and Marx, 2010; Yuan et al., 2010). One such site is the Ca^{2+} bowl (889-QFLDQDDDD-897) in RCK2 with a Ca^{2+} concentration at half-maximal effect (EC_{50}) in the 10^{-6} M range (Xia et al., 2002; Bao et al., 2004). The other site (D367/E535/R514) is located in RCK1 (**Figure 1**; Zeng et al., 2005; Zhang et al., 2010b). The RCK1s and RCK2s of four BK-α subunits form an octameric gating ring that connects to the VSD through a rigid linker (Yuan et al., 2010; Tao et al., 2017). Binding to intracellular free Ca^{2+} and membrane depolarization activate BK channels through allosteric changes in the gating ring.

In addition to Ca^{2+}- and voltage-dependent activation, BK-α activity is tightly regulated by its accessory subunits, BK-β and BK-γ (Li and Yan, 2016; Gonzalez-Perez and Lingle, 2019). Four isoforms of β subunits (BK-β1-4, encoded by the *KCNMB1-4* genes) and γ subunits (BK-γ1-4, encoded by the *LRRC26*, *LRRC38*, *LRRC52*, and *LRRC55* genes) have been cloned in mammalian cells (Li and Yan, 2016; Gonzalez-Perez and Lingle, 2019).

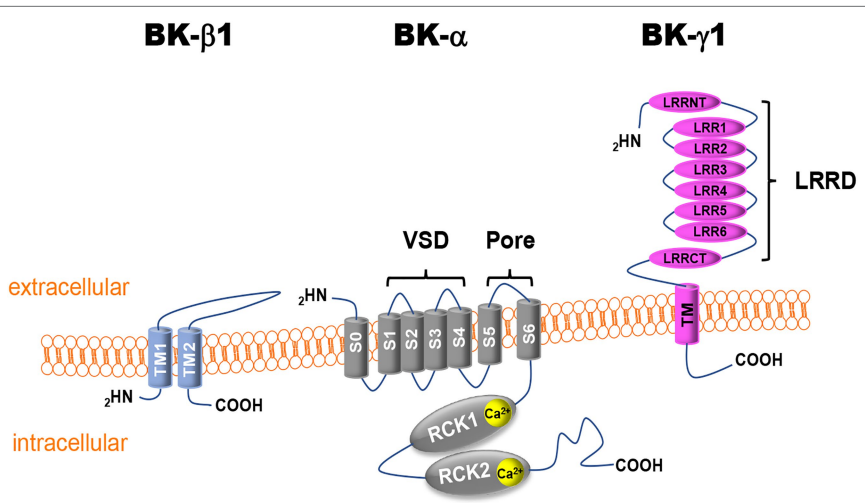

FIGURE 1 | Schematic illustration of vascular Ca^{2+}-activated K^+ (BK) channel subunits. BK-α, BK channel α-subunit; BK-β1, BK channel β1-subunit; BK-γ1, BK channel γ1-subunit; S or TM, transmembrane domain segment; VDS, voltage-sensor domain; RCK, regulator of K^+ conductance; LRR, leucine-rich repeat; LRRD, leucine-rich repeat domain; LRRCT, leucine-rich repeat C-terminus; LRRNT, leucine-rich repeat N-terminus; COOH, C-terminus; and NH_2, N-terminus.

In vascular SMCs, BK-β1 is the predominant vascular isoform. It contains two transmembrane domains (TM1 and TM2) with a relatively large extracellular loop that can reach the inner mouth of the BK-α channel pore and modulates the binding of iberiotoxin (IBTX) and the effects of fatty acids on BK channel activity (Torres et al., 2014). The TM1 is thought to interact with the S2 of an adjacent BK-α subunit and the TM2 with the S0 of another adjacent BK-α subunit (Liu et al., 2010). The presence of the BK-β1 subunit enhances channel sensitivity to Ca^{2+} activation.

BK-γ1 is also expressed in vascular SMCs (Evanson et al., 2014). BK-γ1 shares the structure of the leucine-rich repeat (LRR) protein superfamily and contains an extracellular N-terminus with six LRRs, a single transmembrane domain, and a short intracellular C-terminus (**Figure 1**). The effects of BK-γ1 on BK-α regulation can be reproduced by a 40-amino acid peptide containing the transmembrane domain of BK-γ1, suggesting that this is an important structure in the regulation of BK channel physiology (Li et al., 2016). BK-γ1 is known to enhance BK-α sensitivity to Ca^{2+} and voltage stimuli by magnitudes similar to those of BK-β1, allowing BK channel activation in the physiological range of intracellular free Ca^{2+} concentrations and membrane potentials of vascular SMCs (Tanaka et al., 1997; Cox and Aldrich, 2000; Yan and Aldrich, 2012). In heterologous expression systems, BK-β and BK-γ subunits can co-exist in the same functional BK channel complex. Their effects on the intrinsic properties of the channel were additive, suggesting that the multiplicity of BK-β/BK-γ combinations would generate a range of BK channels with distinct functional properties according to the specific stoichiometry of the contributing subunits (Gonzalez-Perez et al., 2015). Since nothing is known about the role of BK-γ in the regulation of coronary BK channels in DM, this review will focus on the findings regarding BK-α and BK-β1 pathophysiology in DM.

Intracellular Ca^{2+} homeostasis in vascular SMCs is regulated by the balance between sarcolemmal Ca^{2+} entry (L-type Ca^{2+} channels and the transient receptor potential channels; TRP, etc.), release of Ca^{2+} from the endoplasmic reticulum/sarcoplasmic reticulum, uptake of cytoplasmic Ca^{2+} into intracellular stores, and extrusion through the sarcolemmal Ca^{2+} pump and Na^+/Ca^{2+} exchanger (Leopold, 2015). In vascular SMCs, BK channels link Ca^{2+} homeostasis with cellular excitability and regulate vascular tone through membrane hyperpolarization, providing a negative feedback mechanism on Ca^{2+} entry. BK channels are colocalized with L-type Ca^{2+} channels and TRPC/TRPV channels to form BK channel-Ca^{2+} signaling complexes in the sarcolemma of vascular SMCs, allowing channel regulation in the local cellular milieu (Earley et al., 2005; Kwan et al., 2009; Suzuki et al., 2013; Hashad et al., 2018). Activation of L-type Ca^{2+} channels and TRP channels in vascular SMCs produces Ca^{2+} sparklets and triggers Ca^{2+} release from the SR to generate Ca^{2+} sparks (Nelson and Quayle, 1995; Takeda et al., 2011). With a single channel conductance of ~300 pS, BK channels contribute to 50% of the total K^+ currents in coronary arterial SMCs (Wang et al., 2011; Sun et al., 2020). Activation of vascular BK channels by Ca^{2+} sparks/sparklets in their vicinity gives rise to spontaneous transient outward currents (STOCs), which hyperpolarize the cellular membrane potentials, inactivate L-type Ca^{2+} channels and TRP channels, reduce intracellular Ca^{2+} concentrations, and lead to vasorelaxation (Nelson et al., 1995; Ledoux et al., 2006). In addition, BK channels are also expressed in vascular endothelial cells (ECs). Activation of endothelial BK channels may hyperpolarize adjacent SMCs, bestowing EDHF effects (Bryan et al., 2005; Hughes et al., 2010). Nevertheless, activation of BK channels contributes to more than 70% of total vasodilation induced by bradykinin (Miura et al., 1999) and 40% of total vasodilation induced by shear stress in human coronary resistance vessels (Lu et al., 2019).

CORONARY BK CHANNEL DYSFUNCTION IN DM

Both T1DM and T2DM are known to be independent risk factors for cardiovascular diseases, and cardiovascular diseases continue to be a leading cause of mortality in diabetic patients (Dhalla et al., 1985; Stone et al., 1989; Brindisi et al., 2010; Leon and Maddox, 2015). Although, the prevalence of cardiovascular disease in the general population has decreased by 35–40% over recent decades, such a decline has not been observed in patients with DM (Gregg et al., 2007; Beckman and Creager, 2016; Cefalu et al., 2018). Endothelial dysfunction has been recognized as the mechanism that underlies vascular pathology of DM. Subsequent findings confirm that vascular smooth muscle dysfunction is equally important in the pathophysiology of diabetic cardiovascular complications (Creager et al., 2003).

Impaired BK channel-induced vasodilation was first discovered in the cerebral arteries of fructose-rich diet-induced insulin-resistant rats (Dimitropoulou et al., 2002; Erdos et al., 2002). Patch clamp studies provided direct evidence of BK channel dysfunction in freshly isolated coronary arterial SMCs from Zucker diabetic fatty (ZDF) rats, a genetic animal model of T2DM (Lu et al., 2005). Abnormal vascular BK channel function was also found in other diabetic animal models, including streptozotocin (STZ)-induced T1DM rodents, db/db T2DM mice, high fat diet (HFD)-induced obesity/diabetic mice and swine (Dimitropoulou et al., 2002; Pietryga et al., 2005; Burnham et al., 2006; McGahon et al., 2007; Yang et al., 2007; Dong et al., 2008; Lu et al., 2008, 2010, 2012, 2016, 2017a; Borbouse et al., 2009; Navedo et al., 2010; Zhang et al., 2010a; Mori et al., 2011; Nystoriak et al., 2014; Yi et al., 2014). It is worth noting that diabetic vascular BK channel dysfunction is a common finding in most vascular beds, but the results can vary in different species, animal models, and disease status (Mokelke et al., 2003, 2005; Christ et al., 2004; Pietryga et al., 2005; Burnham et al., 2006; Davies et al., 2007; McGahon et al., 2007; Lu et al., 2008; Borbouse et al., 2009; Navedo et al., 2010; Mori et al., 2011; Rueda et al., 2013; Nystoriak et al., 2014; Nieves-Cintron et al., 2017). It has been found that in freshly isolated coronary arterioles from patients with T2DM, BK channel sensitivity to Ca^{2+} and voltage activation was reduced, indicating that the intrinsic biophysical properties of BK channels were altered in diabetic patients (**Figure 2**; Lu et al., 2019).

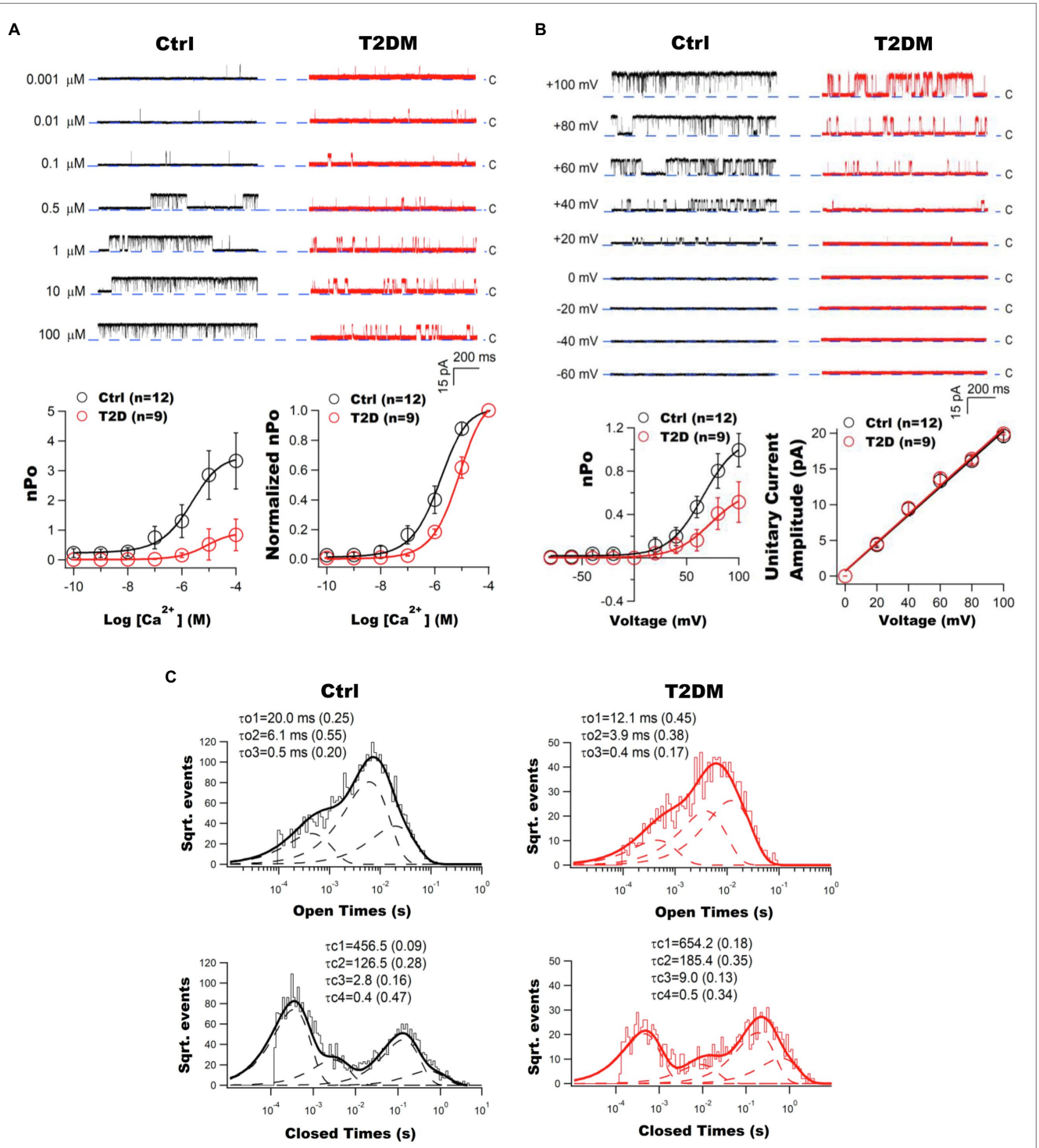

FIGURE 2 | Impaired vascular BK channel function in patients with T2DM. **(A)** Coronary arterioles of T2DM patients exhibit diminished BK channel Ca^{2+} sensitivity. Left panel: Representative tracings of inside-out single BK channel currents recorded at +60 mV in an excised patch of freshly isolated atrial coronary arteriolar myocytes from non-diabetic (Ctrl) and T2DM patients. With an increase in free Ca^{2+} concentration, BK channel open probability (nPo) was robust in controls but not in T2DM patients. Dashed lines indicate the closed state (c) of channel. Right panel: The nPo plotted against logarithm Ca^{2+} concentrations (nPo-log[Ca^{2+}] curve) was fitted using the Hill equation. There were significant reductions in Ca^{2+} log[EC_{50}] and BK channel maximal nPo in T2DM patients ($n = 9$) compared to those in non-diabetic controls ($n = 12$). A rightward shift on the normalized nPo-log[Ca^{2+}] curve of T2DM patients. Data are presented as mean ± SEM. The BK channel maximal nPo and log[EC_{50}] were significantly reduced in diabetic patients. **(B)** Impaired BK channel voltage sensitivity in the coronary arterioles of T2DM patients. Left panel: Representative tracings of inside-out single BK channel currents elicited at different testing

(Continued)

FIGURE 2 | voltages in the presence of 200 nM free Ca^{2+} in freshly isolated coronary arteriolar smooth muscle cells (SMCs) from non-diabetic controls and T2DM patients. BK channel was activated by membrane depolarization with reduced effects in diabetes mellitus (DM). The dashed line indicates the closed state (c) of channel. Right panel: BK channel open probability and voltage (nPo–V) relationships were fitted using the Boltzmann equation. The maximal nPo and voltage at half of maximal channel activation ($V_{0.5}$) were significantly decreased in T2DM patients ($n = 9$), compared with controls ($n = 12$). BK channel unitary current amplitude plotted against membrane voltages (i–V curves) were fitted using a linear equation. The unitary conductance of BK channels was not different between controls and T2DM patients. Data are presented as mean ± SEM. There was a significant decrease in BK channel maximal nPo and $V_{0.5}$ in diabetic patients. **(C)** Altered BK channel kinetics in the coronary arterioles of T2DM patients. Typical histograms of BK channel open and closed dwell-time durations are illustrated. Data were obtained from inside-out patches at +60 mV in the presence of 200 nM free Ca^{2+} in the bath solution. Dwell-time distributions were best fitted by the sum of exponential probability density functions with three open time constant components (the slow τo1, the intermediate τo2, and the fast τo3) and four closed time constant components (the very slow τc1, the slow τc2, the intermediate τc3, and the fast τc4). Dashed lines represent the distribution of exponential components determined by the logarithm likelihood ratio test. The values of each time constant component and its relative weight (in parentheses) are given above each histogram. This figure was adapted from published results with the permission of Cardiovascular Research (Lu et al., 2019).

This finding supports the observation that the BK channel response to Ca^{2+} sparks was diminished in human diabetic vessels. The significance of coronary BK channel dysfunction in DM is underscored by the finding that ischemia–reperfusion-mediated myocardial infarction is exacerbated in STZ-induced T1DM mouse hearts and can be reproduced in non-diabetics hearts after exposure to the BK channel specific inhibitor, iberiotoxin (IBTX; Lu et al., 2016). Since IBTX is membrane impermeable and cardiac myocytes do not have BK-a expression on the sarcolemma, this finding provides evidence of the role of coronary vascular BK channels on cardioprotection during ischemia–reperfusion insults, as well as the loss of its protection in DM.

Altered BK Channel Protein Expression in Diabetic Vessels

Altered coronary vascular BK channel expression is common in DM (Burnham et al., 2006; McGahon et al., 2007). However, diverse levels of vascular BK channel expression in DM have been observed. In most case, the protein expressions of BK channels are downregulated in coronary arteries (Burnham et al., 2006; Dong et al., 2008; Lu et al., 2008, 2017a; Zhang et al., 2010a; Rueda et al., 2013; Nystoriak et al., 2014; Li et al., 2017), but it was reportedly increased, despite impaired BK channel function in the coronary arteries of Ossabaw miniature swine with metabolic syndrome (Borbouse et al., 2009). Recently, human BK channel expression was examined in coronary arterioles obtained from atrial biopsies of patients who underwent coronary artery bypass grafting surgery. Protein downregulation was found in both BK-α and BK-β1 in patients with T2DM, compared to age-matched non-diabetic subjects (Lu et al., 2019). However, the mRNA levels of BK-β1 were (McGahon et al., 2007) not reduced in the coronary arteries of STZ-induced T1DM rats (Zhang et al., 2010a), db/db T2DM mice (Li et al., 2017) and HFD-induced diabetic mice (Lu et al., 2017a). The varied reports of BK channel expression suggest that a complex assortment of mechanisms exist in the regulation of vascular BK channel expression and function in DM. Reduced BK channel expression leads to impaired Ca^{2+} sparks/STOCs coupling, albeit the Ca^{2+} spark amplitudes and intracellular Ca^{2+} concentrations are known to be elevated in diabetic vascular SMCs.

Impaired BK Channel Biophysical Properties and Kinetics in Coronary Arterial SMCs in DM

Ca^{2+}-activated K^+ channel currents (I) are determined by the number of activated channels (N), open probability (Po), and channel unitary conductance (i), where $I = N^*Po^*i$. BK channel current density is reduced in the coronary arteries of T1DM and T2DM animal models and in humans with DM (Lu et al., 2005, 2008, 2010, 2012, 2016, 2017a, 2019; Pietryga et al., 2005; Burnham et al., 2006; McGahon et al., 2007; Dong et al., 2008; Zhang et al., 2010a; Nystoriak et al., 2014; Yi et al., 2014; Li et al., 2017; Nieves-Cintron et al., 2017; Tang et al., 2017; Zhang et al., 2020). BK channels are activated by intracellular free Ca^{2+} concentration and by membrane depolarization (Cox et al., 1997; Lu et al., 2008), and these are impaired in DM (Lu et al., 2008, 2019). BK channel sensitivity to voltage- and Ca^{2+}-mediated activation can be measured by using inside-out patch clamp studies in which the excised cell membrane can be clamped to various voltages and the cytoplasmic surface of the cell membrane directly exposed to bath solutions containing various free Ca^{2+} concentrations. In freshly isolated coronary arterial SMCs of ZDF rats at 8 months after the development of hyperglycemia, BK channels had a rightward-shifted Ca^{2+} concentration-dependent curve, with increased EC_{50} for Ca^{2+} activation and decreased Ca^{2+} cooperativity, compared to those of Lean control rats (Lu et al., 2008). Moreover, BK channel activation by membrane depolarization was also abnormal in coronary arterial SMCs of ZDF rats. The channel open probability–voltage (Po-V) relationships were rightward and downward shifted, with the voltage at 50% maximal Po increased by 40 mV. These results indicate that a higher cytoplasmic Ca^{2+} concentration and a more depolarized membrane potential are required to activate BK channels in DM. Changes in the intrinsic free energy of Ca^{2+}-binding ($\Delta\Delta Ca^{2+}$) that contributes to BK channel activation can be estimated based on the shift of Po-V relationship from 0 to 1 μM free Ca^{2+} in Lean and ZDF rats using the equation: $\Delta\Delta Ca^{2+} = -\Delta(zeV_{0.5})$, where z is the number of equivalence charge movement, e is the elementary charge, and $V_{0.5}$ is the voltage at half maximal activation (Shi et al., 2002). There was a 62.3% decrease in the $\Delta\Delta Ca^{2+}$ in ZDF rats, suggesting a less favorable condition for Ca^{2+} binding to

vascular BK channel Ca^{2+} sensors in ZDF rats (Lu et al., 2008). Similar results were also observed in BK channels in freshly isolated coronary microvascular SMCs from the atrial appendages of patients with T2DM. Ca^{2+}- and voltage sensitivity were significantly impaired in diabetic patients, with the maximal BK channel activity to free Ca^{2+} and voltage activation reduced by 70 and 50%, respectively (**Figure 2**; Lu et al., 2019). Such dysregulation contributed to a 27.4% attenuation in shear stress-mediated coronary arteriolar vasorelaxation in diabetic patients compared with non-diabetic controls (Lu et al., 2019). In addition, single BK channel current amplitudes were unaltered in DM, indicating that the conductance property of vascular BK channels is normal in DM.

Vascular large conductance Ca^{2+}-activated K^+ channel gating kinetics contain multiple components of open and closed states and dwell-times (McManus and Magleby, 1988, 1991). In coronary arterial SMCs, the open and closed dwell-time histograms of single BK channels were best fitted with three open-time constants: fast (τo_1), intermediate (τo_2), and slow (τo_3), along with four closed-time constants: fast (τc_1), intermediate (τc_2), slow (τc_3), and very slow (τc_4). DM affects both channel open dwell-times and channel closed dwell-times. The BK channel mean closed-time constant and the individual closed-time constants were significantly prolonged. At the same time, the channel mean open-time constant and individual open-time constants were significantly reduced in DM. These findings were seen in both ZDF rats and in diabetic patients (Lu et al., 2008, 2019). These changes in BK channel gating kinetics suggest that channel openings are abbreviated, and closures prolonged in DM, with reduced channel Po and maximal activation. Hence, diabetes not only affects BK channel expression, but also alters the intrinsic biophysical properties of the channel.

KCBMA1 and *KCNMB1* Variations Associated With Obesity and DM

Genome-wide association studies (GWASs) are a powerful tool to find genetic variations associated with diseases. Results from a few studies have shown a strong association between *KCNMA1* splicing variants and the incidence of obesity or DM. The results from case–control cohorts involving 4,838 obese and 5,827 control subjects suggested that the *KCNMA1* rs2116830*G variant was associated with obesity with a p value of 2.82×10^{-10} (Jiao et al., 2011). A recent study reported that a *de novo* missense variant in *KCNMA1* (c.1123G > A) was identified in an adult male patient with a plethora of developmental phenotypes including neonatal DM. This loss-of-function polymorphism (p. G375A) of BK channel is located in the S6 transmembrane domain of BK channel (Liang et al., 2019). In addition, it is well known that BK-α and BK-β1 undergo extensive alternative pre-mRNA splicing and that these splice variants have significant changes in BK channel intrinsic properties and surface expression (Poulsen et al., 2009). However, the pathophysiological roles of BK channel variants in the development of BK channelopathy in DM are largely unexplored and warrant further investigation.

SIGNALING MOLECULES AND PATHWAYS MEDIATING VASCULAR BK CHANNEL DYSFUNCTION IN DM

Effects of Reactive Oxygen Species on Vascular BK Channel Redox Modification

Increased reactive oxygen species (ROS) production is a hallmark of diabetic pathophysiology, and the role of ROS on vascular dysfunction has been extensively reviewed (Inoguchi et al., 2003; Konior et al., 2014). ROS is represented by a group of highly reactive molecules that include superoxide anion ($O_2^{\bullet -}$), peroxide ion (O_2^{2-}), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$). In vascular SMCs, multiple enzymatic systems such as the NADPH oxidases (NOXs), xanthine oxidase (XO), nitric oxide synthases (NOS), and the mitochondrial electron transport chain are known to produce $O_2^{\bullet -}$ and H_2O_2 (Taniyama et al., 2004; Byon et al., 2016). The NOXs, in particular NOX1 and NOX4, are the most important because they are commonly expressed in vascular cells and are the major source of ROS generation in vessels (Clempus and Griendling, 2006; Konior et al., 2014; Burtenshaw et al., 2017). $O_2^{\bullet -}$ is converted to H_2O_2 by superoxide dismutases (SODs) or reacts with nitric oxide (NO) to form $ONOO^-$. H_2O_2 is further reduced to H_2O by catalase (CAT) and glutathione peroxidase (GPx; Taniyama and Griendling, 2003). Oxidative stress due to ROS production outweighing their scavenging is implicated in vascular dysfunction associated with T1DM and T2DM. It is well documented that elevated glucose increases the production of intracellular advanced glycation end-products (AGEs), stimulates the protein kinase C (PKC)-dependent activation of NOX1 and NOX4 (Inoguchi et al., 2000; Lu et al., 2006; Deluyker et al., 2017), and reduces the activity and bioavailability of antioxidant enzymes, such as SODs, GSH, CAT, and GPx, which results in higher ROS levels in both vascular ECs and SMCs in DM (Szaleczky et al., 1999; Lu et al., 2012; Tiwari et al., 2013).

Reactive oxygen species triggers many signaling pathways and promotes redox-mediated protein posttranslational modification. We found that redox modification is involved in BK channel dysfunction through hyperglycemia. High glucose culture of HEK293 cells stably expressing BK-α resulted in altered BK-α activity and channel kinetics that were mimicked by the effects of exogenously applied H_2O_2 in BK-α expressing cells cultured in normal glucose (Lu et al., 2006). A 1-week culture with 22 mM glucose markedly downregulated the protein expression of CAT and CuZn-SOD in HEK293 cells, leading to a 3.3-fold increase of H_2O_2 concentration to the 10^{-3} M range. Consequently, high glucose culture produced a 50% reduction of BK-α current density, prolonged the channel activation and deactivation time constants (τ_A and τ_D), and upward shifted the τ-V curve, indicating that BK-α activation is suppressed in high glucose conditions (Lu et al., 2006). The effects of high glucose on BK-α voltage-dependent activation were mimicked by acute exposure to 2 mM H_2O_2. Furthermore, the cysteine residue at 911 (C911) in BK-α is particularly vulnerable to H_2O_2-mediated regulation (Tang et al., 2001), and a single substitution of C911 by alanine (C911A) eliminated

most of the inhibitory effects of BK-α under high glucose conditions and to exogenously applied H_2O_2 (Lu et al., 2006). In addition, acute exposure to $ONOO^-$ (5–100 μM) significantly suppressed BK channel activity in vascular SMCs (Brzezinska et al., 2000; Liu et al., 2002), but did not alter BK-α voltage-dependent activation (Lu et al., 2006), suggesting that the molecular mechanisms underlying BK channel regulation by H_2O_2 and $ONOO^-$ are different. Further studies revealed a 3- to 4-fold increase of 3-nitrotyrosine levels on BK-α protein in freshly isolated aortas from STZ-induced T1DM rats compared to non-diabetic controls, suggesting that $ONOO^-$-induced modification of BK-α may be mediated through protein tyrosine nitration rather than protein oxidation (Lu et al., 2010). The precise amino acid residue(s) in BK-α modified by $ONOO^-$ has not been identified. Nevertheless, an increase of ROS accumulation is the culprit for the development of BK channel dysfunction in DM.

Angiotensin II Signaling and Vascular BK Channel Regulation

Angiotensin II (Ang II) is an oligopeptide hormone, exerting its physiological and pathophysiological effects through binding to Ang II type 1 (AT1R) and type 2 (AT2R) receptors and activating their downstream signaling pathways (Dasgupta and Zhang, 2011). In vascular SMCs, where AT1R is predominantly expressed, Ang II causes vasoconstriction and promotes vascular wall remodeling (Ribeiro-Oliveira et al., 2008). In contrast, activation of AT2R produces vasodilatation and impairs vascular remodeling, effects opposite to those of AT1R (Danyel et al., 2013). AT1R is a G-protein-coupled receptor, which is coupled to Gαq, Gβγ, Gαi, and β-arrestin (Kawai et al., 2017; Wang et al., 2018). Binding of Ang II to AT1R in vascular SMCs activates Gαq which in turn activates the phospholipase C (PLC)-dependent inositol-1,4,5-triphosphate (IP_3)/diacylglycerol (DAG)-mediated Ca^{2+} signaling cascades, causing an increase in protein kinase C (PKC) activity (De Gasparo et al., 2000; Touyz and Schiffrin, 2000). Activation of PKCβ stimulates NOXs with ROS overproduction under hyperglycemic conditions (Inoguchi et al., 2000; Evcimen and King, 2007) and is a cause of impaired vascular BK channel function in diabetic vessels (**Figure 3**; Zhou et al., 2006; Lu et al., 2012; Zhang et al., 2020). In addition to redox-mediated modification of BK-α, it has been shown that PKC-induced serine phosphorylation at 695 (S695) and 1151 (S1151) in the C-terminus of BK-α inhibits BK channel current density by 50%, and S1151 phosphorylation by PKC also abolishes BK-α activation by protein kinase A (PKA) and protein kinase G (PKG; Zhou et al., 2001, 2010). On the other hand, the activity of tyrosine-protein kinase is regulated by Gαi and β-arrestin upon AT1R stimulation, causing BK channel dysfunction (Ma et al., 2000; Alioua et al., 2002; Fessart et al., 2005; Tian et al., 2007). Another study reported that the C-terminus of AT1R physically interacts with the C-terminus of BK-α in heterologous expression system, and such protein–protein interaction between AT1R and BK-α directly inhibits BK-α activity, independent of G-protein mediated processes (Zhang et al., 2014).

However, AT1R expression, Ang II bioavailability, and tissue sensitivity to Ang II are upregulated in diabetic vessels (Arun et al., 2004; Kawai et al., 2017). The pathophysiological importance of Ang II-mediated BK channel regulation in diabetic coronary

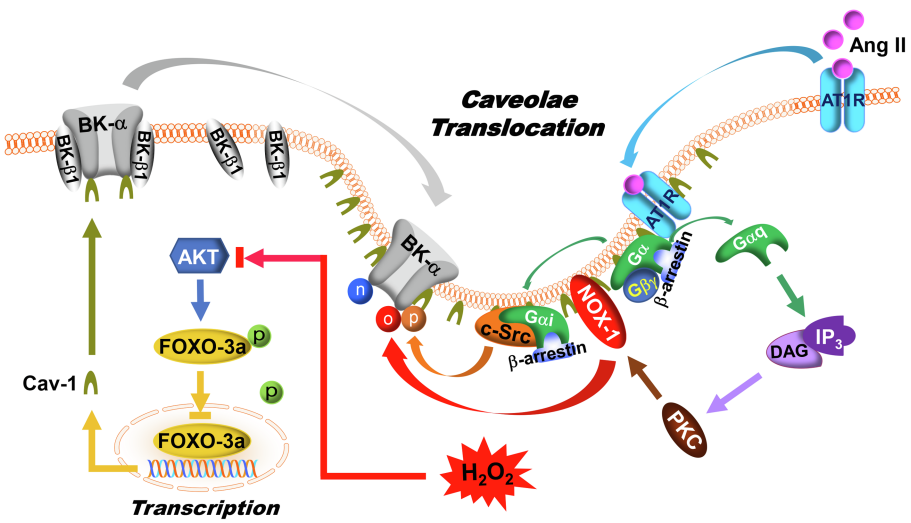

FIGURE 3 | Regulation of BK channels by AT1R signaling and caveolae compartmentalization. In DM, AT1R expression, and caveolae formation are upregulated in vascular SMCs. Upon Ang II activation, AT1R translocates to caveolae, where G-proteins, BK-α, NOX-1, and c-Src are colocalized. In caveolae, AT1R interacts with Gαq to activate PKC and NOX-1 through IP_3/DAG signaling pathway, leading to an increase of ROS production. Meanwhile, the Gαi and β-arrestin complex induces c-Src activation. As a result of AT1R activation, BK-α protein oxidation, tyrosine phosphorylation, and tyrosine nitration are enhanced. In addition, AKT phosphorylates FOXO-3a, which in turn suppresses FOXO-3a nuclear translocation and reduces its transcriptional activities. With high glucose, increased ROS production inhibits AKT function, which promotes FOXO-3a nuclear translocation and facilitates Cav-1 expression. Since BK-β1 is not present in the caveolae, an increase in BK-α compartmentalization in caveolae may lead to physical uncoupling between BK-α and BK-β1 in vascular SMCs. The symbols "n," "o," and "p" represent protein nitration, oxidation, and phosphorylation, respectively.

arteries is supported by the evidence that cardiac infarct size induced by experimental ischemia/reperfusion in STZ-induced T1DM mice was twice as large as non-diabetic mice (Lu et al., 2016). The effects of DM on myocardial ischemia/reperfusion injury can be reproduced by infusion of 2 μM Ang II or 0.1 μM membrane impermeable BK channel inhibitor, IBTX, but attenuated by the BK channel activator, NS-1619 (Lu et al., 2016). Similar results were observed in Akita T1DM mice with exacerbated cardiovascular complications and cardiac and vascular dysfunction, from an imbalance of Ang II/AT1R signaling in DM (Patel et al., 2012). Most importantly, the pathological roles of Ang II signaling are supported by clinical outcomes showing that treatment with AT1R blockers and ACE inhibitors reduced cardiovascular complications and cardiovascular death in patients with DM by 25–30% (Niklason et al., 2004; Abuissa et al., 2005; Cheng et al., 2014; Lv et al., 2018).

Caveolae Compartmentation and Vascular BK Channel Subcellular Distribution

Caveolae, which are nonclathrin-coated, flask-shaped invaginations of plasma membrane lipid raft subdomains, are characterized by their signature structural protein caveolin, with caveolin-1 (Cav-1) predominantly expressed in the vasculature (Gratton et al., 2004; Krajewska and Maslowska, 2004). Caveolae have emerged as a central platform for signal transduction in many tissues through the interaction between the Cav scaffolding domain and protein partners that contain a Cav-binding motif (ΦxΦxxxxΦ or ΦxxxxΦxxΦ, where Φ is an aromatic amino acid, and x is any amino acid; Okamoto et al., 1998). Many signaling molecules that are associated with BK channel regulation, such as the β-adrenergic receptors (Bucci et al., 2004), AT1R (Ushio-Fukai and Alexander, 2006; Basset et al., 2009), NOX1 (Hilenski et al., 2004; Wolin, 2004), cellular tyrosin protein kinase Src (c-Src; Zundel et al., 2000; Lee et al., 2001), guanylyl cyclase (Linder et al., 2005; Vellecco et al., 2016), PKA (Heijnen et al., 2004; Linder et al., 2005), protein kinase B (PKB or AKT; Sedding et al., 2005), PKC (Zeydanli et al., 2011; Ringvold and Khalil, 2017), PKG (Linder et al., 2005), NOS (Garcia-Cardena et al., 1996; Vellecco et al., 2016), and prostacyclin (PGI_2) synthase (PGIS; Spisni et al., 2001), are found in the low buoyant density, caveolae-rich membrane fractions of vascular ECs and SMCs. The significance of Cav-1 on vascular physiology is demonstrated by findings in Cav-1 knockout (KO) mice that show constitutively activated eNOS with elevated NO production as well as a failure to maintain a constant vasocontractile tone, resulting in the development of cardiovascular pathologies (Drab et al., 2001; Razani et al., 2001). Overgeneration of NO facilitates the production of $ONOO^-$ and contributes to vascular dysfunction with excessive H_2O_2 accumulation (Pacher et al., 2007).

The consensus sequence of the Cav-binding motif is present in BK-α, but not in BK-β1. Indeed, only BK-α but not BK-β1 is detected in the caveolae-rich fractions of SMCs (Lu et al., 2016). Moreover, BK-α is colocalized in the caveolae with other ion channels (Wang et al., 2005; Riddle et al., 2011; Howitt et al., 2012; Lu et al., 2016), especially those associated with Ca^{2+} spark/sparklet generation, such as L-type Ca^{2+} channels (Suzuki et al., 2013; Saeki et al., 2019), T-type Ca^{2+} channels (Hashad et al., 2018), TRPV4 (Goedicke-Fritz et al., 2015; Lu et al., 2017b), TRPC1, TRPC3, and TRPC6 (Bergdahl et al., 2003; Adebiyi et al., 2011; Grayson et al., 2017) in vascular ECs and SMCs. The close proximity of BK channels with Ca^{2+} entry molecules leads to Ca^{2+} spark-coupled STOCs. However, it has been reported that Cav-1 interacts with BK channels and inhibits BK channel activities in coronary ECs (Wang et al., 2005; Riddle et al., 2011). Cholesterol depletion by methyl-β-cyclodextrin and silencing of Cav-1 by small interference RNA enhance BK currents, while exposure to the scaffolding domain peptide of Cav-1 (AP-CAV) inhibits BK currents (Wang et al., 2005; Riddle et al., 2011). Hence, the presence of caveolae may exert an inhibitory effect on BK channel activity.

Increased Cav-1 expression has been found in most diabetic vessels (Hillman et al., 2001; Bucci et al., 2004; Pascariu et al., 2004; Elcioglu et al., 2010; Uyy et al., 2010; Li et al., 2014). Cav-1 expression is directly upregulated by the Forkhead Box O (FOXO) transcription factor (Sandri et al., 2004; Van Den Heuvel et al., 2005). The FOXO-3a phosphorylation levels are significantly reduced in STZ-induced T1DM rat arteries and in cultured human coronary arterial SMCs (Zhang et al., 2010a). This explains the underlying mechanism that leads to Cav-1 upregulation in DM (**Figure 3**). Furthermore, in STZ-induced T1DM rats, our results in co-immunoprecipitation experiments show that AT1R, c-Src, and BK-α are enriched in the low buoyant density, caveolae-rich membrane fractions of aortas, compared to non-diabetic rats (Lu et al., 2010). Infusion with Ang II (0.05 μg/kg) results in markedly enhanced AT1R protein translocation to the low buoyant density fractions of aortas after 1 h (83.4% of total membrane AT1R in STZ-induced T1DM rats vs. 28.5% in controls), suggesting enhanced AT1R translocation into caveolae-rich lipid rafts upon agonist activation in diabetic vessels, consistent with previous report in cultured vascular SMCs (Ishizaka et al., 1998). However, the precise mechanism underlying AT1R translocation is currently unclear. The levels of vascular BK-α protein oxidation, tyrosine phosphorylation, and tyrosine nitration are significantly increased in STZ-induced T1DM rats, likely due to the co-localization of NOS, NOX1 and c-Src in the caveolae. Since BK-α but not BK-β1 is present in caveolae, BK-α translocation into the caveolae of arteries in STZ-induced T1DM mice may promote the physical dissociation of BK-α and BK-β1 (Lu et al., 2016), which may explain the uncoupling of BK-α and BK-β1 in diabetic vessels. A working framework has emerged in caveolae targeting of BK channel regulation, in which caveolae compartmentalize BK-α with AT1R, NOS, NOXs, and c-Src to form BK-α-receptor-enzyme microdomain complexes in vascular SMCs (**Figure 3**). Such caveolae compartmentation is enhanced in diabetic vessels, which facilitates the redox modification of BK-α. Of note, because BK-β1 does not translocate into caveolae, such subcellular distribution of BK-α and BK-β1 may contribute to BK-α and BK-β1 functional uncoupling, thereby exacerbating BK channelopathy in diabetic vessels (**Figure 3**). Additionally, caveolae take part in endosomal trafficking and regulating surface expression of many membrane proteins (Elkin et al., 2016). Taking into account the consequences of upregulation

of caveolae formation in the vascular SMCs in DM, BK-α caveolae translocation may have important pathophysiological implications for vascular BK channel dysfunction in DM.

Ubiquitin Proteasome System and Vascular BK Channel Protein Degradation

Protein homeostasis with a balanced regulation between synthesis and degradation is essential for the maintenance of normal cellular function. Cellular proteins are degraded mainly through the lysosomes and the ubiquitin proteasome system (UPS; Ciechanover, 2005). Lysosomal protein degradation occurs through fusion with endocytotic vesicles. This mechanism of protein degradation is non-specific, and all proteins are digested indiscriminately at the same rate. UPS-mediated protein degradation accounts for 80–90% of protein degradation in mammalian cells and it is substrate-specific (Powell, 2006; Schapira et al., 2019). This process is facilitated by three distinct enzymatic steps that involve an ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). E1 interacts with ubiquitin through an E1-ubiquitin thioester bond in an ATP-dependent manner. It transfers the activated ubiquitin molecule to a cysteine residue on the E2 enzyme to form an E2-ubiquitin thioester-linked intermediate. The E3 ligase facilitates transfer of the E2-ubiquitin moiety to the substrate protein *via* an amide bond between the carboxy terminus of ubiquitin and a lysine side chain of the substrate protein. The E3 ligase is substrate-specific, allowing repeated positioning of the distal end of ubiquitin molecule for ubiquitin chain assembly with high precision. The poly-ubiquitinated protein is then recognized for enzymatic degradation in the 26S proteasome (Powell, 2006; Schapira et al., 2019). Hence, the E3 reaction is critical for determining the turnover of specific proteins. There are 617 E3 ligases functionally annotated in the human genome (Li et al., 2008). It is known that F-box (FBXO) proteins are a key component of the Skp1-Cullin-F-box (SCF)-type ubiquitin ligase complex (SCF^{FBXO}) and serve as sites for enzyme-substrate interaction (Kipreos and Pagano, 2000). FBXO proteins contain several functional domains such as the F-box domain, the LRRs, and the WD40 repeats for protein-protein interaction. Two muscle-specific FBXO proteins, FBXO-9 and FBXO-32 (also known as atrogin-1), have been found to be upregulated in diabetic vessels. They mediate BK-β1 protein ubiquitination in coronary arterial SMCs (Zhang et al., 2010a). The molecular basis of FBXO-32 and BK-β1 interaction was identified using site-directed mutagenesis and co-immunoprecipitation approaches, which showed that the PDZ-binding motif (ETSV) on BK-β1 is critical for FBXO-32-dependent ubiquitination (Zhang et al., 2010a). Deletion of the consensus sequence of the PDZ-binding motif in BK-β1 significantly decreases BK-β1 protein ubiquitination (**Figure 4**; Zhang et al., 2010a). Activation of FBXO proteins reduces BK-β1 expression, while knockdown of *FBXO* and proteasomal inhibition enhances BK-β1 levels, suggesting that accelerated UPS-mediated degradation of BK-β1 is an important mechanism of BK channel regulation in DM.

The muscle RING-finger protein 1 (MuRF1) is another E3 ligase involved in UPS-dependent vascular BK-β1 degradation (Yi et al., 2014). Nuclear factor-κB (NF-κB) sites in the MuRF1 promoter are required for transcriptional activation, while FOXO sites are not (Wu et al., 2014). Overexpression of MuRF1 downregulates BK-β1 expression, impairs BK-β1-mediated BK channel activity, and reduces BK channel-induced vasodilation in mouse coronary arteries. We found that the N-terminus of BK-β1 and the coiled-coil region of MuRF1 are necessary for BK-β1 and MuRF1 interaction (Yi et al., 2014). Importantly, the protein expressions of FBXO-9, FBXO-32, and MuRF1 are unregulated in the arteries of STZ-induced T1DM animals and in primary human coronary arterial SMCs cultured with high glucose (Zhang et al., 2010a, 2020; Lu et al., 2012; Yi et al., 2014). Such upregulation of FBXO expression is mediated through the suppression of PI3K/AKT-dependent phosphorylation in FOXO-3a, thereby promoting FOXO-3a nuclear translocation and binding to the consensus sequence [GTAAA(C/T)A] in the promoter of *Fbxo* gene, activating its transcription (Furuyama et al., 2000). However, activation of MuRF1 is due to an increase of NF-κB-mediated *Trim63* (encoding MuRF1) transcription (Wu et al., 2014). In DM or hyperglycemia, the activity of AKT is reduced (Okon et al., 2005), while that of NF-κB is augmented (Narayanan et al., 2014), thereby promoting FBXO and MuRF1 expression (**Figure 4**). Indeed, inhibition of PKCβ activity by ruboxistaurin, NF-κB activity by TPCA-1, and proteasomal activity by MG132 downregulates BK-β1 ubiquitination, preserves BK-β1 expression, and improves BK channel function in coronary arterial SMCs (Zhang et al., 2010a; Lu et al., 2012; Yi et al., 2014).

BK-α protein expression is also regulated by lysosome and UPS degradation (Wang et al., 2013; Liu et al., 2014; Leo et al., 2015; Song et al., 2018). It has been found that the CRL4A and its substrate cereblon (CRBN) complex ($CRL4A^{CRBN}$) serves as the ubiquitin ligase that interacts with the C-terminus of BK-α and induces BK-α protein degradation in neurons (Liu et al., 2014). A recent study reported that both CRBN and BK-α proteins were targeted by $SCF^{FBXO\text{-}7}$ ubiquitin ligase complex for ubiquitination and proteolysis, controlling BK-α function and regulating the learning and memory processes in the brain (Song et al., 2018). However, the specific E3 ligase(s) responsible for BK-α protein ubiquitination in blood vessels is unknown, and how the BK-α-specific E3s are regulated in DM remains to be determined.

Effects of Nuclear Factor Erythroid-2-Related Factor 2 Signaling on Vascular BK Channel Expression

Nuclear factor erythroid-2-related factor 2 (Nrf2) plays a critical role in the maintenance of intracellular redox homeostasis by regulating multiple downstream antioxidant enzymes and phase II detoxifying enzymes, which include NADPH dehydrogenase quinone 1 (NQO1), glutathione-disulfide reductase (GSR), glutathione translocase (GSTA), thioredoxin (TXN), thioredoxin reductase 1 (TXNRD1), heme oxygenase-1 (HO-1), SODs, CAT, and GPx (Gao and Mann, 2009; Chen et al., 2014). In addition, Nrf2 negatively regulates the expression of NOXs (McSweeney et al., 2016). The function of Nrf2 is principally regulated by the kelch-like ECH-association protein 1 (Keap1), which mediates

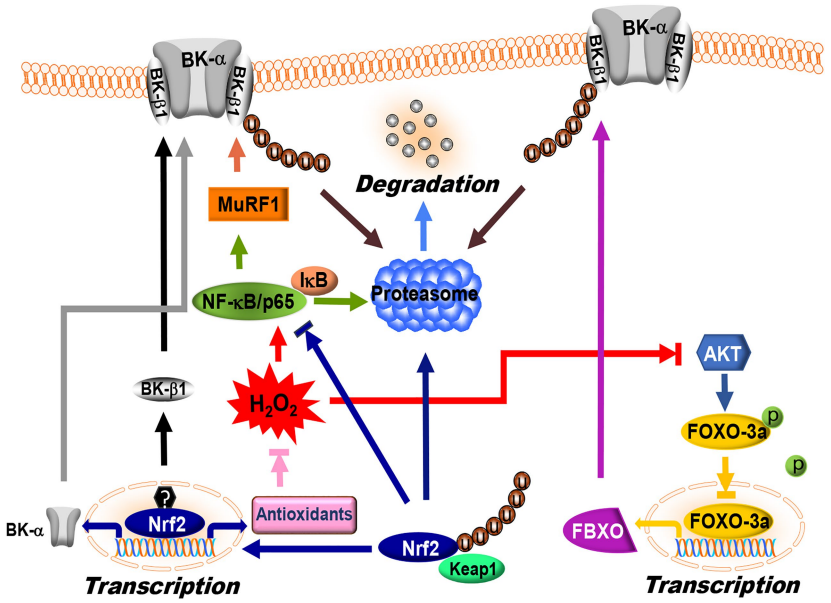

FIGURE 4 | Regulation of BK channel expression by ubiquitin proteasome system (UPS) and nuclear factor erythroid-2-related factor 2 (Nrf2) signaling. FBXO and MuRF1 are the E3 ligases targeting BK-β1 protein degradation *via* the UPS in vascular SMCs. FBXO is one of downstream targets of FOXO-3a. FOXO-3a activity is negatively controlled by AKT-dependent phosphorylation, while MuRF1 expression is controlled by NF-κB/p65. Under baseline conditions, p65 is bound to an inhibitory subunit, IκB that keeps it sequestered in an inactive state in the cytoplasm. Phosphorylation of IκB by IκB kinase promotes IκB degradation through the UPS, which in turn releases p65 and facilitates nuclear translocation. Under hyperglycemic conditions, overproduction of ROS inhibits AKT and activates NF-κB/p65, which in turn promotes FBXO and MuRF1 expression, leading to BK-β1 ubiquitination and accelerated degradation in vascular SMCs. Nrf2 is the master regulator of the antioxidant response. Under normal conditions, each molecule of Nrf2 interacts with two molecules of Keap1 resulting in UPS-mediated degradation. ROS modifies specific cysteine residues in Keap1 and releases Nrf2 from binding with Keap1. The unbound Nrf2 translocates into the nucleus and binds to the promoter region of target genes. Nrf2 directly upregulates BK-α mRNA expression *via* binding to the promoter region of *KCNMA1*. However, BK-β1 mRNA expression is not regulated by Nrf2 but by other transcription factor(s). In DM, Nrf2 expression and function is significantly downregulated, leading to a decrease in BK-α expression through reduced transcription and a decrease in BK-β1 expression through accelerated UPS degradation. The symbols "u" and "p" represent protein ubiquitination and phosphorylation, respectively.

Nrf2 ubiquitination and subsequent proteasomal degradation (Canning et al., 2015; Suzuki and Yamamoto, 2015). In the nuclei, Nrf2 binds to the promoters of antioxidant response elements (AREs) and electrophile response elements (EpREs) through interaction with the Nrf2-binding motif [TGA(G/C) xxxGC], where x represents any amino acid (Chorley et al., 2012). Both the *KCNMA1* and *KCNMB1* genes contain the consensus sequences of Nrf2-binding motifs in their promoter regions. Using promoter luciferase reporter assays, we confirmed that Nrf2 binds to the ARE of the *KCNMA1* promoter, but not to that of *KCNMB1* promoter. Mutation of the Nrf2-binding motif in the *KCNMA1* promoter abolishes the transcription response to Nrf2 (Sun et al., 2020). In addition, adenoviral expression of Nrf2 significantly augmented the mRNA levels of BK-α and BK-β1 in coronary arterial SMCs (Lu et al., 2017a; Sun et al., 2020). These results suggest that Nrf2 facilitates BK-α mRNA expression through activation of *KCNMA1* transcription, whereas the stimulatory effect of Nrf2 on BK-β1 mRNA expression is indirect and may be achieved by activating other transcription factor(s) or signaling mechanisms that upregulate *KCNMB1* transcription and expression in vascular SMCs.

Nuclear factor erythroid-2-related factor 2 deficiency has been implicated in diabetic complications including those associated with the heart (Tan et al., 2011; Bai et al., 2013), blood vessels (Ungvari et al., 2011; Miao et al., 2012; Li et al., 2017; Lu et al., 2017a), kidneys (Zheng et al., 2011; Cui et al., 2012), and the brain (Pu et al., 2018; Tarantini et al., 2018). The expression of Nrf2 and its downstream genes is slightly increased in the cardiovascular systems of STZ-induced T1DM mice at 2–3 months after the onset of hyperglycemia, but then becomes significantly downregulated at 5–6 months after the development of hyperglycemia (Tan et al., 2011; Miao et al., 2012; Bai et al., 2013), suggesting the burnout of an important redox protective mechanism in the advanced stages of DM. In db/db and HFD-induced diabetic mice 6 months after the development of hyperglycemia, BK channel activity and BK channel-mediated vasodilation in coronary arteries are impaired, accompanied by a remarkable reduction in Nrf2 and its associated antioxidant enzymes (Li et al., 2017; Lu et al., 2017a). Nrf2 KO mice show excessive ROS production, as well as diminished BK channel expression and function in vascular SMCs (Ashino et al., 2013; Sun et al., 2020). Both mRNA and protein expression of BK-α are downregulated, whereas BK-β1 proteins but not mRNA levels are decreased in the arterial SMCs of Nrf2 KO mice, consistent with the notion that Nrf2 regulates BK-α *via* transcription, and BK-β1 through posttranscriptional mechanisms (**Figure 4**; Sun et al., 2020). Administration of dimethyl formamide (DMF, an FDA-approved Nrf2 activator) preserves

BK channel protein expression, BK channel activity, and BK channel-mediated vasodilation in the coronary arteries of db/db and HDF-induced diabetic mice (Li et al., 2017; Lu et al., 2017a). Currently, Nrf2 activators such as DMF and sulforaphane (SFN) are being used in clinical trials for cardiovascular diseases and metabolic disorder (Yagishita et al., 2020), but it has not been administered for diabetic patients with coronary heart disease (Houghton, 2019). Whether the beneficial effects of Nrf2 activators observed in animal studies would translate into better outcomes in diabetic patients with cardiovascular complications needs to be determined.

Effects of Calcineurin-Nuclear Factor of Activated T Cells Cytoplasmic 3 Isoform Pathway on BK-β1 Transcription

Nuclear factor of activated T cells cytoplasmic 3 isoform (NFATc3) belongs to the nuclear factor of activated T cells (NFAT) family of transcription factors that were originally discovered in resting T cells and is important in immune response (Rao et al., 1997). NFATc3 is also involved in the development of skeletal muscle and of the cardiovascular systems (Crabtree and Olson, 2002). The activity of NFATc3 is modulated by the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin. Elevation of the intracellular Ca^{2+} concentration activates calmodulin and promotes its binding to calcineurin, leading to calcineurin activation. Activated calcineurin dephosphorylates NFATc3, which in turn induces NFATc3 nuclear translocation. Calcineurin binds to the scaffolding protein A-kinase anchoring protein 150 (AKAP150), corresponding to AKAP79 in humans, which also anchors PKA and L-type Ca^{2+} channel to form a dynamic Ca^{2+} signaling complex (Oliveria et al., 2007). AKAP79/150 strongly suppresses PKA-mediated L-type Ca^{2+} channel phosphorylation and is required for the activation of NFAT by local Ca^{2+} influx through L-type channels (Oliveria et al., 2007).

Nuclear factor of activated T cells share a conserved DNA-binding domain that specifically binds to the DNA core sequence [(A/T)GGAAA] at the promoter region of target genes, activating gene transcription (Rao et al., 1997). Human and mouse *KCNMA1* and *KCNMB1* contain at least one NFAT-binding motif in their promoters. Inhibition of vascular BK channels by NFATc3 has been reported, while upregulation of NFATc3 expression by Ang II results in decreased BK channel activity in mouse arteries due to the downregulation of BK-β1 mRNA expression (Nieves-Cintron et al., 2007). The effects of NFATc3 on BK channel activity and BK-β1 mRNA expression are abolished by calcineurin inhibitors, FK506 and cyclosporin A, in the presence of Ang II, a finding that has been confirmed in NFATc3 KO mice (Nieves-Cintron et al., 2007). AKAP150 also participates in NFATc3-mediated BK channel downregulation in HFD-induced diabetic mice (**Figure 5**; Nystoriak et al., 2014). In HFD-induced diabetic mice, the activity of the AKAP150-NFATc3 signaling pathway is upregulated, contributing to impaired BK channel function with reduced BK-β1 expression and increased vascular tone in the mesenteric arteries. However, in AKAP150 KO mice with HFD consumption, the deleterious effects of HFD on BK channels are not observed

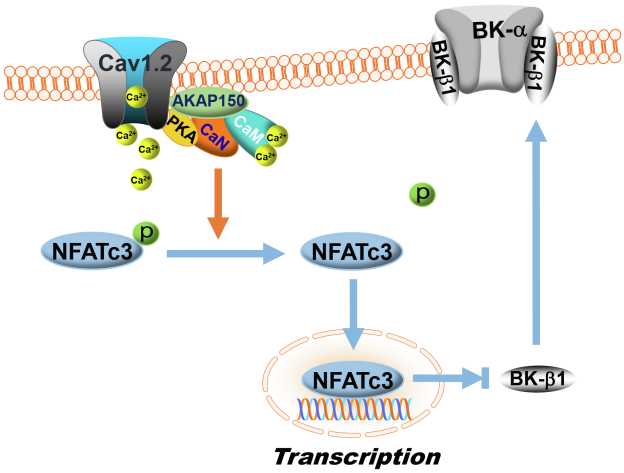

FIGURE 5 | Regulation of BK-β1 expression by NFATc3 signaling. Calcineurin is a Ca^{2+}/calmodulin (CaM)-activated phosphatase. In the membranes of vascular SMCs, AKAP150 proteins anchor calcineurin (CaN) with PKA and L-type Ca^{2+} channels (Cav1.2) to form dynamic Ca^{2+} signaling complexes. L-type Ca^{2+} channel activity is upregulated by PKA, which increases Ca^{2+} influx. Upon Ca^{2+} binding to calmodulin, calcineurin is activated, which then dephosphorylates NFATc3 and promotes NFATc3 nuclear translocation, inhibiting BK-β1 mRNA expression. In DM, the activity of the AKAP150-NFATc3 signaling pathway is upregulated, resulting in enhanced suppression of BK-β1 expression and impaired BK channel function in vascular SMCs. The symbol "p" represents protein phosphorylation.

(Nystoriak et al., 2014). Recently, *in vivo* administration of a NFATc3 inhibitor (A285222, Abbott Labs) in Akita T1DM mice is found to improve vascular endothelial function, enhance eNOS activity and NO production, reduce endothelin-1 secretion, lower blood pressure, and improve survival (Garcia-Vaz et al., 2020). The beneficial effects of NFATc3 inhibitors on coronary BK channel function in DM warrant further investigation.

Arachidonic Acid and Its Metabolites on BK Channel Regulation

Arachidonic acid (AA), a polyunsaturated omega-6 fatty acid, is abundant in normal human diet and in membrane phospholipids. It is an important precursor to a wide range of bioactive mediators and eicosanoids that regulate a multitude of essential functions in the body (Tallima and El Ridi, 2018). AA is metabolized by three major enzyme systems: It is converted by 12-lipoxygenase (12-LOX) into leukotrienes and 12-hydroxyeicosatetraenoic acid (12-HETE), by cytochrome P-450 (CYP-450) epoxygenase into epoxyeicosatrienoic acids (EETs), and by cyclooxygenases (COX) into prostaglandins, including PGI_2 and thromboxane A2 (TXA_2; Brash, 2001; Vila, 2004). Additionally, AA can be metabolized by CYP-450 omega-hydroxylase to produce 20-hydroxyeicosatetraenoic acid (20-HETE).

Arachidonic acid (Lu et al., 2005; Kur et al., 2014; Martin et al., 2014, 2021) and its metabolites (EETs, PGI_2, 12-HETE, and 20-HETE; Li and Campbell, 1997; Yamaki et al., 2001; Zhang et al., 2001; Zink et al., 2001; Lauterbach et al., 2002; Morin et al., 2007) are known to activate vascular BK channels and promote vasodilation through endothelium-dependent

hyperpolarization mechanisms. Direct exposure to 10μM AA robustly increases BK channel activity in inside-out excised patches from human umbilical arterial SMCs, suggesting activation of BK channels directly by AA (Martin et al., 2021). Extracellular application of AA results in BK channel activation and hyperpolarization of resting membrane potentials in vascular SMCs (Kur et al., 2014; Martin et al., 2021). These changes can be blocked by LOX, CYP, and COX inhibitors, suggesting that AA metabolites affect BK channels. The effects of AA on BK channels require the presence of BK-β1 (Sun et al., 2007; Martin et al., 2021).

The activation of vascular BK channels by PGI_2 is associated with cAMP-dependent, PKA-mediated phosphorylation. EETs and their metabolites dihydroxyeicosatrienoic acids (DHETs) are also potent BK channel activators and vasodilators, including the human coronary microvessels and internal mammary arteries (Quilley et al., 1997; Archer et al., 2003; Feletou and Vanhoutte, 2006; Larsen et al., 2006). Several different mechanisms of EET- and DHET-mediated BK channel activation have been proposed, including direct activation (Wu et al., 2000; Lu et al., 2001), ADP-ribosylation of Gsα (Fukao et al., 2001; Li et al., 2002), and stimulation of PKA-mediated phosphorylation (Dimitropoulou et al., 2007; Imig et al., 2008). However, AA-induced vasodilation of coronary arterioles *via* BK channel activity is impaired in high glucose conditions and DM (Lu et al., 2005; Zhou et al., 2005, 2006; Yousif and Benter, 2007; Tsai et al., 2011). PGI_2 and EET levels are decreased in patients with cardiovascular diseases (Theken et al., 2012; Mokhtar et al., 2013; Schuck et al., 2013) and DM (Lane et al., 1982; Kazama et al., 1987; Migdalis et al., 2001; Duflot et al., 2019). As a result of these findings, AA metabolites and analogues have been developed as potential therapeutic agents for cardiovascular diseases and diabetic vascular complications (Campbell et al., 2017; Wang et al., 2021).

FUTURE DIRECTIONS IN DIABETIC BK CHANNEL RESEARCH

Studies of the regulation of BK channel function and expression have greatly advanced our understanding on the role of BK channels in diabetic cardiovascular complications. DM involves a plethora of signaling abnormalities including those pertaining to insulin, ROS generation, Ang II signaling, and Ca^{2+} regulation. Thus, it is not surprising that DM affects vascular BK channel expression and function in many different ways, including transcription, translation, post-translation, surface trafficking, and channel degradation. Whether surface trafficking dysregulation of BK channel subunits contributes to BK channelopathy of the vascular SMCs in DM is unknown. Moreover, BK channels do not exist as isolated proteins but are assembled in membrane microdomains of vascular ECs and SMCs. Studies of BK channel organization by scaffolding proteins in close proximity with receptors, enzymes, and Ca^{2+} sources in blood vessels will provide further insights into BK channel physiology and into the molecular mechanisms underlying BK channelopathy in DM. In addition, our knowledge on BK-γ1 in diabetic BK channel dysregulation is very limited. Little is known about the regulation of vascular BK-γ1 expression and function in hyperglycemia and DM. Since the results of BK channel pathology from diabetic animal models are diverse, it is critical to study vascular BK channel biology and dysfunction using human tissues, which serve as the gold standard for diabetic BK channel research.

Ca^{2+}-activated K^+ channels are important regulators of vascular physiology and are critical determinants coronary circulation and cardioprotection. Preservation of BK channel expression and activities protects vascular function in DM. Hence, a better understanding of BK channelopathy and prevention of BK channel abnormalities in DM may lead to better vascular therapeutics and care for patients with DM.

AUTHOR CONTRIBUTIONS

TL and HL wrote the manuscript and critically reviewed the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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BK in Double-Membrane Organelles: A Biophysical, Pharmacological, and Functional Survey

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In the 1970s, calcium-activated potassium currents were recorded for the first time. In 10 years, this Ca^{2+}-activated potassium channel was identified in rat skeletal muscle, chromaffin cells and characterized in skeletal muscle membranes reconstituted in lipid bilayers. This calcium- and voltage-activated potassium channel, dubbed BK for "Big K" due to its large ionic conductance between 130 and 300 pS in symmetric K^+. The BK channel is a tetramer where the pore-forming α subunit contains seven transmembrane segments. It has a modular architecture containing a pore domain with a highly potassium-selective filter, a voltage-sensor domain and two intracellular Ca^{2+} binding sites in the C-terminus. BK is found in the plasma membrane of different cell types, the inner mitochondrial membrane (mitoBK) and the nuclear envelope's outer membrane (nBK). Like BK channels in the plasma membrane (pmBK), the open probability of mitoBK and nBK channels are regulated by Ca^{2+} and voltage and modulated by auxiliary subunits. BK channels share common pharmacology to toxins such as iberiotoxin, charybdotoxin, paxilline, and agonists of the benzimidazole family. However, the precise role of mitoBK and nBK remains largely unknown. To date, mitoBK has been reported to play a role in protecting the heart from ischemic injury. At the same time, pharmacology suggests that nBK has a role in regulating nuclear Ca^{2+}, membrane potential and expression of eNOS. Here, we will discuss at the biophysical level the properties and differences of mitoBK and nBK compared to those of pmBK and their pharmacology and function.

Keywords: BK channel, mitoBK, nBK, BK pharmacology, mitochondria, nucleus

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INTRODUCTION

In the 1980s, the calcium-activated potassium channel was identified for the first time in rat skeletal muscle (Pallotta et al., 1981), chromaffin cells (Marty, 1981), and skeletal muscle membranes incorporated in lipid bilayers (Latorre et al., 1982). The BK channel has a large ionic conductance (~250 pS in symmetrical 100 mM K^+) and an exceptional K^+ selectivity, hallmarks that established the name of BK "big K^+" (Marty, 1983) or MaxiK (Latorre and Miller, 1983).

The BK channel is regulated by intracellular Ca^{2+} concentration and the membrane potential difference (Marty, 1981; Pallotta et al., 1981; Latorre et al., 1982). Both properties allow it to work in a wide range of membrane potentials and intracellular Ca^{2+} concentrations. BK has

been described in different cell types and organelles (Singh et al., 2012; Li and Gao, 2016). Given the ubiquitous distribution of the BK channel and the variety of physiological roles in which it is involved, it is reasonable to think that channel alteration may have severe consequences in various channelopathies.

The BK channel is a member of the super family of K^+ voltage-dependent channels (Kv) encoded by the KCNMA1 gene (Latorre et al., 2010). BK is a homotetramer, and each of the α subunits consists of seven transmembrane segments (S0–S6). Segments S0–S4 constitute the voltage sensor domain (VSD) and segments S5–S6 the pore domain (PD). The C-terminal region located on the intracellular side contains two K^+-conductance regulators (RCK1 and RCK2) where the Ca^{2+}-binding sites reside (Yuan et al., 2010; Hite et al., 2017; Tao et al., 2017).

Although our knowledge of ion channel biophysics and pharmacology has increased enormously in recent years, the biophysical properties and pharmacology of different variants of BK that are expressed in organelles, especially in double-membrane organelles, need more detailed studies. However, despite the obvious structural and functional importance of the nucleus in gene expression and regulation, the role of nuclear BK channels (nBK) in intracellular signaling pathways is not fully understood (Li et al., 2014; Selezneva et al., 2021).

To understand the nBK functional importance, we need to comprehend Ca^{2+} storage and signaling in nuclei and how the nuclear envelope (NE) is involved. First of all, the NE consists of two concentric lipid bilayers. The outer nuclear membrane (ONM), which is continuous with the endoplasmic reticulum membrane, and the inner nuclear membrane (INM). Within the NE, InsP3R, which is a Ca^{2+} permeable channel, can be found in both the ONM and the INM (Leite et al., 2003). There is the perinuclear space located between the ONM and the INM, which is a crucial source of Ca^{2+} that can be released into the nucleoplasm not only through InsP3R, but also using of ryanodine receptors (RyR; see **Figure 1B**; Zahradníková and Mészáros, 1998). A critical effect of nuclear Ca^{2+} increase is phosphorylation and activation of cAMP response element-binding protein (CREB), which regulates many genes of different cell types, such as neurons that elicits transcription of genes that promotes neuronal survival (Papadia et al., 2005).

Multiple investigations suggest that both mitoBK and nBK have the same structure as pmBK, and they share biophysical and pharmacological properties (Singh et al., 2012; Balderas et al., 2015; Li and Gao, 2016). Although it has been assumed that the same pharmacology for pmBK applies to BK channels contained in organelle membranes, some examples show unexpected effects of BK blocking agents. For instance, charybdotoxin (ChTX), a high-affinity BK blocker, could not block a BK like-channel characterized in mitochondria (Singh et al., 2012), as similarly reported by Meera et al. (2000) for coexpression of α with the β4 subunit, where β4 renders the BK channel insensitive to ChTX (Meera et al., 2000; Torres et al., 2014).

This review will summarize all the biophysical, pharmacological, and functional information that exists to date on the mitoBK and nBK channels, with a comparative perspective over pmBK features.

MitoBK AND nBK LOCALIZATION IN ORGANELLES AND TISSUES

Mitochondria are crucial for cell survival, and vital cellular processes occur in this organelle. Therefore, understanding the different ion channels interplay in the mitochondrial membranes could be helpful in the modulation of diverse mitochondrial-related molecular mechanisms and thus cellular processes such as the apoptosis or hypoxia response (Wallace, 1999; Kim et al., 2006; Papandreou et al., 2006). Impairment of the mitochondrial membrane potential leads to the release of cytochrome c from the mitochondrial membrane, an essential process for the induction of cell death. Therefore, the study of ion channels in the mitochondrial membranes became an exciting subject at the end of the 20th century (Borecký et al., 1997; Siemen et al., 1999).

Important diseases that include mitochondria failures may well involve the presence of potassium channels. In the search for anti-ischemic drugs, Xu et al. (2002) were the first to find clear electrophysiological evidence aiming to an isoform of the BK channel within the cardiac myocyte inner mitochondrial membrane (IMM) of guinea pig hearts. This channel carried a large portion of the K^+ uniport activity and led to the finding of the ischemic insult-protecting role of the mitoBK. Likewise, the mitoBK channel was also found in rat hearts, specifically in cardiac ventricular myocytes (Ohya et al., 2005).

Using western-blot, immunocytochemistry, and inmuno-gold electron microscopy, Douglas et al. (2006) showed that mitoBK is present in the rat's brain mitochondria. Considering that ischemic-brain injury-related hypoxia has substantial effects on neuron metabolism and survival, it is remarkable that hypoxic conditions activated mitoBK from rat brain astrocytes (Cheng et al., 2008). Similarly, it has been demonstrated that hypoxia activates the BK channels present in mitoplast derived from the human glioma LN-229 cells (Gu et al., 2007), and the

Abbreviations: Aβ, Amyloid β-sheet fibrils; BAX, Bcl-2-associated X; Bcl-XL, B-cell lymphoma-extra-large; BK, Large-conductance calcium- and voltage-activated potassium channel; CaMKII, Calmodulin kinase II; CCO, Cytochrome C oxidase; CGS718, Ethyl2-hydroxy-1-[[(4-methylphenyl)amino]oxo]-6-trifluoromethyl-1H-indole-3-carboxylate; CGS7184, Ethyl 1-[[(4-chlorophenyl)amino]oxo]-2-hydroxy-6-trifluoromethyl-1H-indole-3-carboxylate; ChTX, Charybdotoxin; CREB, cAMP response element-binding protein; CS, Cold storage; diCl-DHAA, 12,14-dichloro dehydroabietic acid; eNOS, Endothelial nitric oxide synthase; EP_3, Perinuclear prostaglandin receptors; ETC, Electron transport chain; H_2O_2, Hydrogen peroxide; I/R, Ischemia and reperfusion injuries; IbTX, Iberiotoxin; IMM, Inner mitochondrial membrane; INM, Inner nuclear membrane; InsP3R, Inositol-1,4,5 trisphosphate (InsP3) receptors; Kv, K+voltage-dependent channels; LPS, Lipopolysaccharides; mdivi-1, Mitochondrial division inhibitor; mitoBK, Mitochondrial BK; mPTP, Mitochondrial permeability transition pore; nBK, Nuclear BK; NE, Nuclear envelope; NF-kB, Nuclear factor kappa B; NPo, Absolute open probability; ONM, Outer nuclear membrane; PD, Pore domain; pmBK, BK channel in the plasma membrane; P_o, Open probability; RCK, K+−conductance regulators; ROS, Reactive oxygen species; RW, Rewarming; RyR, Ryanodine receptors; S0-S6, Transmembrane segments; TLR4, Toll-like receptors 4; TMPD, Tetramethyl-p-phenylenediamine; VSD, Voltage sensor domain; γ, Unitary conductance; ΔΨ, Electrochemical membrane potential.

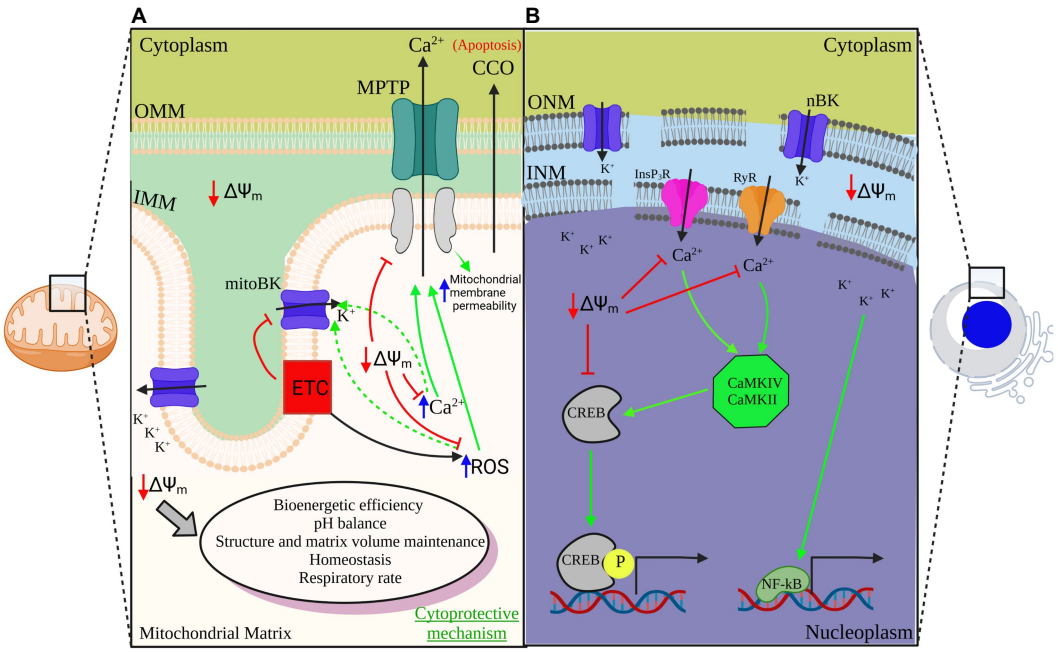

FIGURE 1 | Proposed scheme for the functional role of the BK channel in double-membrane organelles. **(A)** Mitochondria, **(B)** Nucleus. In both organelles, BK plays a fundamental role in maintaining the transmembrane potential and in regulating the movement of the calcium ion between the cytosol and the organelle, and between the lumen and the internal membrane of the same. MitoBK is involved in mitochondrial function, structure, homeostasis, and volume, as well as pH control, bioenergetic efficiency, respiratory rate (through a structural and functional assembly with the chain electron transporter (ETC)), in the closure of the mitochondrial permeability transition pore (MPTP) and with it, indirectly, in the release of cytochrome C oxidase (CCO). Thus, it would ultimately be involved in apoptosis and death cells. nBK is involved in pCREB-dependent gene regulation (in principle regulated by Calmodulin-dependent kinases (CaMKIV and CaMKII)) and NF-kB, mechanisms by which, to date, nBK has been associated with neuronal survival and response inflammatory mediated by macrophages, respectively.

same effect was also found in the mitoplast of liver mitochondria (Cheng et al., 2008). In addition, submitochondrial particles extracted from rat hippocampal neurons were reconstituted into lipid bilayer membranes. Thus, electrophysiological recordings and confocal immunohistochemical images confirmed the presence of the mitoBK channel, including its accessory β4 subunit with an apparent molecular weight of ~26 kDa (Skalska et al., 2009). Recalling here that β4 is present in the plasma membrane of neurons in the brain (Weiger et al., 2000), it was demonstrated, using western blot analysis, that β4 and β2 subunits are present in brain homogenates and mitochondrial fractions (Piwonska et al., 2008). Additionally, the β2 subunit was also identified in human epithelial cell line mitoplasts; however, the idea that it does not form a complex with mitoBK is disputed (Bednarczyk et al., 2013a) since it does not show the time-dependent inactivation that this subunit confers on membrane BK channels (Wallner et al., 1999; Benzinger et al., 2006; Torres et al., 2014).

Later on, more studies confirmed mitoBK presence in cardiomyocytes (Singh et al., 2013; Soltysinska et al., 2014; Frankenreiter et al., 2017) and in brain tissues (Fahanik-Babaei et al., 2011a,b; Singh et al., 2012; Augustynek et al., 2018).

Recently, mitoBK channels were well-described in human glioma cell lines (Gu et al., 2007; Walewska et al., 2018; Gałecka et al., 2021), supporting the data of Siemen et al. (1999) in their first attempt to obtain mitoBK currents from human glioma cells. Further, mitoBK was characterized in other human cell lines from the endothelium, fibroblast, and glioblastoma to detect the expression of different splice variants and the co-assembly with different types of auxiliary β subunits that may affect the complexity of the mitoBK channel gating (Wawrzkiewicz-Jałowiecka et al., 2021). β4 expression with mitoBK has been found in H9c2 cell line derived from heart (Fretwell and Dickenson, 2009), in human astrocytoma cells U-87 MG rat skeletal muscle (Bednarczyk et al., 2013b), and in thalamus and brainstem (Piwonska et al., 2008). On the other hand, the β2 subunit has been reported in the human endothelium EA.hy926 cell line (Bednarczyk et al., 2013a) and in rat brain (Piwonska et al., 2008), while β3 is highly expressed in human fibroblasts, in which β2 and β4 subunits were also found (Kicinska et al., 2016).

Nonmammalian cells have been a point of interest for the search of mitochondrial potassium channels. More precisely, a mitoBK channel-like protein in potato tuber cells was characterized by obtaining similar properties as the pmBK channel with a remarkably exception of the single-channel conductance of about ~600 pS (Koszela-Piotrowska et al., 2009). The functional and pharmacological features of the mitoBK channel were also characterized in the IMM of *Dictyostelium discoideum* a unicellular ameboid protozoon that forms multicellular structures. The mitoBK of this protozoon was characterized using electrophysiological measurements, immunoblotting, and functional measurements of oxygen uptake and Δψ changes (Laskowski et al., 2015).

Like the mitochondria and other membrane-bound organelles, as already mentioned, the nuclear envelope (NE) is also made up of two separated membranes: the inner nuclear membrane (INM), which interacts with the nuclear skeleton, and the outer nuclear membrane (ONM), which is continuous with the endoplasmic reticulum (Fedorenko et al., 2010; Singh et al., 2012). The presence of a potassium channel activated by Ca^{2+} and voltage was reported in the outer nuclear membrane of pancreatic acinar cells, with a single-channel conductance between 180 and 200 pS (Maruyama et al., 1995). Later, the presence of nBK channels was also confirmed in isolated nuclei from brain microvessel endothelial cells (Gobeil et al., 2002).

Further, the nBK channel was found in the nuclear membrane of mouse hippocampal neurons, and immunohistochemical assays clarified that BK is not present in the nucleus of BK-knockout mouse neurons (*KCNMA1* −/−; Li et al., 2014). Single-channel recordings in isolated nuclei from hippocampal neurons further confirmed the presence of nBK channels (Li et al., 2014). The single-channel conductance obtained was similar to that reported for the pmBK channel present in neurons (Salkoff et al., 2006).

Like neuronal pmBK channels, nBKs form complexes with β4 helper subunits (Shruti et al., 2012; Li et al., 2014). More recently, Chen et al. reported the presence of the nBK channel in Ampullae of Lorenzini cells (an electroreceptor organ of cartilaginous fish) from *in situ* assays using confocal microscopy and immunostaining (Chen et al., 2020).

Altogether, the data suggest that the mitoBK and nBK channels have the same mammalian-tissue localization as the pmBK channel (Dworetzky et al., 1994; Knaus et al., 1996; Poulsen et al., 2009; Chen et al., 2010).

BIOPHYSICAL PROPERTIES AND DIFFERENCES TO PMBK

Mitochondrial BK Biophysical Properties

mitoBK single-channel recordings were reported for the first time in mitoplasts from glioma human cell line LN229 mitochondria (Siemen et al., 1999). Since then, patch-clamp experiments from mitoBK have been carried out not only using mitoblasts (Xu et al., 2002; Ohya et al., 2005; Gu et al., 2007; Cheng et al., 2008, 2011; Bednarczyk et al., 2013a,b; Soltysinska et al., 2014; Frankenreiter et al., 2017; Walewska et al., 2018; Balderas et al., 2019) but also lipid bilayers (Skalska et al., 2009; Fahanik-Babaei et al., 2011a,b). Overall, it has been found that mitoBK shares similar behavior to pmBK (see **Table 1**), with a unitary conductance (γ) around 282 ± 23 pS in multiple K^+ conditions, a voltage-dependent open probability (P_o) (Siemen et al., 1999; Bednarczyk et al., 2013b) which shows a leftward shift in the P_o – Voltage curves when increasing Ca^{2+} levels (Xu et al., 2002; Balderas et al., 2019) and sensitivity to negative hydrostatic pressure (Walewska et al., 2018). Interestingly, mitoBK P_o increases under hypoxic conditions (Gu et al., 2007; Cheng et al., 2008). BK localization in mitochondria is a result of VEDEC splice variant from *KCNMA1* gene, which has been described with the aforementioned properties (Singh et al., 2013; Gałecka et al., 2021).

Nuclear BK Biophysical Properties

The first identified calcium- and voltage-activated potassium channel (nBK) in nucleus was characterized in rat pancreatic acinar cells at the single-channel level using the patch-clamp technique (Maruyama et al., 1995). Although there was no pharmacology approaches or microscope imaging, this study was pioneer in the search of BK channels in other intracellular organelles membranes. It has been found that nBK shares similar behavior to pmBK (Singh et al., 2012).

Almost 20 years later, functional nBK channels were described using a set of different techniques, including immunoelectron microscopy and confocal fluorescence. Most importantly, single-channel recordings in isolated nuclei showed a P_o of 0.3 using 5 μM Ca^{2+}, increasing to ~0.8 at 10 uM Ca^{2+}, indicating the presence of a $Ca^{2+}-$activated channel with a $\gamma = 217$ pS (**Table 1**; Li et al., 2014).

Regulation of mito-BK and nBK by Auxiliary β Subunits

Likewise, as in pmBK, the accessory β1 subunit can assemble with the α subunit of mitoBK and nBK (Ohya et al., 2005; Li et al., 2014; Balderas et al., 2020). This subunit modify the pharmacological characteristics and gating of the channel. Recent findings have revealed the presence of mitoBK channels formed by the β1/α complex in mammalian myocyte mitochondria (Ohya et al., 2005; Testai et al., 2017; Balderas et al., 2020). β1 regulates expression and targets mitoBK to the IMM and changes the channel voltage sensitivity (Balderas et al., 2020). These results could explain how is possible to activate mitoBK in the mitochondrial environment ($\Delta\Psi \sim -200$ mV, $[Ca^{2+}]_{mit} \approx 200$ μM). Under these conditions, the β1/α mitoBK conductance–voltage curve is leftward shifted and the channel shows an appreciable P_o (Ohya et al., 2005; Bautista et al., 2009; Balderas et al., 2020). The accessory β1 subunit detected in mitoplast from rat ventricular myocytes interacts with the cytochrome C oxidase (CCO), confirming the mitoBK-β1 complex association with the respiratory electron transport chain in heart mitochondria (Ohya et al., 2005). In addition, the mitoBK-β1 complex was also found in cultured pulmonary artery smooth muscle mitochondria (Loot et al., 2012).

mitoBK was characterized in other human cell lines from the endothelium, fibroblast, and glioblastoma where multiple splice variants were found that co-assemble with different types of auxiliary β subunits that may affect the complexity of the mitoBK channel pharmacology and gating (Wawrzkiewicz-Jałowiecka et al., 2021).

At present, only β4 expression has been reported in nuclear membranes co-localizing with B-type lamin (Li et al., 2014.). However, there are many questions that still need to be answered regarding the detailed mechanism of how accessory subunits are directed and assembled in IMM and ONM, as well as the modulatory effect they exert on mitoBK and nBK. Regarding γ subunits, the association with the mitoBK-α and nBK-α is still to be addressed (González-Cota et al., 2021).

TABLE 1 | Biophysics and Pharmacology of mitoBK and nBK.

Tissue/organism	Methodology	Unitary conductance (pS) and conditions	Biophysical parameters	Pharmacology	References
Potato tuber (mitoBK)	Lipid bilayer	615 ± 12 – gradient cis/trans 50/450 mM K^+	V_r = +34 mV, P_0 = 0.49 (0 Ca^{2+}; 0 mV)/0.84 (300 μM Ca^{2+}; 0 mV)	NS1619 Iberiotoxin	Koszela-Piotrowska et al., 2009
Rat brain (mitoBK)	Lipid bilayer	~565 – gradient cis/trans 200/50 mM K^+	V_r = −30 mV	Iberiotoxin charybdotoxin	Fahanik-Babaei et al., 2011b; Singh et al., 2012
Drosophila melanogaster (mitoBK)	Single channel in mitoplast	382 ± 8 – symmetric 150 mM K^+	N/A	NS1619 Paxilline	Gururaja et al., 2019
Guinea pig cardiomyocytes (mitoBK)	Single channel in mitoplast	307 ± 4.6 – symmetric 150 mM K^+	N/A	NS1619 NS11021 Paxilline	Xu et al., 2002; Aon et al., 2010
Rat cardiomyocites (mitoBK)	Single channel in mitoplast	303 ± 19 – symmetric 140 mM K^+	$V_{1/2}$ = −55 mV (12 μM Ca^{2+})	diCl-DHAA paxilline	Sakamoto et al., 2008; Balderas et al., 2019
Rat astrocytes (mitoBK)	Single channel in mitoplast	296 ± 18 – symmetric 150 mM K^+	N/A	CGS7181 CGS7184 paxilline charybdotoxin	Cheng et al., 2008; Augustynek et al., 2018
Human glioma (mitoBK)	Single channel in mitoplast	295 ± 18 – symmetric 150 mM K^+	V_r = +70 mV (150 mM Na^+), $EC_{50}(Ca^{2+})$ = 900 nM (+60 mV)/6.9 μM (−20 mV)	Charybdotoxin	Siemen et al., 1999
Rat non-neoplastic astrocytes (mitoBK)	Single channel in mitoplast	~290 – symmetric 140 mM K^+	N/A	Iberiotoxin	Cheng et al., 2011
HEK293T (DEC splice variant; mitoBK)	Single channel in mitoplast	290 ± 3 – symmetric 150 mM K^+	$V_{1/2}$ = +19.2 mV (100 μM Ca^{2+}), t_0 = 10.2 ms (+60 mV)	Paxilline	Gałecka et al., 2021
Human glioma (mitoBK)	Single channel in mitoplast	276 ± 9 – symmetric 150 mM K^+	V_r = +9.3 ± 2.4 mV; O_2: 21.1 ± 1.2 nmol/ml	Charybdotoxin	Gu et al., 2007
Rat ventricular myocytes (mitoBK)	Single channel in mitoplast	~270 – symmetric 140 mM K^+	N/A	17B-estradiol paxilline	Ohya et al., 2005
Human endothelium (mitoBK)	Single channel in mitoplast	270 ± 10 – symmetric 150 mM K^+	t_0 = 0 ms (−60 mV)/~70 ms (+60 mV)	NS1619 NS11021 paxilline iberiotoxin	Bednarczyk et al., 2013a
Rat brain (mitoBK)	Lipid bilayer	265 ± 5 – gradient cis/trans 50/450 mM K^+	V_r = +50 mV, P_o (+70 mV) = 0.5 (0 Ca^{2+})/0.77 (300 μM Ca^{2+})	NS1619 iberiotoxin charybdotoxin	Skalska et al., 2009
Human glioma (mitoBK)	Single channel in mitoplast	262 ± 12 – symmetric 150 mM K^+	N/A	Charybdotoxin	Walewska et al., 2018
Dictyostelium discoideum (mitoBK)	Lipid bilayer	258 ± 12 – gradient cis/trans 50/150 mM K^+	V_r = +27.6 ± 0.5 mV, P_0 = 0.14 (1 μM Ca^{2+}; 0 mV)/0.48 (100 μM Ca^{2+}; 0 mV)	NS1619 NS11021 paxilline iberiotoxin	Laskowski et al., 2015
Rat hippocampal neurons (nBK)	Single channel in nuclear envelope	217 pS – symmetric 135 mM K^+	V_r = 0 mV, P_0 = 0.3 (5 μM Ca^{2+})/0.78 (10 μM Ca^{2+})	Paxilline	Li et al., 2014
Rat brain (mitoBK)	Lipid bilayer	~211 – gradient cis/trans 200/50 mM K^+	V_r = −30 mV	Iberiotoxin	Fahanik-Babaei et al., 2011a; Singh et al., 2012
Rat pancreas (nBK)	Single channel in nuclear envelope	200 ± 25 pS – symmetric 148 mM K^+	V_r = 0 mV	N/A	Maruyama et al., 1995
Mice cardiomyocytes (mitoBK)	Single channel in mitoplast	~190 – internal/external = 130:10 mM K^+	t_0 = 9.23 ms, P_0 = 0.79 (+80 mV)	NS11021 Paxilline	Soltysinska et al., 2014
Mice cardiomyocytes (mitoBK)	Single channel in mitoplast	~145 – symmetric 150 mM K^+	P_0 = 0.28 (1 μM Ca^{2+}); 0.54 (100 μM Ca^{2+})	NS11021 Paxilline	Frankenreiter et al., 2017

PHARMACOLOGICAL PROPERTIES

Mitochondrial BK Pharmacological Properties

The basic pharmacology properties of mitochondrial potassium channels like mitoK_{ATP}, mitoBK, and mitoKv1.3 are similar to their equivalents in plasma membrane from different cell types (Szewczyk et al., 2006; Laskowski et al., 2016). Therefore, activators and inhibitors previously described for the pmBK channel can exert the same effect on mitoBK (O'Rourke, 2007; Szewczyk et al., 2010). Different reports indicate that nonspecific interactions of potassium channel modulators may occur, indicating that these compounds may influence cell and mitochondrial function regardless of their main targets (Szewczyk et al., 2010; Laskowski et al., 2016; Augustynek et al., 2018).

CGS7181(ethyl2-hydroxy-1-[[(4-methylphenyl)amino]oxo]-6-trifluoromethyl-1H-indole carboxylate) is an indole carboxylate derivative that, just as its analog CGS7184 (ethyl 1-[[(4-chlorophenyl)amino]oxo]-2-hydroxy-6-trifluoromethyl-1H-indole-3-carboxylate), activates mitoBK in single-channel

recordings from astrocytoma (Augustynek et al., 2018). Using the inside-out patch-clamp configuration, they report that the open probability (NPo) increases from 0.09 in the control to 0.55 in the presence of 1 μM of CGS7181. This activity was subsequently inhibited by adding 10 μM of paxilline to the bath (mitochondrial matrix; Augustynek et al., 2018). Augustynek et al. (2018) proposed that activation of mitoBK by CGS7184 induces an influx of potassium ions into the negatively charged mitochondrial matrix and promotes a light uncoupling of mitochondria. This uncoupling stimulates the activity of the mitochondrial respiratory chain to restore the potential of the mitochondrial membrane by pumping protons from the matrix into the mitochondrial intermembrane space (Augustynek et al., 2018). The agonistic effect observed in the presence of CGS7184 is dependent on potassium and charybdotoxin, indicating that the target of this compound is mitoBK (Augustynek et al., 2018).

Sakamoto demonstrated that 12,14-dichloro dehydroabietic acid (diCl-DHAA) activates mitoBK (Sakamoto et al., 2008) similarly as it activates the pmBK channel (Ohya et al., 2005). Additionally, adding 3 μM of paxilline eliminates channel opening events, allowing the authors to confirm that diCl-DHAA activates the mitoBK channel, likewise the pmBK channel (Sakamoto et al., 2008). Finally, they evaluated the protective effects of diCl-DHAA against ischemic cell death in cardiomyocytes by using the simulated ischemia procedure. diCl-DHAA has protective effects on cardiac myocytes against ischemic injury through the opening of mitoBK channels, supporting the idea that the opening of mitoBK is a novel way to protect cardiac myocytes from ischemic and reperfusion injury (Sakamoto et al., 2008).

Ohya et al. (2005) showed that 17β-estradiol could increase the mitoBK channel P_o, activation that is inhibited by paxilline (Ohya et al., 2005). Importantly, in the presence of 17β-estradiol, cell death decreased significantly during simulated ischemia, and that this cardioprotective effect was eliminated by 3 μM paxilline (Ohya et al., 2005). They concluded that this cardioprotective effect is due to the activation of mitoBK by 17β-estradiol, and since 17β-estradiol activates BK only in the presence of the β1 subunit (Valverde et al., 1999; Granados et al., 2019), this result confirms the presence of mitoBK-β1 in rat ventricular myocytes.

The benzimidazole derivatives BK activator family includes NS1619, NS004, NS1604, NS11021, and NS1643 that can activate mitoBK (Skalska et al., 2009; Szewczyk et al., 2010). NS1619 activates mitoBK at micromolar concentrations (Szewczyk et al., 2006). Moreover, the activation of mitoBK by NS1619 has a cytoprotective effect in guinea pig heart before simulated ischemia; this effect was antagonized by paxilline (Xu et al., 2002; Stowe et al., 2006; Singh et al., 2013). MitoBK activators have been reported to protect the heart against ischemic injury (Shintani et al., 2004). Furthermore, like the effect of mitoK$_{ATP}$ activation, mitoBK opening has been implicated in preconditioning. For example, preconditioning of hearts with mitoBK activators such as NS1619 or NS11021 reduced myocardial infarction and this beneficial effect could be antagonized by co-administration with paxilline (Bentzen et al., 2009). The activation of mitoBK by NS1619 leads to cytoprotection of cardiomyocytes during ischemia/reperfusion or treatment with ouabain (Augustynek et al., 2018). However, it should considered that NS1619, like NS004, may present non-mitoBK-dependent effects in the mitochondria (Debska et al., 2003; Heinen et al., 2007b).

NS11021 exerts other protective effects by activating mitoBK channels, which are abolished in the presence of paxilline. For example, nanomolar concentrations of NS11021 improve the bioenergetic performance of the mitochondria of the heart (Aon et al., 2010; Testai et al., 2014). NS11021 also protects against ischemic injury when applied prior to ischemia or when applied immediately after reperfusion. These findings support the idea that ischemia and reperfusion-induced tissue damage can be reduced by pharmacological activation of cardiac mitoBK channels (Bentzen et al., 2009).

The following compounds are mitoBK inhibitors: charybdotoxin (Gu et al., 2007; Skalska et al., 2009; Augustynek et al., 2018), iberiotoxin (Cheng et al., 2011), and paxilline (Xu et al., 2002; Ohya et al., 2005; Sakamoto et al., 2008; Augustynek et al., 2018; Balderas et al., 2020) y Ba^{2+} (Xu et al., 2002). These compounds have been characterized previously in the pmBK having similar effects to those found in mitoBK (Szabo and Zoratti, 2014). MitoBK is inhibited by the blockers charybdotoxin, iberiotoxin, and paxilline at concentrations in the nanomolar range (O'Rourke, 2007; Singh et al., 2012). Adding 100 nM of paxilline to the bath in the inside-out configuration decreases the P_o of the mitoBK and increases the mean close time with no effects on the mean open time. These results suggest that paxilline decreases the probability of opening by stabilizing the closed state of the channel (Balderas et al., 2020). We note here that the Lingle group who proposed that paxilline binding is state-dependent binding preferentially to the closed state of the pmBK (Zhou and Lingle, 2014).

Recently, Kravenska et al. (2020) reported in human astrocytoma cell mitoplasts that different forms of Aβ (a self-aggregating peptide) produced by cleavage of a transmembrane glycoprotein (the amyloid precursor protein involved in Alzheimer's disease), including monomers, oligomers, and fibrils, inhibit mitoBK in a concentration-dependent manner. Five μM of Aβ fibrils, oligomers or monomers produced 80, 70, and 50% inhibition, respectively. All forms of Aβ inhibited mitoBK channel activity when applied to both sides of the membrane, indicating an indirect effect on the channel (Kravenska et al., 2020).

Nuclear BK Pharmacological Properties

Paxilline- and iberiotoxin-specific pmBK channel inhibitors block nBK. nBK is activated by NS1619, a specific activator of pmBK. Therefore, nBK channels share similar pharmacological properties with the pmBK and mitoBK channels, targeting the same compounds (Gobeil et al., 2002; Singh et al., 2012; Li et al., 2014; Du et al., 2020).

Experiments in isolated nuclei of brain endothelial cells using NS1619 as an activator of nBK and iberiotoxin as a blocker showed that nBK is coupled to the activity of perinuclear prostaglandin receptors (EP3). Iberiotoxin abolished K^+-dependent membrane potential changes and the expression of eNOS transcription induced by the activation of agonists of

the prostanoid EP_3-receptor, M&B 28767, while NS1619 produced Ca^{2+} transients and alterations in the perinuclear membrane potential (Gobeil et al., 2002; Singh et al., 2012).

Li et al. (2014) showed that nBKs in the nuclear envelope of hippocampal cells are functional and sensitive to pharmacological inhibition by paxilline. This compound's blockage of nBK causes transient increases in Ca^{2+} and depolarization of the nucleoplasm relative to the perinuclear lumen, thus affecting the transcription of calcium-dependent genes, neuronal activity, and dendritic arborization in these neurons (Li et al., 2014).

On the other hand, treatment with paxilline, both in isolated RAW264.7 macrophage nuclei and whole cells, resulted in a dose-dependent increase in the phosphorylation of CREB in the nucleus (Selezneva et al., 2021). We recall here that treatment of the nucleus with high concentrations of Ca^{2+} also causes CREB phosphorylation. These results do not exclude a role for the BK channels located in other cell membranes, due to the high membrane permeability of paxilline, which would allow it to block the BK channels of both the plasma membrane and intracellular organelles (Selezneva et al., 2021).

FUNCTION

Mitochondrial BK Function

The functions of the mitoBK channel can be easily studied using isolated mitochondria. However, we cannot apply these studies' results directly to intact cells (Li and Gao, 2016). It has been hypothesized that the activity of this channel is essential for mitochondrial function and homeostasis. mitoBK is expressed in IMM, in which they could regulate ion and protein movement involved cell apoptosis and the electron transport chain (ETC), respectively (Szabo and Zoratti, 2014; Li and Gao, 2016). Most studies have mainly focused on the cytoprotective effect on cardiac and neuro ischemia of mitoBK channels. Still, they have also shown significant evidence regarding mitochondrial structure and function, reactive oxygen species (ROS) regulation, mitochondrial Ca^{2+} retention capacity, and permeability transition pore (mPTP) activation in cellular respiration and cancer as well (see **Figure 1A**).

MitoBK Channels in Cardioprotection

So far, the physiological role of mitoBK has been reported mainly by pharmacology or using genetic models (Szabo and Zoratti, 2014). Most studies have primarily focused on the cytoprotective effect on cardiac ischemia and reperfusion (I/R) injuries, to which mitoBK has been associated after the pioneering work of Xu et al. (2002). Using pharmacological agents to open and block the channel, mitoBK shows to be involved in such cardioprotection (Wang et al., 2004; Stowe et al., 2006; Bentzen et al., 2009, 2010; Borchert et al., 2013; Singh et al., 2013; Testai et al., 2013; Schmitt et al., 2014).

The cardioprotective effect mediated by the mitoBK channel is attributed mainly to (a) an increase in K^+ in the mitochondrial matrix, (b) the retention of Ca^{2+}, (c) a decrease in ROS, and (d) closure of the mPTP (Hermann et al., 2015). The flux of K^+ from the cytosol to the negatively charged mitochondrial matrix is caused by the opening of mitoBK channels, which depolarizes the organelle (Szewczyk et al., 2006). The opening of mitoBK reduces the influx of Ca^{2+}, decreasing the Ca^{2+} overload in the mitochondria (Xu et al., 2002; Du et al., 2020). Therefore, the functional effect of mitoBK channel activators is to reduce ROS production and Ca^{2+} overload, improving homeostasis and mitochondrial redox status after I/R as seen in isolated guinea pig hearts (Heinen et al., 2007a,b; Bentzen et al., 2009).

Due to nonspecific effects of drugs, the role of mitoBK in protection against I/R injury has been questioned, invoking biochemical and molecular reasons (see Gaspar et al., 2009; Szewczyk et al., 2009; Wojtovich et al., 2011, 2013). Conclusive evidence for the role of mitoBK in cardioprotection comes from studies using BK knockout mouse models (Kcnma1 −/−). The hearts of these mice are not protected from ischemic injury under treatment with NS1619 or NS11021. This lack of protection is revealed by measurements of cardiac function and infarct size in isolated perfused hearts (Singh et al., 2013; Wojtovich et al., 2013; Soltysinska et al., 2014). These experiments demonstrate that BK activator-mediated cardioprotection requires KCNMA1 expression and that mitoBK activation protects cardiomyocytes from ischemia and reperfusion injury (Singh et al., 2013; Goswami et al., 2019). Nonetheless, in vascular smooth muscle myocytes, the evidence suggests that mitoBK channels are not involved in protection against I/R injury (Frankenreiter et al., 2017).

MitoBK Channel in Neuroprotection

Strong evidence shows that mitoBK channels located in IMM in neurons are associated with neuroprotective effects (Kulawiak and Szewczyk, 2012; Singh et al., 2013; Wojtovich et al., 2013; Soltysinska et al., 2014; Li and Gao, 2016; Krabbendam et al., 2018; Du et al., 2020). Kulawiak et al. (2008) demonstrated that opening of mitoBK located in IMM of rat brain, stimulated by CGS7184 and NS1619, inhibits hydrogen peroxide production by 20%. This effect is sensitive to BK channel blockers iberiotoxin and charybdotoxin. These results suggest that the opening of mitoBK inhibits ROS, promoting neuronal survival and neuroprotection (Kulawiak et al., 2008). However, Gaspar et al. (2009) proposed that the protective effect of NS1619 may not be mediated by mitoBK in every system. They studied primary rat cortical neurons and found that preconditioning with NS1619 caused mitochondrial depolarization, an effect that was displayed even with preincubation with paxilline (Gaspar et al., 2009). A possible explanation for this negative result could be that paxilline takes longer to diffuse through the plasma membrane and reach mitoBK in IMM (Balderas et al., 2015).

Subsequently, NS11021 was used to evaluate its cytoprotective effect on primary cortical neurons of rats with glutamate-induced excitotoxicity. On the one hand, due to the suppression of glutamate excitotoxicity, attenuation of oxidative stress, and preservation of mitochondrial function, mitoBK-dependent neuroprotection is induced (Borchert et al., 2011). On the other hand, the mitochondrial division inhibitor, mdivi-1,

exhibited protective effects in ischemic injury by regulating the activation of mitoBK in mitochondria of cardiac neurons due to an increase in BK channel expression levels and attenuation of oxidative stress, mitochondrial dysfunction, and neuronal apoptosis (Liu et al., 2012). This neuroprotective effect is associated with the increase of mitochondrial Ca^{2+} and the decrease in ROS production mediated by mitoBK (Kulawiak and Szewczyk, 2012; Krabbendam et al., 2018; Du et al., 2020).

MitoBK Channel in Mitochondrial Structure and Function

Opening of mitoBK channels has been found to regulate the respiratory rate, mitochondrial depolarization, matrix volume, and ROS production (Heinen et al., 2007a,b; Kulawiak et al., 2008; Hermann et al., 2015).

In muscle mitochondria from *Drosophila* mutants slo1- / - severe defects were found in terms of the mitochondrial ultrastructure, aberrations in the arrangement of ridges, an increased size (swollen) of the organelle, and loss of continuity of IMM compared to wild type cells expressing mitoBK (Gaspar et al., 2009). Meanwhile, Du et al. (2020) analyzed mitochondria of HEK and PC12 cells transfected with mutant BK channels (BKG354S, mutation that affects the selectivity filter). This mutation caused a selective loss of BK channels in the mitochondrial membrane and the loss of mitochondrial content, ranging from the loss of voltage-gated anion channel (VDAC) proteins to a reduction in every component mitochondrial oxidative phosphorylation (OXPHOS). This led to depolarized and dysfunctional mitochondria and the loss of the cytoprotective effect due to the activation of mitoBK (Du et al., 2020). Therefore, the mitoBK channel plays a crucial role in maintaining mitochondrial structure, function, and content.

That said, it has been found that mitoBK present in IMM contributes to the regulation of volume of the mitochondrial matrix, influences uptake of K^+, mitochondrial transmembrane potential, pH balance, Ca^{2+} transportation, ROS production, mitochondrial dynamics in general and it has also been proposed to participate in increasing bioenergetic efficiency (Aon et al., 2010; Leanza et al., 2019). These may be considered as the mechanisms proposed for the cytoprotection above (Testai et al., 2015; Wawrzkiewicz-Jałowiecka et al., 2020).

MitoBK Channel and ROS Regulation

The cardio- and neuroprotection conferred by mitoBK activators appears to be associated with the modulation of the rate of mitochondrial reactive oxygen species (ROS) generation in brain and heart cells (Andrukhiv et al., 2006; Facundo et al., 2006; Heinen et al., 2007a; Kulawiak et al., 2008; Krabbendam et al., 2018; Kshatri et al., 2018; Gururaja et al., 2019). The conclusive evidence of the key role of mitoBK channels in ROS generation comes from the use of genetic models, which demonstrated that the absence of BK channels increases ROS production. However, Soltysinska et al. (2014) reported that the knockout of mitoBK channels increased postanoxic ROS production in ventricular mitochondrial cells. This result strongly suggests that mitoBK channels regulate the production of ROS, as well as the oxidative state in hypoxia and reoxygenation of mitochondria. Moreover, Gururaja et al. (2019) found that in *Drosophila* mitoplasts that genetically blocking mitoBK channels increases ROS production, the consumption of O_2 and the respiratory rate (Gururaja et al., 2019).

There is no consensus in the literature regarding the effect of mitoBK in the ROS production. Several reports demonstrate that MitoBK activation after I/R injury causes a reduction in ROS levels. ROS production increases when channel blockers are applied (Szewczyk et al., 2009; Cordeiro et al., 2015; Goswami et al., 2019). On the contrary, the activation of mitoBK in isolated and I/R injury-induced ventricular myocytes with NS11021, caused an increase in ROS levels. Addition of antioxidants, which decrease the open probability of mitoBK, abolished the increase in ROS production (Borchert et al., 2013). This increase in ROS production after mitoBK activation was also observed in a liver cancer cell line (Booth et al., 2016).

Ambivalence in responses after mitoBK activation could be related to some coupling between the channel and ROS generation sites. In the case that mitoBK is coupled to the mitochondrial complex I (reverse electron flow), the production of ROS should decrease upon mitoBK activation. However, if the channel is coupled to the mitochondrial complex III (direct electron flow), activation of mitoBK should lead to an increase in ROS (Krabbendam et al., 2018). In this regard, Stowe et al. (2006) showed in isolated mitochondria from cardiac cells that the succinate and rotenone-dependent H_2O_2 production that blocked reverse electron flow increased slightly after the activation of the mitoBK channel. On the other hand, Heinen et al. (2007a,b) demonstrated that in the absence of rotenone, under substrate conditions that allow reverse electron flow, mitoBK activation reduces H_2O_2 production by 73% by accelerating forward electron flow.

We note here that hemin, a by-product of hemoglobin with oxidative properties, can inhibit the electrical activity of BK channels. Therefore, the mitoBK channel can be considered a redox sensor. (Augustynek et al., 2014). Moreover, mitochondria of ventricular muscle fibers lacking mitoBK channels (by knockout) showed an increase in the production of postanoxic ROS, indicating that these channels regulate the oxidative state in hypoxia and reoxygenation (Soltysinska et al., 2014).

MitoBK Channel and Mitochondrial Ca^{2+} Retention Capacity and mPTP Activation

MitoBK channel regulation of mitochondrial Ca^{2+} retention capacity could be observed pharmacologically activating the channel with NS1619, increasing the number of Ca^{2+} pulses necessary to cause a massive release of Ca^{2+} from the mitochondria (Singh et al., 2013). Ca^{2+} retention capacity in mitochondria is closely related to mPTP activation, which mediates Ca^{2+} release from mitochondria to the cytosol (Singh et al., 2013). In fact, in a study with rat liver, mitoplasts, and astrocytes, hypoxia inhibited mPTP but substantially increased mitoBK activity, with an increase in Ca^{2+} retention capacity, which was reduced using iberiotoxin (Cheng et al., 2008). This finding

may suggest a functional link between mitoBK and mPTP, where the reduction of the activity of the mitoBK channel by mitochondrial substrates can support the activation of mPTP, leading to cell death by apoptosis (Laskowski et al., 2016).

Possibly by the opening of mPTP, apoptosis results from the complex interaction between Ca^{2+} and ROS. The activation of mitoBK is involved in both processes, linking this channel to a delay in the formation and/or closure of mPTP (Goswami et al., 2019). In single-channel recordings in rat astrocyte mitoplasts and hepatic mitochondria, inhibition of mitoBK channels by the pro-apoptotic protein BAX (B-cell lymphoma (Blc) -2-associated X) was observed, which in turn activated mPTP and induced cytochrome C release (an effect like that obtained using iberiotoxin (Cheng et al., 2008)). Conversely, BCL-Xl (an anti-apoptotic protein) inhibited the impact of BAX on mitoBK and mPTP blockade. mitoBK channel is related to apoptotic mechanisms mediated by BAX, which exerts its pro-apoptotic effect by inhibiting mitoBK and thus promotes the opening of mPTP (Cheng et al., 2008, 2011).

MitoBK in Cellular Respiration

In the mitochondria of the human glioblastoma cell line U-87 MG, the substrates of the ETC (NADH, succinate, and malate or glutamate) and artificial donors of electrons (tetramethyl-p-phenylenediamine TMPD/ascorbate) inhibited the mitoBK channel (Bednarczyk et al., 2013b). These results suggest that the mitoBK channel is regulated by the cytochrome C oxidase and that a redox signal is "transferred" from ETC to mitoBK through CCO (Ohya et al., 2005). Together, these observations suggest a structural and functional coupling of the respiratory chain and mitoBK channels, although the underlying molecular mechanisms are still unknown (Laskowski et al., 2016).

Cytoprotection induced by mitoBK activators may also be mediated by inhibiting the mitochondrial respiratory chain (Kicinska and Szewczyk, 2004). Activating the mitoBK channel sing NS1619 in IMM of isolated rat brain mitochondria inhibited ROS production of the respiratory chain using the complex I (Kulawiak et al., 2008). In cultures of hippocampal sections exposed to glutamate, preincubation with NS1619 showed an increase in basal respiration (Piwońska et al., 2016). Activating cardiac mitoBK channels produced an improvement in mitochondrial respiration due to a decrease in state 4 respiration (characterized as a state without any ATP usage/production), while state 3 of respiration (described as a state with saturating ATP usage/production) was unchanged (Aon et al., 2010). These findings suggest a probable mitoBK-dependent mechanism for both cardiac and neuronal cytoprotection (Testai et al., 2015; Wawrzkiewicz-Jałowiecka et al., 2020).

MitoBK and Cancer

To date, a possible role for mitoBK in cancer development has not been reported; despite that, it has been related to the survival and motility of glioma cells after irradiation (Steinle et al., 2011). Irradiation and hypoxia (Gu et al., 2014) have been found to increase the P_o of mitoBK, which in turn activates Calmodulin kinase II (CaMKII), leading to increased migration of glioblastoma cells (Steinle et al., 2011; Peruzzo et al., 2016), as well as resistance to hypoxic conditions (Gu et al., 2014). MitoBK in gliomas may also regulate the respiratory chain and confer cytoprotection, which may be one reason that makes this type of cancer incurable (Wawrzkiewicz-Jałowiecka et al., 2020).

MitoBK and Kidney Transplantation

Shrum et al. (2019) demonstrated that mitoBK channels might represent a therapeutic target to prevent cold storage (CS) preservation and rewarming (RW)-induced kidney injury that is very common in kidneys routinely subjected to transplant. To do this, they added NS11021 to the CS solution and evaluated the effect on normal rat kidney proximal tubular epithelial cells. The addition of this activator of mitoBK prevented the deterioration induced by CS + RW in the uptake of K^+ mediated by mitoBK, as well as a reduction in cell death and mitochondrial damage. In addition, they observed mitigation in respiratory dysfunction, depolarization, and superoxide production (Shrum et al., 2019).

Nuclear BK Function

Even though the presence of BK channels has been reported in the NE of many cell types such as pancreatic cells, brain endothelial cells and macrophages (Maruyama et al., 1995; Gobeil et al., 2002; Selezneva et al., 2021), little is known about its functional role.

nBK and Nucleoplasmic Ca^{2+} Signaling

In pancreatic acinar cell nuclei, nBK channels only localize in ONM, and their activation is sensitive to Ca^{2+} lumen levels (Maruyama et al., 1995). Whether nBK can regulate the nuclear transmembrane potential was proven in mice hippocampal neurons using a potentiometric probe. This experiment showed that the perinuclear lumen got more negative when nBK was blocked by paxilline. Usage of paxilline also indicated an increase in nuclear Ca^{2+} through RyR, mainly due to intracellular BK inhibition without pmBK being involved (Li et al., 2014). We recall here that RyR is sensitive to changes in nuclear transmembrane potential (Zahradníková and Mészáros, 1998). This increase in nuclear Ca^{2+} due to nBK inhibition showed to activate CREB through phosphorylation in a nuclear Ca^{2+}/CaMKIV-dependent manner, which also causes changes in neuronal dendritic arborization (Li et al., 2014).

Not only nBK but also pmBK are found in macrophages from the nervous system (microglia). These channels are involved in pro-inflammatory mechanisms induced by Toll-like receptors 4 (TLR4) activated by lipopolysaccharides (LPS). pmBK is activated through TLR4, which induces translocation of NF-kB (nuclear factor kappa B) to the nucleus, where it prompts gene expression regarding cytokine production. Treatment with paxilline at different times after LPS application showed that after 6 h, paxilline did not affect cytokine production, indicating the existence of BK modulation on gene expression NF-kB-independent. This result becomes clear by considering that the nBK expression

is induced by LPS long-lasting activation on TLR4 (Yang et al., 2019). Another signaling mechanism in macrophages regarding nBK was described using the RAW264.7 cell line. The blockage of nBK using paxilline in preparations of the cell line and isolated nuclei showed an increase in CREB phosphorylation due to CaMKII Calmodulin kinase II) and CaMIV (Calmodulin kinase IV) activity (see **Figure 1B**; Selezneva et al., 2021). It is important to note that CREB is related to many roles for macrophages, particularly preventing apoptosis (Park et al., 2005).

DISCUSSION AND CONCLUDING REMARKS

Compared to the vast information available for pmBK, one may get the impression that there is a lack of evidence for mitoBK and nBK. Nonetheless, there has been an increase in studies regarding their biophysical and pharmacological properties during recent years (Ohya et al., 2005; Li et al., 2014) and how these studies relate to their functional role in different cell types (Gobeil et al., 2002; Selezneva et al., 2021; Wawrzkiewicz-Jałowiecka et al., 2021).

mitoBK is sensitive to multiple stimuli regarding mitochondrial function (like Ca^{2+}, membrane potential and O_2). It also regulates ETC, ROS production, and apoptosis (Siemen et al., 1999; Heinen et al., 2007a,b; Cheng et al., 2008; Kulawiak et al., 2008; Hermann et al., 2015). On the other hand, nBK plays a role in nuclear Ca^{2+} signaling and induction of gene expression under the effect of different drugs (Yang et al., 2019; Selezneva et al., 2021). However, there is a lack of research regarding nBK biophysical properties and how these can determine the underlying mechanisms (Li et al., 2014).

Both in nucleus and mitochondria membranes K^+ flow is essential to maintain ionic homeostasis and hence a myriad of cell functions. The electrochemical driving force for ion movement across membranes varies in different intracellular organelles. In case of the nucleus, K^+ concentration is higher than in the cytoplasm, while in mitochondria it is lower, which causes a large influx of K^+ toward the perinuclear space and into the mitochondrial matrix, respectively. Even though other potassium channels are expressed in both the mitochondrial and nuclear membranes, BK channels are high conductance, where a single BK channel can transport up to 10^8 ions per second, generating a significant change in K^+ flux and, therefore, changes in membrane potential in the different organelles (Singh et al., 2012). Thus, mitoBKs as well as the other mitochondrial K^+ channels participate in the mitochondrial K^+ cycle, which consists in a balance between the electrophoretic uptake of K^+ in the mitochondrial matrix and the diffusive leakage of this ion, mediated by the K^+/H^+ exchanger (Garlid and Paucek, 2003; Szabo et al., 2012; Schulz and Di Lisa, 2016).

As we mentioned the pmBK channels regulate membrane potential, ionic homeostasis, calcium signaling, and cell volume (Latorre et al., 2017). Functions that are also reported for mitoBK and nBK in the mitochondria and nucleus, respectively. For this reason, it would be expected that the pharmacological or genetic modulation of these channels would serve as therapeutic targets. Pharmacological and genetic activation of mitoBK results in cellular and organic protection against I/R injury, giving this channel a promising therapeutic approach as a potential target in the treatment of cardiovascular and neurodegenerative diseases, as well as a potential drug target in organ transplant and cancer medicine (Singh et al., 2012; Laskowski et al., 2016; Leanza et al., 2019). Conversely, nBK represents a new strategy to develop effective therapies in neurodegenerative diseases such as Alzheimer and autism (Li et al., 2014). However, despite the obvious structural and functional importance of the nucleus, nuclear ion channels, their characteristics, and potential therapeutic targets remain largely unknown.

It is unfortunate that exclusive modulators of mitoBK have not yet been reported and the low selectivity and pleiotropic effects of its agonists have hindered the development of a treatment that exclusively involves the activation of mitoBK (Gururaja et al., 2019). The molecular identification of the regulatory and pore-forming subunits of mitoBK channels would provide more possibilities for the development of therapeutic strategies based on the selective modulation of mitoBK in various tissues (Wrzosek et al., 2020).

Overall, the study of BK role in double-membrane organelles such as mitochondria and nucleus is in the need of a more detailed research regarding the differences between organelle BK channels and pmBK concerning their biophysics and pharmacological properties. This knowledge can determine the still unknown molecular mechanisms involving their functional role in their respective organelles and how they can work as possible targets in different pathological conditions.

AUTHOR CONTRIBUTIONS

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

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Calcium-Dependent Ion Channels and the Regulation of Arteriolar Myogenic Tone

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Arterioles in the peripheral microcirculation regulate blood flow to and within tissues and organs, control capillary blood pressure and microvascular fluid exchange, govern peripheral vascular resistance, and contribute to the regulation of blood pressure. These important microvessels display pressure-dependent myogenic tone, the steady state level of contractile activity of vascular smooth muscle cells (VSMCs) that sets resting arteriolar internal diameter such that arterioles can both dilate and constrict to meet the blood flow and pressure needs of the tissues and organs that they perfuse. This perspective will focus on the Ca^{2+}-dependent ion channels in the plasma and endoplasmic reticulum membranes of arteriolar VSMCs and endothelial cells (ECs) that regulate arteriolar tone. In VSMCs, Ca^{2+}-dependent negative feedback regulation of myogenic tone is mediated by Ca^{2+}-activated K^+ (BK_{Ca}) channels and also Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels (VGCC). Transient receptor potential subfamily M, member 4 channels (TRPM4); Ca^{2+}-activated Cl^- channels (CaCCs; TMEM16A/ANO1), Ca^{2+}-dependent inhibition of voltage-gated K^+ (K_V) and ATP-sensitive K^+ (K_{ATP}) channels; and Ca^{2+}-induced-Ca^{2+} release through inositol 1,4,5-trisphosphate receptors (IP_3Rs) participate in Ca^{2+}-dependent positive-feedback regulation of myogenic tone. Calcium release from VSMC ryanodine receptors (RyRs) provide negative-feedback through Ca^{2+}-spark-mediated control of BK_{Ca} channel activity, or positive-feedback regulation in cooperation with IP_3Rs or CaCCs. In some arterioles, VSMC RyRs are silent. In ECs, transient receptor potential vanilloid subfamily, member 4 (TRPV4) channels produce Ca^{2+} sparklets that activate IP_3Rs and intermediate and small conductance Ca^{2+} activated K^+ (IK_{Ca} and sK_{Ca}) channels causing membrane hyperpolarization that is conducted to overlying VSMCs producing endothelium-dependent hyperpolarization and vasodilation. Endothelial IP_3Rs produce Ca^{2+} pulsars, Ca^{2+} wavelets, Ca^{2+} waves and increased global Ca^{2+} levels activating EC sK_{Ca} and IK_{Ca} channels and causing Ca^{2+}-dependent production of endothelial vasodilator autacoids such as NO, prostaglandin I_2 and epoxides of arachidonic acid that mediate negative-feedback regulation of myogenic tone. Thus, Ca^{2+}-dependent ion channels importantly contribute to many aspects of the regulation of myogenic tone in arterioles in the microcirculation.

Keywords: ion channels, calcium ions, arterioles, microcirculation, vascular smooth muscle, endothelial cells

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INTRODUCTION

Arterioles are prominent resistance vessels that regulate blood flow to and within tissues and organs; determine capillary blood pressure and fluid exchange in the microcirculation; and contribute to the regulation of systemic blood pressure (Renkin, 1984). A defining characteristic of arterioles is pressure-dependent myogenic tone, the steady state vascular smooth muscle cell (VSMC) contractile activity that is induced and maintained by pressure-dependent mechanisms (Jackson, 2020, 2021). Myogenic tone sets resting arteriolar internal diameter such that these microvessels can dilate or constrict to maintain homeostasis by meeting the blood flow and pressure needs of the tissues and organs that they perfuse.

Arterioles express numerous ion channels that are essential to their function (**Figure 1**). Plasma membrane and endoplasmic reticulum (ER) ion channels in VSMCs are a major source of Ca^{2+} triggering contractile machinery activation and increased arteriolar tone (vasoconstriction). In endothelial cells (ECs), ion channels provide a key Ca^{2+}source controlling EC autacoid production including prostacyclin (PGI_2), nitric oxide (NO) and epoxides of arachidonic acid (EETs; Jackson, 2016). Intracellular Ca^{2+} also controls gene expression and cell proliferation in VSMCs (Cartin et al., 2000; Stevenson et al., 2001; Barlow et al., 2006) and in ECs (Quinlan et al., 1999; Nilius and Droogmans, 2001; Munaron, 2006; Minami, 2014). Ion channels play a major role in cell volume regulation in all cells (Hoffmann et al., 2009). Finally, ion channels help set and modulate VSMC and EC membrane potential (Jackson, 2016, 2020, 2021; Tykocki et al., 2017). Membrane potential, in turn, regulates the open state probability of voltage-gated Ca^{2+} channels (VGCCs) which provide a major source of activator Ca^{2+} in VSMCs (Tykocki et al., 2017), but probably not most ECs (Jackson, 2016). The electrochemical gradient

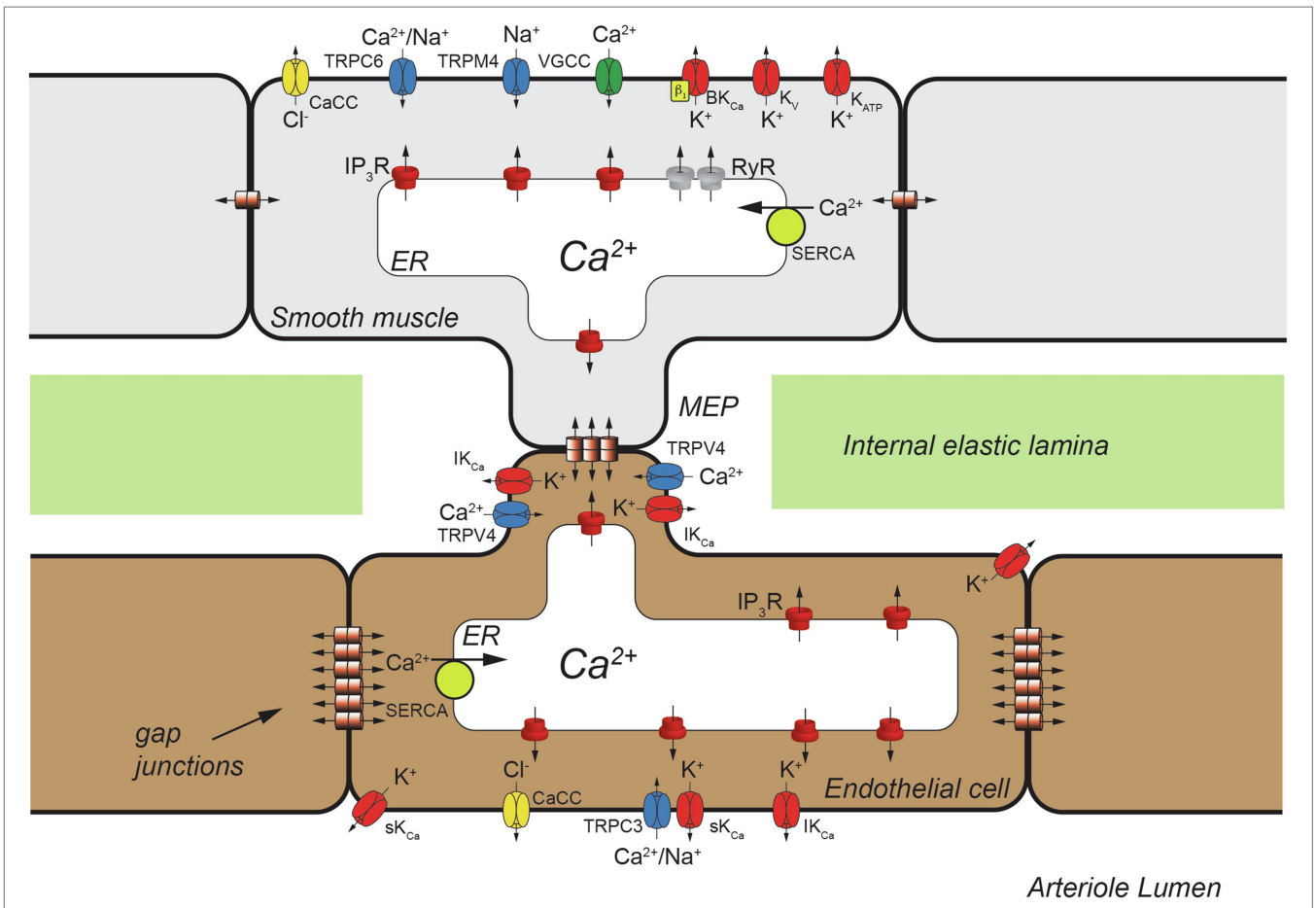

FIGURE 1 | Schematic representation of a cross section of one wall of an arteriole showing a myoendothelial projection (MEP) passing through a hole in the internal elastic lamina (IEL). Heterocellular gap junctions are present allowing electrical and chemical (Ca^{2+}, IP_3, etc.) communication between ECs and VSMCs. Also shown are homocellular (EC-EC and VSMC-VSMC) gap junctions that also allow electrical and chemical communication as shown. Only a few classes of ion channels expressed by arteriolar VSMCs and ECs are shown for clarity. TRPC6, transient receptor potential channel C family member 6; CaCC, Ca^{2+}-activated Cl^- channels; TRPM4, transient receptor potential channel melanostatin family member 4; VGCC, voltage-gated Ca^{2+} channels, BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channels; K_V, voltage-gated K^+ channels; K_{ATP}, ATP-sensitive K^+ channels; IP_3R, inositol 1,4,5 trisphosphate receptor; RyR, ryanodine receptor; SERCA, smooth endoplasmic reticulum Ca^{2+} ATPase; IK_{Ca}, intermediate-conductance Ca^{2+}-activated K^+ channel; TRPV4, Transient Receptor Potential Vanilloid-family 4 channels; TRPC3, transient receptor potential channel C family member 3; sK_{Ca}, small-conductance Ca^{2+}-activated K^+ channel.

for diffusion of Ca^{2+} and other ions depends on membrane potential in all cells (Tykocki et al., 2017). Membrane potential also has been proposed to affect Ca^{2+} release from ER Ca^{2+} stores and may influence the Ca^{2+} sensitivity of Ca^{2+}-dependent processes [see (Tykocki et al., 2017) for references]. Lastly, membrane potential serves as an essential signal for cell–cell communication, because VSMCs and ECs express both homocellular and heterocellular gap junctions allowing electrical and chemical communication among cells in the arteriolar wall (de Wit and Griffith, 2010; Bagher and Segal, 2011; Dora and Garland, 2013; Garland and Dora, 2017; Schmidt and de Wit, 2020). Thus, arteriolar function critically depends on ion channels.

Calcium-dependent ion channels in both VSMCs and ECs play a central role in the generation and modulation of myogenic tone and maintenance of homeostasis (**Figure 1**). These channels provide both positive- and negative-feedback control of intracellular Ca^{2+} in VSMCs that allows fine tuning of arteriolar tone as will be outlined in Section VSMC Ca^{2+}-Dependent Ion Channels, below.

The arteriolar endothelium provides negative-feedback signals to overlying VSMCs through Ca^{2+}-dependent autacoid production and direct electrical communication *via* myoendothelial gap junctions (MEGJs; Lemmey et al., 2020). Endothelial Ca^{2+}-dependent ion channels contribute to these processes (**Figure 1**) as outlined in Section Endothelial Ca^{2+}-Dependent Ion Channels and Arteriolar Tone, below.

Section Integration of Ca^{2+}-Dependent Ion Channels Into the Mechanisms Underlying Pressure-Induced Myogenic Tone then will integrate the VSMC and EC Ca^{2+}-dependent ion channels into the mechanisms that establish, maintain, and modulate pressure-dependent myogenic tone in resistance arteries and arterioles.

VSMC Ca^{2+}-DEPENDENT ION CHANNELS

Arteriolar VSMCs express at least six different Ca^{2+}-dependent ion channels (Tykocki et al., 2017) that participate in the generation, maintenance and modulation of myogenic tone. Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels provide negative-feedback regulation of myogenic tone. Ryanodine receptors (RyRs) can be both inhibitory (negative feedback) or excitatory (positive feedback) dependent on where in the ER they are expressed and with which ion channels they interact. Inositol 1,4,5-trisphosphate receptors (IP_3Rs), transient-receptor potential melanostatin member 4 (TRPM4) channels, Ca^{2+}-activated Cl^- channels (CaCCs) and transient receptor potential polycystin-family member 1 [TRPP1 (PKD2)] channels are excitatory and contribute to the positive-feedback regulation of myogenic tone. In addition, VGCCs (Shah et al., 2006), voltage-gated K^+ (K_V) channels (Gelband et al., 1993; Ishikawa et al., 1993; Gelband and Hume, 1995; Post et al., 1995; Cox and Petrou, 1999) and ATP-sensitive K^+ (K_{ATP}) channels (Wilson et al., 2000) are inhibited in a Ca^{2+}-dependent fashion and will be briefly discussed.

VSMC BK_{Ca} Channels and the Regulation of Arteriolar Tone

Arteriolar VSMCs express BK_{Ca} channels that provide negative-feedback regulation of myogenic tone (**Figure 1**). Both membrane depolarization and increases in intracellular Ca^{2+} activate BK_{Ca} (Tykocki et al., 2017), and because of their large conductance (~200 pS), they powerfully dampen the excitation of VSMCs, preventing vasospasm. BK_{Ca} channels consist of a tetramer of $K_{Ca}1.1$ α-pore-forming subunits (gene = KCNMA1) which have seven transmembrane spanning domains (Meera et al., 1997; **Figure 2A**). Voltage is sensed by positively charged amino acids in membrane spanning domains S2, S3, and S4 (Ma et al., 2006; **Figure 2A**), while Ca^{2+} is sensed by two regulator of conductance of K^+ (RCK) domains (RCK1 and RCK2) in the long, cytosolic C-terminus of the α-subunit (see (Hoshi et al., 2013a) for references; **Figure 2A**).

Vascular smooth muscle cells express both β and γ subunits that modulate the function of the BK_{Ca} channel α-pore-forming subunits (**Figure 2A**). The primary β subunits in VSMCs are β1 (KCNMB-1, $K_{Ca}\beta1$; Tykocki et al., 2017; **Figure 2A**). These subunits modulate channel gating kinetics and increase the Ca^{2+} sensitivity of the α-subunit (McCobb et al., 1995; McManus et al., 1995; Meera et al., 1996; Tseng-Crank et al., 1996). They also are dynamically trafficked to the cell membrane from Rab11A-positive recycling endosomes, providing the ability of VSMCs to tune BK_{Ca} channel function (see (Leo et al., 2014, 2017) for details). The expression of β1-subunits may be downregulated during disease states like hypertension (Amberg et al., 2003; Tajada et al., 2013) and diabetes (McGahon et al., 2007), decreasing the ability to activate VSMC BK_{Ca} channels, increasing myogenic tone. The BK_{Ca} channel agonists dehydrosoyasaponin I (McManus et al., 1995) and 17β-estradiol require expression of β1-subunits (Valverde et al., 1999). Thus, β1-subunits control the Ca^{2+} sensitivity and the pharmacology of BK_{Ca} channels in VSMCs.

Arteriolar VSMC BK_{Ca} channels have a high Ca^{2+} setpoint requiring >3 μM cytosolic Ca^{2+} ($[Ca^{2+}]_{in}$) to open at negative, physiological membrane potentials (−30 to −40 mV; Jackson and Blair, 1998). For reference, global $[Ca^{2+}]_{in}$ measured with Fura-2 in arterioles with myogenic tone is on the order of 300–400 nM (Brekke et al., 2006). Patch clamp studies have shown that arteriolar BK_{Ca} channels are silent when VSMCs are dialyzed with solutions containing 300 nM $[Ca^{2+}]_{in}$ (Jackson, 1998), consistent with a high $[Ca^{2+}]_{in}$ threshold for their activation. The high Ca^{2+} setpoint (threshold) in arteriolar VSMCs may be due to lower expression of the β_1-subunits (Yang et al., 2009, 2013) and possible differences in expression of spliced variants of the α-pore-forming subunit (Nourian et al., 2014) compared to VSMCs in larger arteries.

There also are γ-subunits associated with BK_{Ca} channels that are leucine-rich-repeat-containing proteins (LRRCs; Yan and Aldrich, 2010; Almassy and Begenisich, 2012; Evanson et al., 2014; Gonzalez-Perez et al., 2014; **Figure 2A**). LRRCs allow activation of BK_{Ca} channels at negative membrane potentials, even in the absence of Ca^{2+}, by shifting their voltage vs. activity relationships to the left (increasing their voltage-sensitivity), facilitating their negative feedback function (Yan and Aldrich, 2010; Gonzalez-Perez et al., 2014). The BK_{Ca} channel sensitivity

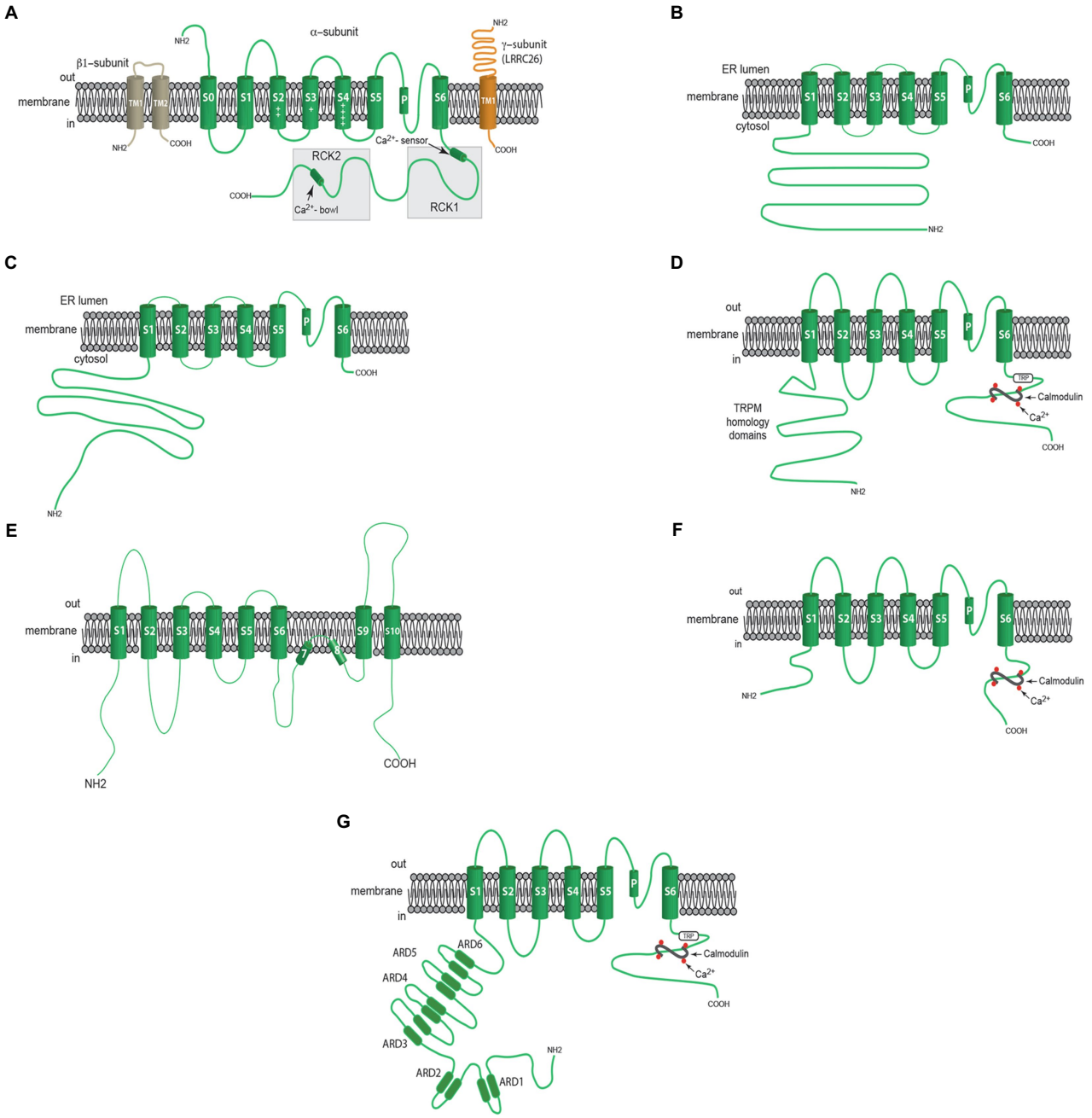

FIGURE 2 | Membrane topology of Ca^{2+}-dependent ion channels involved in the regulation of myogenic tone. **(A)** Components of VSMC BK_{Ca} channels including a β_1- subunit with two membrane-spanning domains, one pore-forming α-subunit with seven membrane-spanning domains and a γ-subunit (LRRC26, for example) with one membrane-spanning domain. **(B)** Shows one α-subunit of an RYR with a large cytosolic N-terminal domain, 6 membrane spanning domains and a short C-terminal sequence. **(C)** Shows one α-subunit of an IP_3R with a large cytosolic N-terminal domain, 6 membrane spanning domains and a short C-terminal sequence. **(D)** Shows one α-subunit of a TRPM4 channel including an N-Terminal domain with a TRPM homology sequence, 6 membrane spanning domains, and a C-terminal domain containing a TRP sequence and binding sites for calmodulin. **(E)** Shows one α-subunit of ANO1 (TMEM16A) CaCC with 10 membrane spanning domains. **(F)** Shows α-subunit of either sK_{Ca} or IK_{Ca} channels with 6 membrane spanning domains and a C-terminal domain with bindings sites for calmodulin. **(G)** Shows one α-subunit of a TRPV4 channel with N-terminal sequence containing ankyrin repeat domains (ARDs), 6 membrane spanning domains and a C-terminal domain with TRP sequence and calmodulin binding sites. See text for more information.

to activation by docosahexaenoic acid (DHA) also is increased by LRRCs (Hoshi et al., 2013b). The role played by LRRCs in arteriolar VSMCs has not been studied.

BK_{Ca} channels provide strong negative feedback regulation of both pressure-induced and agonist-induced tone in resistance arteries and arterioles [see (Tykocki et al., 2017) for numerous

references]. However, there is regional heterogeneity in the source of Ca^{2+} that activates BK_{Ca} channels in resistance arteries versus arterioles. In most resistance arteries, BK_{Ca} channels are controlled by Ca^{2+} sparks which represent the simultaneous release of Ca^{2+} from the ER through small, clustered groups of RyRs (Nelson et al., 1995). Vascular smooth muscle cells that utilize this mechanism of BK_{Ca} channel activation display the so-called spontaneous-transient-outward currents (STOCs): bursts of activity of small groups of BK_{Ca} channels coinciding with the RyR-based Ca^{2+} sparks [(Nelson et al., 1995), see (Tykocki et al., 2017) for additional references]. In VSMCs where this mechanism is active, pharmacological block of RyRs produces the same effect as block of BK_{Ca} channels.

In contrast to many larger resistance arteries, Ca^{2+} influx through VGCCs directly activates BK_{Ca} channels in skeletal muscle arteriolar VSMCs; RyRs are silent, at least under the conditions studied (Westcott and Jackson, 2011; Westcott et al., 2012). In resistance arteries immediately upstream from skeletal muscle arterioles, both RyR-dependent and VGCC-dependent control of BK_{Ca} channels is apparent (Westcott and Jackson, 2011; Westcott et al., 2012). These data suggest that there may be a spectrum of control mechanisms that are involved in Ca^{2+}-dependent control of BK_{Ca} channels in the resistance vasculature. In cerebral penetrating arterioles, both RyRs and BK_{Ca} channels are silent at rest, but both can be activated by low pH (Dabertrand et al., 2012). The molecular mechanisms underlying pH-sensitive recruitment of RyR-control of BK_{Ca} channels has not been established. The mechanisms responsible for the differences in Ca^{2+} sources that control BK_{Ca} channels are not known, but likely relate to the number and location of BK_{Ca} channels expressed relative to RyRs, VGCCs and other ion channels.

VSMC Ryanodine Receptors and Arteriolar Tone

Ryanodine receptors are composed of four, >500 kDa subunits that form ryanodine-sensitive Ca^{2+} channels in ER membranes (**Figure 2B**; Van Petegem, 2015; Yan et al., 2015; Zalk et al., 2015). Increases in $[Ca^{2+}]_{in}$ from resting levels [~300 nM in VSMCs with tone (Brekke et al., 2006).] up to ~10 μM activate release of Ca^{2+} through RyRs, although high levels of $[Ca^{+}]_{in}$ (>10 μM) are inhibitory (Tykocki et al., 2017). Ryanodine receptors also serve as scaffolds for a plethora of signaling proteins [see (Tykocki et al., 2017) for numerous references]. There are three isoforms of RyRs, RyR1, RyR2 and RyR3 [genes = RYR1, RYR2 and RYR3, respectively (Lanner et al., 2010)]: RyR1 is predominantly expressed in skeletal muscle, RyR2 is expressed in cardiac muscle and RyR3 is expressed in the brain and other tissues (Ledbetter et al., 1994; Giannini et al., 1995; Reggiani and te Kronnie, 2006). Vascular smooth muscle expresses multiple isoforms of RYRs with considerable vessel-to-vessel heterogeneity (Vallot et al., 2000; Yang et al., 2005; Salomone et al., 2009; Vaithianathan et al., 2010; Westcott and Jackson, 2011; Westcott et al., 2012). In VSMCs of skeletal muscle arterioles, RyR2 is predominate, and RyR1 is absent (Westcott et al., 2012).

Ryanodine receptors are highly regulated proteins that are modulated by phosphorylation, cellular redox status and interactions with many binding partners in addition to $[Ca^{2+}]_{in}$ (see Tykocki et al., 2017). The overall function of RyRs depends on exactly where they are located in cells and with which ion channels and other proteins they interact.

The elemental Ca^{2+} signal generated by RyRs is the Ca^{2+} spark which represents the simultaneous release of Ca^{2+} from small clusters of RyRs as noted in Section VSMC BK_{Ca} Channels and the Regulation of Arteriolar Tone. Calcium influx through VGCCs has been shown to indirectly regulate Ca^{2+} spark frequency and amplitude by effects on global $[Ca^{2+}]_{in}$ and ER Ca^{2+} store loading (Essin et al., 2007). Subsequent studies have shown that the magnitude of Ca^{2+} influx through the persistent activity of membrane clusters of VGCCs, that can be recorded as VGCC Ca^{2+} sparklets (Navedo et al., 2005; Amberg et al., 2007), controls the amplitude of Ca^{2+} sparks (Tajada et al., 2013). These data suggest that local influx of Ca^{2+} is a major determinant of RyR activity in VSMCs.

In skeletal and cardiac muscle, RyRs act in a positive-feedback manner through Ca^{2+}-induced-Ca^{2+}-release (CICR) to cause explosive release of Ca^{2+} from the ER and subsequent muscle contraction. In both skeletal muscle and cardiac muscle, Ca^{2+} sparks form the basis of this positive feedback process. A similar positive feedback role for Ca^{2+} sparks has been proposed for some arteriolar VSMCs (Curtis et al., 2004, 2008; Fellner and Arendshorst, 2005, 2007; Balasubramanian et al., 2007; Tumelty et al., 2007; Kur et al., 2013). In addition to Ca^{2+} sparks, RyRs can cooperate with IP_3Rs and contribute to Ca^{2+} waves and the positive regulation of myogenic tone in some resistance arteries (Jaggar, 2001; Mufti et al., 2010, 2015; Westcott and Jackson, 2011; Westcott et al., 2012). In other VSMCs, RyR-dependent Ca^{2+} sparks may also act in an excitatory fashion by activating plasma membrane CaCCs producing the so-called spontaneous transient inward currents (STICs) that cause membrane depolarization, VGCC activation and an increase in tone (ZhuGe et al., 1998; Cheng and Lederer, 2008).

As outlined in Section VSMC BK_{Ca} Channels and the Regulation of Arteriolar Tone, in many resistance arteries upstream from the microcirculation, RyRs function as part of a negative-feedback process limiting VSMC excitability. In these vessels, RyR-dependent Ca^{2+} sparks are functionally coupled to BK_{Ca} channels producing membrane hyperpolarization, VGCC deactivation and a decrease in tone (Nelson et al., 1995; Jaggar et al., 1998; Cheng and Lederer, 2008).

However, in skeletal muscle (Westcott and Jackson, 2011; Westcott et al., 2012), cerebral (Dabertrand et al., 2012), and ureteral (Borisova et al., 2009) arterioles downstream from resistance arteries, RyRs are not active and do regulate myogenic tone. Low pH has been shown to recruit RyR-dependent Ca^{2+} sparks in cerebral arterioles, thereby activating BK_{Ca} channels and mediating dilation (Dabertrand et al., 2012). Whether RyRs can be recruited by pH or other conditions in skeletal muscle or ureteral VSMCs has not been studied.

The mechanisms responsible for the heterogeneity in RyR function are not known but most likely result from the specific pattern and magnitude of RyR isoform expression, their cellular

localization, and the expression and localization of other ion channels (for example, CaCC vs. BK_{Ca} channels) in the plasma membrane over RyRs. This area of research should be explored in more detail in the future.

VSMC IP_3Rs and Arteriolar Tone

Inositol 1,4,5 trisphosphate receptors are homotetramers that, like RyRs, form large (~310 kDa) Ca^{2+} release channels in ER membranes (Foskett et al., 2007; **Figure 2C**). There is one binding site for IP_3 on each IP_3R monomer (Foskett et al., 2007; Seo et al., 2012, 2015; Taylor et al., 2014).

Three isoforms of IP_3Rs (IP_3R1, IP_3R2, and IP_3R3) arise from three genes (ITPR1, ITPR2 and ITPR3 respectively; Foskett et al., 2007). There is regional heterogeneity in VSMC IP_3R expression and multiple isoforms are usually expressed in a given VSMC (see (Narayanan et al., 2012) for review). In VSMCs from skeletal muscle resistance arteries and downstream arterioles, we have found expression of $IP_3R1 > IP_3R2 >> IP_3R3$ (Westcott et al., 2012).

Like RyRs, IP_3Rs can be triggered to open by increases in $[Ca^{2+}]_{in}$, with IP_3 affecting the sensitivity of the channels to CICR [see (Tykocki et al., 2017) for review]. In the presence of IP_3, IP_3Rs display a bell shaped $[Ca^{2+}]_{in}$-response relationship with high $[Ca^{2+}]_{in}$ (>1 μM) inhibiting Ca^{2+} release through these channels (Tu et al., 2005). IP_3Rs serve as amplifiers of Ca^{2+} signals generated by other ion channels. They have a number of protein binding partners that modulate their function including FKBP12 (MacMillan et al., 2005), RACK1 (Patterson et al., 2004; Foskett et al., 2007), ankyrin (Hayashi and Su, 2001), Homer (Tu et al., 1998; Foskett et al., 2007), Bcl family members (Bcl-x_L, Mcl and Bcl-2; Li et al., 2007; Eckenrode et al., 2010) and, importantly, a number of TRPC channels including TRPC1 (Boulay et al., 1999), TRPC3 (Boulay et al., 1999; Kiselyov et al., 1999), TRPC4 (Mery et al., 2001), TRPC6 (Boulay et al., 1999) and TRPC7 (Vazquez et al., 2006), either directly (Boulay et al., 1999) or as a component of larger protein complexes (Yuan et al., 2003).

Vascular smooth muscle IP_3Rs are essential for the initiation and maintenance of myogenic tone in resistance arteries (Osol et al., 1993; Gonzales et al., 2010, 2014; Garcia and Earley, 2011) and some, but not all arterioles (Jackson and Boerman, 2017). Three mechanisms have been proposed to account for pressure-dependent activation of IP_3Rs in resistance arteries including angiotensin receptor-mediated (Gonzales et al., 2014), or integrin-mediated (Mufti et al., 2015) activation of $PLC\gamma_1$, angiotensin receptor-mediated activation of PLCβ (Mederos y Schnitzler et al., 2008; Schleifenbaum et al., 2014), or mechanisms involving membrane depolarization-induced activation of G_q-coupled receptors (Ganitkevich and Isenberg, 1993; del Valle-Rodriguez et al., 2003; Urena et al., 2007; Mahaut-Smith et al., 2008; Liu et al., 2009; Fernandez-Tenorio et al., 2010; Yamamura et al., 2012).

In contrast, myogenic tone in hamster cheek pouch arterioles (Jackson and Boerman, 2017) and in murine 4th-order mesenteric arteries (Mauban et al., 2015) does not depend on IP_3 and activation of IP_3Rs. Phospholipase-mediated hydrolysis of phosphatidylcholine and subsequent production of diacylglycerol was proposed to participate in the generation and maintenance of myogenic tone in murine 4th-order mesenteric arteries (Mauban et al., 2015).

Myogenic tone in rat cerebral resistance arteries is accompanied by an increase in the frequency of Ca^{2+} waves (Jaggar, 2001; Mufti et al., 2010, 2015) that involve both IP_3Rs (Mufti et al., 2015) and RyRs (Jaggar, 2001; Mufti et al., 2010, 2015). Similarly, Ca^{2+} waves in skeletal muscle resistance arteries depend on both RyRs and IP_3Rs (Westcott and Jackson, 2011; Westcott et al., 2012). In contrast, Ca^{2+} waves in downstream skeletal muscle arterioles depend only on Ca^{2+} release from IP_3Rs (Westcott and Jackson, 2011; Westcott et al., 2012) that may amplify Ca^{2+} influx through VGCCs (Jackson and Boerman, 2018). However, in rat (Miriel et al., 1999) and mouse (Zacharia et al., 2007) mesenteric resistance arteries, Ca^{2+} waves were inhibited as myogenic tone developed. Thus, there appears to be regional heterogeneity in the role played by IP_3R in the development and maintenance of myogenic tone. The mechanisms responsible for the heterogeneity in function of IP_3Rs among blood vessels has not been established but likely stems from differences in the IP_3R isoforms that are expressed; their localization and interactions with other proteins; and their proximity to other ion channels.

VSMC Ca^{2+}-Activated Cl^- Channels and Arteriolar Tone

VSMCs also express CaCCs that may contribute to myogenic tone. The protein anoctamin-1 (gene = ANO1), also known as transmembrane member 16A (TMEM16A), appears to be the molecular basis of CaCCs in VSMCs (Ji et al., 2019). This protein exists as a homodimer with each monomer having 10 membrane spanning domains (S1-S10), with the pore being formed by S3-S7 helices which also contains a Ca^{2+} binding domain (Ji et al., 2019; **Figure 2E**). TMEM16A demonstrates a synergistic dependence on voltage and Ca^{2+} to control its activity, with depolarization and increases in $[Ca^{2+}]_{in}$ leading to opening of these channels (Ji et al., 2019). In vascular smooth muscle, $[Cl^-]_{in}$ is elevated due to intracellular Cl^- accumulation from the activities of the Cl^-/HCO_3^- exchanger and the $Na^+/K^+/Cl^-$ co-transporter (Matchkov et al., 2013). The elevated $[Cl^-]_{in}$ sets the equilibrium potential for Cl^- [−40 to −25 mV, (Matchkov et al., 2013)] to be positive to the resting membrane potential [−45 to −30 mV, (Tykocki et al., 2017)] of VSMCs that develop myogenic tone. Therefore, opening of a Cl^- channel results in an outward Cl^- current (an inward current in electrophysiological terms), membrane depolarization, activation of VGCCs and an increase in tone (Matchkov et al., 2013).

Calcium-activated chloride channels contribute to agonist-induced tone in a variety of arteries (Bulley and Jaggar, 2014). In addition, STICs carried by Cl^- and coupled to RyR-mediated Ca^{2+} sparks or IP_3-based Ca^{2+} waves have been reported (Bulley and Jaggar, 2014). Cerebral resistance artery VSMCs express TMEM16A that are functionally coupled to transient receptor potential C-family member 6 (TRPC6) channels. Calcium influx

through TRPC6 activates TMEM16A contributing to the membrane depolarization, VGCC activation and pressure-induced myogenic tone in these vessels (Bulley et al., 2012; Wang et al., 2016). In hamster cheek pouch arterioles, CaCCs appear to contribute to myogenic tone when VGCCs are active (Jackson, 2020), suggesting that CaCCs may be functionally coupled to VGCCs in those VSMCs. The molecular identity of CaCCs in hamster cheek pouch arteriolar VSMCs has not been established. Additional research on expression and function of CaCCs in resistance arteries and arterioles appears warranted.

VSMC TRPM4 Channels and Arteriolar Tone

VSMCs express many members of the transient receptor potential (TRP) family of ion channels that contribute to myogenic tone [see (Earley and Brayden, 2015; Tykocki et al., 2017) for more information; **Figures 1, 3**]. Of these, TRPM4 channels are Ca^{2+}-activated and are essential for pressure-induced myogenic tone in cerebral resistance arteries (Gonzales et al., 2014). Like all TRP channels, the pore-forming subunit of TRPM4 channels has six transmembrane domains (S1–S6) which assemble as a tetramer to form a functional ion channel with residues in the intramembrane loop between S5 and S6 forming the channel's pore (Earley and Brayden, 2015; **Figure 2D**). A conserved TRP domain located distal to S6 and a TRPM homology region in the NH2 terminus (Earley and Brayden, 2015) distinguish all members of the TRPM family (Earley and Brayden, 2015; **Figure 2D**). TRPM4 channels selectively conduct monovalent cations such that opening of these channels produces membrane depolarization due primarily to the influx of Na^+ (Earley and Brayden, 2015). Calmodulin binding sites in the C-terminus of TRPM4 are essential for Ca^{2+}-dependent activation and the Ca^{2+}-sensitivity of these channels is increased by protein kinase C-dependent phosphorylation in their amino terminus (Earley, 2013). Rho kinase also has been reported to increase the Ca^{2+}-sensitivity of TRPM4 channels in cerebral parenchymal arterioles (Li and Brayden, 2017).

In cerebral resistance arteries and arterioles, TRPM4 channels are part of the signal transduction pathway for pressure-dependent myogenic tone (Gonzales et al., 2014; Li et al., 2014; Li and Brayden, 2017; see **Figure 3** and Section Integration of Ca^{2+}-Dependent Ion Channels Into the Mechanisms Underlying Pressure-Induced Myogenic Tone for more details). In this scheme, TRPM4 channels are activated by release of Ca^{2+} through IP_3Rs into the subplasmalemmal space (Gonzales et al., 2010), with the IP_3Rs being activated by IP_3, formed by mechanosensitive G-protein coupled receptor-mediated stimulation of phospholipase C (PLC)γ_1, and Ca^{2+} entry through TRPC6 channels, likely activated by both pressure and PLCγ_1-production of diacylglycerol (DAG; Gonzales et al., 2014; **Figure 3**). As noted above, in cerebral parenchymal arterioles, rho-kinase, which also is activated and contributes to myogenic tone, appears to modulate the Ca^{2+} sensitivity of TRPM4 channels (Li and Brayden, 2017; **Figure 3**). The Na^+ entry through TRPM4 channels, along with the entry of Ca^{2+} and Na^+ through TRPC6 channels produces membrane depolarization and activation of Ca^{2+} entry through VGCCs, hallmark elements of pressure-dependent myogenic tone (see (Tykocki et al., 2017) for numerous references; **Figure 3**). The role of TRPM4 in myogenic tone of vessels in other vascular beds has been questioned because global knockout of TRPM4 has no effect on pressure-induced tone in hind limbs of mice (Mathar et al., 2010). However, the details of the mechanisms responsible for pressure-induced tone in the TRPM4 knockout animals was not determined, such that compensation for the global knockout of TRPM4 channels may have occurred. Additional research on TRPM4 and myogenic tone appears warranted.

VSMC TRPP1 (PKD2) Channels and Myogenic Tone

Another potentially Ca^{2+}-activated ion channel that is involved in regulation of myogenic tone are TRPP1 (PKD2) channels. Similar to TRPM4 channels already described, TRPP1 channels are tetramers of 6 membrane spanning domains encoded by the PKD2 gene that have coiled-coil domains in their C-termini and a Ca^{2+}-binding EF-hand motif that may be involved in Ca^{2+}-dependent activation of these channels (Giamarchi and Delmas, 2007). The channels formed from TRPP1 are non-selective cation channels that conduct both Ca^{2+} and Na^+ (Giamarchi and Delmas, 2007). The function of TRPP1 in regulation of myogenic tone is unclear. In murine mesenteric arteries, VSMC TRPP1 channels appear to inhibit myogenic tone (Sharif-Naeini et al., 2009), whereas in rat cerebral arteries VSMC TRPP1 channels significantly contribute to myogenic tone (Narayanan et al., 2013). Conditional knockout of TRPP1 from VSMCs decreases blood pressure and substantially reduces myogenic tone in murine skeletal muscle resistance arteries (Bulley et al., 2018). The plasma membrane expression of TRPP1 in VSMCs is controlled by recycling of sumoylated channels and SUMO1 modification of TRPP1 channels is required for pressure-induced myogenic tone (Hasan et al., 2019). How TRPP1 channels "fit" with other channels that have been shown to be involved in initiation and maintenance of myogenic tone (TRPC6 and TRPM4, for example) remains to be established. Nor has it been established that VSMC TRPP1 channels are activated by Ca^{2+} or that Ca^{2+}-dependent activation is part of their role in pressure-dependent myogenic tone. It is known that TRPP1 channels can heterodimerize with other members of the TRP family (Giamarchi and Delmas, 2007) such that it is feasible that TRPP1 channels may be part of a multi-channel complex. Additional research will be required to determine how TRPP1 channels and all of the other VSMC ion channels implicated in the generation and maintenance of myogenic tone fit together.

Inhibition of VSMC Ion Channels by Ca^{2+}

Voltage-gated Ca^{2+} channels composed of CaV1.2 α-subunits (gene = CACNA1C) play a central role myogenic tone as these channels provide the main source of intracellular Ca^{2+}, the primary trigger of VSMC contraction (Tykocki et al., 2017). Calcium-dependent inhibition of VGCCs is mediated by calmodulin that binds to the C-terminus of CaV1.2 channels

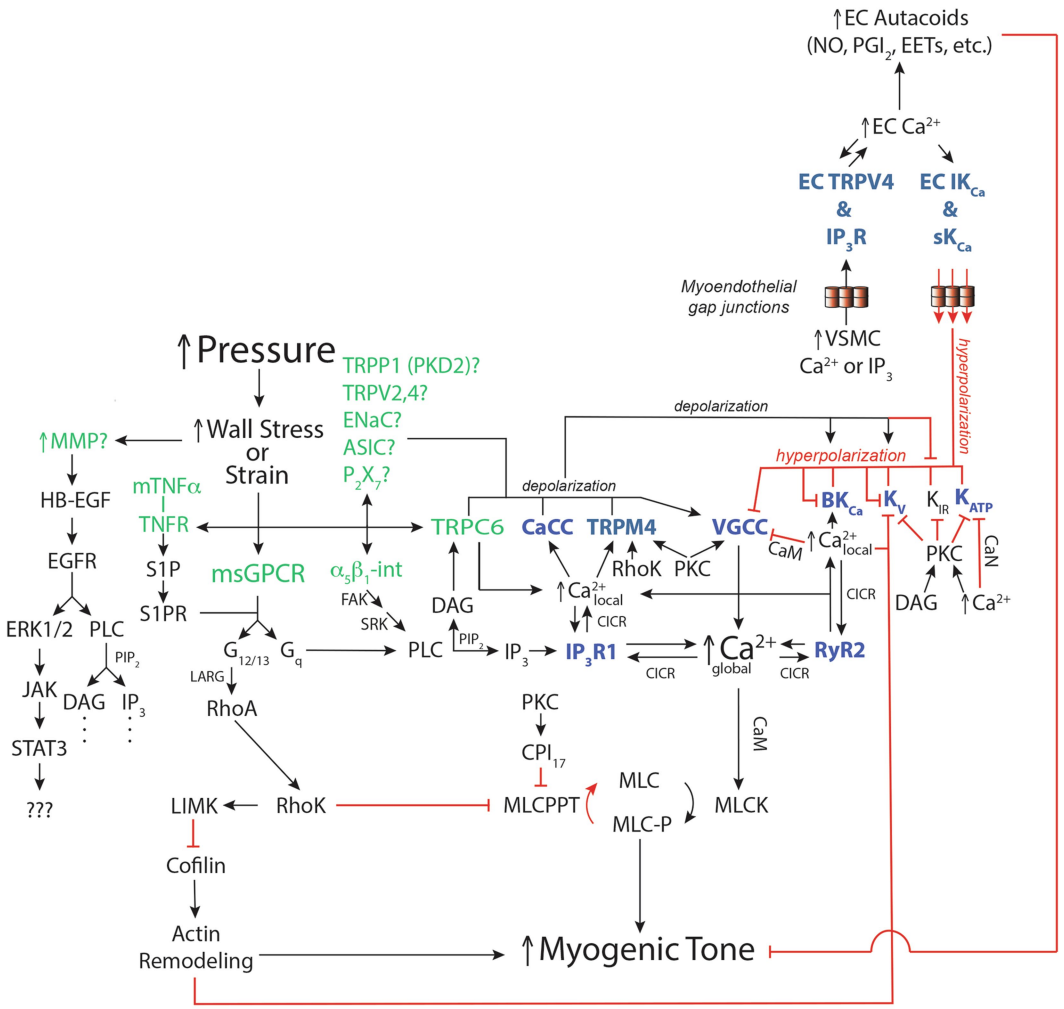

FIGURE 3 | Ca^{2+}-dependent ion channels and vascular smooth muscle signaling pathways for pressure-induced myogenic tone. Schematic diagram [modified from Jackson (2020), (2021)] of reported signaling pathways involved in myogenic tone in resistance arteries and arterioles highlighting the roles played by Ca^{2+}-dependent ion channels. See Section Integration of Ca^{2+}-Dependent Ion Channels Into the Mechanisms Underlying Pressure-Induced Myogenic Tone of text for more details and references. Green font color depicts putative mechanosensors in pressure-induced myogenic tone. Blue font color depicts Ca^{2+}-dependent ion channels involved in regulation of myogenic tone. Black arrows show stimulation, increases or activation of signaling molecules, ion channels or enzymes that participate in myogenic tone. Red capped lines indicate inhibition, decreases or deactivation of signaling molecules, ion channels or enzymes involved in myogenic tone. EC, endothelial cell; VSMC, vascular smooth muscle cell; IK_{Ca}, intermediate conductance Ca^{2+}-activated K^+ channel; sK_{Ca}, small conductance Ca^{2+}-activated K^+ channel; MMP, matrix metalloproteinase; HB-EGF, heparin-bound epidermal growth factor; EGFR, Epidermal Growth Factor Receptor; ERK1/2, Extracellular-Signal-Related Kinases 1 or 2; JAK, Janus Kinase; STAT3, Signal Transducer and Activator of Transcription 3; mTNFα, membrane-bound Tumor Necrosis Factor α; TNFR, TNFα Receptor; S1P, Sphingosine-1-phosphate; S1PR, S1P Receptor; $\alpha_5\beta_1$-int, $\alpha_5\beta_1$ Integrin: FAK, Focal Adhesion Kinase; SRK, Src-related kinases; CaCC, Ca^{2+}-activated Cl^- channel; TRPP1 (PKD2), Transient Receptor Potential Polycystin family member 1; TRPV2,4, Transient Receptor Potential Vanilloid-family 2 or 4 channels; ENaC, Epithelial Na^+ Channel; ASIC, Acid Sensing Ion Channel; P_2X_7, P_2X_7 Purinergic Receptor; TRPC6, transient receptor potential C family member 6; TRPM4, transient receptor potential melanostantin member 4; VGCC, voltage-gated Ca^{2+} channel; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channel; K_V, voltage-gated K^+ channel; K_{IR}, inwardly-rectifying K^+ channel; K_{ATP}, ATP-sensitive K^+ channel; msGPCR, mechanosensitive G-protein-coupled receptor; DAG, diacylglycerol; PKC, protein kinase C; PLC, phospholipase C; PIP_2, phosphatidylinositol bisphosphate; IP_3, inositol, 1,4,5 trisphosphate; IP_3R1, IP_3 receptor 1; RyR, ryanodine receptor; CICR, Ca^{2+}-induced-Ca^{2+} release; LARG, Guanine Nucleotide Exchange Factor LARG; RhoA, small G-protein Rho; RhoK, Rho kinase; LIMK, LIM kinase; CPI_{17}, C-kinase potentiated Protein phosphatase-1 Inhibitor; MLCPPT, myosin light-chain phosphatase; MLC, myosin light-chain; MLCK, myosin light-chain kinase; CaN, calcineurin; CaM, calmodulin.

that make up VSMC VGCCs (Shah et al., 2006). Thus, VGCCs themselves may contribute to the negative-feedback regulation of myogenic tone through this process (**Figure 3**).

Vascular smooth muscle cells express a diverse array of K_V channels that participate in the negative-feedback regulation of myogenic tone (Tykocki et al., 2017). Early studies showed Ca^{2+}-dependent inhibition of K_V channel currents in VSMCs from large arteries (Gelband et al., 1993; Ishikawa et al., 1993; Gelband and Hume, 1995; Post et al., 1995; Cox and Petrou, 1999). However, the molecular identity of the K_V channel isoform that was inhibited was not identified: it was only suspected to be a channel inhibited by 4-amino pyridine (4-AP).

Block of K_V channels by 4-AP appears to be Ca^{2+}-dependent, making interpretation of 4-AP sensitivity difficult (Baeyens et al., 2014). It is well established that increased $[Ca^{2+}]_{in}$ inhibits K_V7.2-7.5 channels *via* binding to calmodulin associated with these channels (Alaimo and Villarroel, 2018). K_V7 channels contribute substantially to the regulation of myogenic tone in resistance arteries (Mackie et al., 2008; Greenwood and Ohya, 2009; Jepps et al., 2013; Cox and Fromme, 2016). Therefore, it is likely that at least some of the inhibitory effect of elevated $[Ca^{2+}]_{in}$ on whole-cell K_V currents is through inhibition of K_V7 channels. Regardless, Ca^{2+}-dependent inhibition of active K_V channels will cause membrane depolarization, activation of VGCCs and a further increase in $[Ca^{2+}]_{in}$ contributing to the positive-feedback regulation of myogenic tone (**Figure 3**). It should be noted that the density of K_V channels is such that Ca^{2+}-dependent inhibition of these channels serves only to blunt the main, negative-feedback role that K_V channels play in the regulation of myogenic tone (Tykocki et al., 2017; Jackson, 2018).

Elevated $[Ca^{2+}]_{in}$ also inhibits ATP-sensitive K^+ (K_{ATP}) channels through Ca^{2+}-dependent activation of the protein phosphatase, calcineurin (Wilson et al., 2000). These channels are active at rest in the microcirculation of a number of vascular beds (Tykocki et al., 2017). Closure of K_{ATP} channels by increased Ca^{2+} would contribute to membrane depolarization, activation of VGCCs, and a further increase in $[Ca^{2+}]_{in}$, a positive-feedback process that would increase myogenic tone (**Figure 3**).

ENDOTHELIAL Ca^{2+}-DEPENDENT ION CHANNELS AND ARTERIOLAR TONE

Numerous ion channels also contribute to EC function and to the modulation of myogenic tone (Jackson, 2016). Calcium-dependent ion channels in ECs include IP_3Rs, small conductance Ca^{2+}-activated K^+ (sK_{Ca}) channels, intermediate conductance Ca^{2+}-activated K^+ (IK_{Ca}) channels, CaCCs, transient receptor potential vanilloid-family member 4 ($TRPV_4$) channels and TRPP1 channels.

EC IP_3Rs and Arteriolar Tone

Endothelial cells express IP_3Rs that contribute to the negative-feedback regulation of arteriolar myogenic tone. Early EC studies demonstrated that the initial increase in $[Ca^{2+}]_{in}$ in response to agonists of EC $G\alpha_q$-coupled receptors resulted from Ca^{2+} release from ER stores (Hallam and Pearson, 1986; Colden-Stanfield et al., 1987; Busse et al., 1988; Schilling et al., 1992; Sharma and Davis, 1994, 1995). Subsequent studies pinpointed IP_3Rs as the primary Ca^{2+} release channel involved in this response (Sharma and Davis, 1995; Cohen and Jackson, 2005).

Endothelial cells from arteries (Mountian et al., 1999, 2001; Grayson et al., 2004; Ledoux et al., 2008) and arterioles (Jackson, 2016) appear to express all three isoforms of IP_3R. However, the dominant isoform may display regional- or species-dependent heterogeneity. For example, IP_3R2 is the dominant IP_3R expressed in mouse mesenteric artery ECs (Ledoux et al., 2008), whereas IP_3R3 is the dominant IP_3R in mouse cremaster muscle arteriolar ECs (Jackson, 2016). There is little information about the specific localization of IP_3R in native arteriolar ECs. In both EC-VSMC co-cultures and in intact mouse cremaster arterioles, IP_3R1 localizes at sites of MEGJs (Isakson, 2008). Similarly, in mouse mesenteric resistance arteries, EC IP_3Rs cluster near holes in the internal elastic lamina (Ledoux et al., 2008), that are sites of myoendothelial projections (MEPs) and MEGJs (Sandow and Hill, 2000; **Figure 1**). Although the IP_3R isoform(s) expressed in these IP_3R clusters has not been identified, they were demonstrated to be the sites of EC Ca^{2+} pulsars, localized IP_3-dependent Ca^{2+} events arising from clusters of IP_3Rs in the ER that extend into MEPs (Kansui et al., 2008; Ledoux et al., 2008; **Figure 1**).

Myoendothelial projections and MEGJs are important signaling microdomains in resistance arteries and arterioles and contain a growing list of signaling proteins including IP_3Rs (Kansui et al., 2008; Ledoux et al., 2008), IK_{Ca} channels (Sandow et al., 2006), TRPA1 channels (Earley et al., 2009a), TRPV4 channels (Sonkusare et al., 2012, 2014), anchoring proteins [e.g., AKAP150 (Sonkusare et al., 2014)], protein kinases [e.g., PKC (Sonkusare et al., 2014)], NO synthase (Straub et al., 2011; Wolpe et al., 2021), Na^+/K^+ ATPase (Dora et al., 2008) and other proteins (Straub et al., 2014; Wolpe et al., 2021; **Figure 1**). Calcium influx through TRPA1 and TRPV4, which produce small, localized Ca^{2+} events called Ca^{2+} sparklets, likely serves as the source of Ca^{2+} that actually triggers release of Ca^{2+} through IP_3Rs to form both localized Ca^{2+} pulsars (Kansui et al., 2008; Ledoux et al., 2008), Ca^{2+} wavelets (Tran et al., 2012) and larger Ca^{2+} waves (Duza and Sarelius, 2004; Kansui et al., 2008) found in ECs of resistance arteries and arterioles. These Ca^{2+} events are then translated into several signals that are vasodilatory and tend to reduce or temper myogenic tone. Activation of EC sK_{Ca} and IK_{Ca} channels (Section EC sK_{Ca} and IK_{Ca} Channels and Arteriolar Tone, below) leads to EC hyperpolarization, which can be conducted through MEGJs to overlying VSMCs, deactivating VGCCs, reducing VSMC Ca^{2+} influx and decreasing myogenic tone (**Figure 3**). Endothelial cell IP_3R Ca^{2+} signals also activate EC NO synthase and production of other EC autacoids (PGI_2, EETs, H_2O_2, etc.) that diffuse to overlying VSMCS and reduce myogenic tone (**Figure 3**).

Global increases in $[Ca^{2+}]_{in}$ reported for ECs in intact resistance arteries or arterioles exposed to endothelium-dependent vasodilators (Dora et al., 1997; Marrelli, 2000; Cohen and Jackson, 2005; Socha et al., 2011) are a complicated blend of IP_3R-mediated Ca^{2+} pulsars, Ca^{2+} wavelets and Ca^{2+} waves. Both the number and frequency of Ca^{2+} pulsars (Ledoux et al., 2008) and both synchronous (Duza and Sarelius, 2004; Socha et al., 2012) and asynchronous (Ledoux et al., 2008; Socha et al., 2012) Ca^{2+} waves are increased by endothelium-dependent vasodilators, such as acetylcholine (Ledoux et al., 2008; Socha et al., 2012) or adenosine (Duza and Sarelius, 2004). Additional research will be required to discover the precise IP_3R isoform expression, location and function related to endothelium-dependent vasomotor activity and modulation of myogenic tone.

Arteriolar ECs Do Not Express Functional RyRs

Early studies of ECs from large arteries provided evidence for expression of functional RyRs (Lesh et al., 1993; Graier et al., 1994, 1998; Ziegelstein et al., 1994; Rusko et al., 1995; Kohler et al., 2001b). In contrast, there is a lack of evidence for expression of RyRs in resistance artery and arteriolar ECs. Mouse mesenteric resistance artery ECs do not express mRNA for the three RyR isoforms, whereas transcripts for IP_3Rs are readily detected (Ledoux et al., 2008). In addition, resting Ca^{2+} levels or acetylcholine-evoked Ca^{2+} events in mouse (Ledoux et al., 2008) or rat (Kansui et al., 2008) mesenteric resistance artery ECs are unaffected by concentrations of ryanodine that block RyRs. Similarly, mouse cremaster arteriolar ECs do not express message for RyRs (Jackson, 2016), and the RyR agonist, caffeine (10 mM), has no effect on $[Ca^{2+}]_{in}$ in these ECs (Cohen and Jackson, 2005). These data do not support a role for RyRs in resistance artery or arteriolar EC Ca^{2+} signals.

EC sK_{Ca} and IK_{Ca} Channels and Arteriolar Tone

Resistance artery and arteriolar ECs express both sK_{Ca} ($K_{Ca}2.3$; gene = KCNN3) and IK_{Ca} ($K_{Ca}3.1$; gene = KCNN4) channels (Kohler et al., 2001a; Eichler et al., 2003; Taylor et al., 2003; Sandow et al., 2006; Si et al., 2006; Grgic et al., 2009). These channels are a tetramer of six transmembrane domain subunits with cytosolic N- and C-termini (Adelman et al., 2012; **Figure 2F**). The ion conducting pore is formed from a pore loop between membrane spanning domains 5 and 6, as in voltage-gated K^+ channels (Adelman et al., 2012). Calmodulin interacts with the intracellular C-terminus to gate opening of both channels (Xia et al., 1998; Fanger et al., 1999; Adelman et al., 2012; Sforna et al., 2018). The Ca^{2+} sensitivity of sK_{Ca} and IK_{Ca} channels is an order of magnitude higher than for BK_{Ca} channels. The threshold for activation by Ca^{2+} binding to calmodulin occurs at 100 nM, 50% of maximal activation at 300 nM and maximal activation at 1 μM for both sK_{Ca} channels (Xia et al., 1998) and IK_{Ca} channels (Ishii et al., 1997). The distinct pharmacology of sK_{Ca} and IK_{Ca} channels has helped to define their function in intact vessels (Jackson, 2016).

Endothelial cell sK_{Ca} and IK_{Ca} channels are not distributed uniformly in the plasma membrane of ECs: IK_{Ca} channels cluster at MEPs (Sandow et al., 2006; Ledoux et al., 2008; Earley et al., 2009a), the site of MEGJs (Sandow and Hill, 2000), whereas sK_{Ca} channels are more distributed around the cell periphery (Sandow et al., 2006). Both channels appear to reside in macromolecular signaling complexes. At MEPs and near MEGJ's, IK_{Ca} channels localize with IP_3Rs (Ledoux et al., 2008), TRPA1 channels (Earley et al., 2009a), TRPV4 channels (Sonkusare et al., 2012, 2014), anchoring proteins [e.g., AKAP150 (Sonkusare et al., 2014)], protein kinases [e.g., PKC (Sonkusare et al., 2014)], nitric oxide synthase (Straub et al., 2011; Wolpe et al., 2021), Na^+/K^+ ATPase (Dora et al., 2008), likely G-protein coupled receptors (Sonkusare et al., 2014) and other proteins (Straub et al., 2014; Wolpe et al., 2021; **Figure 1**). Local Ca^{2+} signals through TRPA1 channels (Earley et al., 2009a), TRPV4 channels (Sonkusare et al., 2012, 2014), and/or IP_3Rs (Ledoux et al., 2008) activate IK_{Ca} (and sK_{Ca}) channels, leading to EC hyperpolarization and conduction of this signal to overlying VSMCs. Hyperpolarization then deactivates VSMC VGCCs reducing myogenic tone (**Figure 3**). EC hyperpolarization also may amplify Ca^{2+} influx through TRPA1 and TRPV4 channels by increasing the electrochemical gradient for Ca^{2+} influx (Qian et al., 2014).

Endothelial cell sK_{Ca} channels also exist in macromolecular signaling microdomains around the EC periphery. They are found in cholesterol-rich areas (caveolae or lipid rafts) and colocalize with caveolin-1 (Absi et al., 2007). Ca^{2+} influx through TRPC3 channels selectively activates sK_{Ca} channels in rat cerebral arteries (Kochukov et al., 2014), suggesting that TRPC3 and sK_{Ca} channels exist in the same microdomain. In mouse carotid arteries, sK_{Ca} channels are in caveolae adjacent to EC-EC gap junction plaques (Brahler et al., 2009). Conditional knockout of sK_{Ca} channels attenuates shear-stress-induced vasodilation in these arteries, suggesting that sK_{Ca} channel localization has functional consequences (Brahler et al., 2009). The respective EC localization of sK_{Ca} and IK_{Ca} channels and their signaling microdomains explain how these two channels mediate different facets of EC hyperpolarization and the regulation of myogenic tone (Crane et al., 2003; Si et al., 2006).

Because ECs are electrically coupled to VSMCs *via* MEGJs, resting membrane potential of ECs can impact myogenic tone. Resting EC membrane potential is determined, in part, by the activity of sK_{Ca} and IK_{Ca} channels. Overexpression of sK_{Ca} channels (which hyperpolarizes ECs) reduces myogenic tone of mesenteric resistance arteries (Taylor et al., 2003). In contrast, conditional knockout of sK_{Ca} channels has the opposite effect (EC depolarization and an increase in myogenic tone; Taylor et al., 2003). Consistent with these data, pharmacological inhibition of sK_{Ca} and IK_{Ca} channels, or both channels augment(s) myogenic tone in rat cerebral parenchymal arterioles (Cipolla et al., 2009; Hannah et al., 2011). Endothelial cell sK_{Ca} and IK_{Ca} channels seem to play a smaller role in modulating myogenic tone of larger cerebral resistance arteries, although they remain important in endothelium-dependent agonist-induced vasodilation (Cipolla et al., 2009). Nonetheless, sK_{Ca} and IK_{Ca} channels significantly contribute to EC-dependent negative-feedback regulation of myogenic tone.

Endothelium-dependent vasodilators that act through G_q-coupled receptors also activate sK_{Ca} and IK_{Ca} channels. In some vessels, such as guinea-pig carotid artery (Corriu et al., 1996), rat mesenteric arteries preconstricted with phenylephrine (Crane et al., 2003) and porcine coronary arteries (Bychkov et al., 2002) both channels appear to be involved because block of both sK_{Ca} and IK_{Ca} channels is necessary to inhibit agonist-induced EC hyperpolarization. In contrast, IK_{Ca} channels mediate endothelium-dependent hyperpolarization and vasodilation in rat cerebral arteries (Marrelli et al., 2003) and in murine arteries and arterioles (Brahler et al., 2009). The reason for this heterogeneity in the roles played by sK_{Ca} and IK_{Ca} channels between vascular beds is not apparent and will require further research.

EC BK_{Ca} Channels and Arteriolar Tone

The expression and function of BK_{Ca} channels in ECs remains debatable (Sandow and Grayson, 2009). As described for VSMCs, BK_{Ca} channels are activated by both voltage and Ca^{2+}, have a much larger conductance (~250 pS) than sK_{Ca} and IK_{Ca} channels, do not require association with calmodulin, and display pharmacology distinct from sK_{Ca} and IK_{Ca} channels (Hoshi et al., 2013a; Tykocki et al., 2017). Cultured large artery ECs have been reported to express BK_{Ca} channels (see (Sandow and Grayson, 2009) for references). Native ECs isolated from hypoxic rats (Hughes et al., 2010; Riddle et al., 2011) or cholesterol depleted ECs (Riddle et al., 2011) express functional BK_{Ca} channels. In cultured ECs, BK_{Ca} channels are located in caveolae and caveolin inhibits their function (Wang et al., 2005). These studies open the possibility that EC BK_{Ca} channels are normally inhibited. Conversely, chronic hypoxia, and potentially other stresses or pathologies, that alter membrane lipid domains may upregulate EC BK_{Ca} channel function (Sandow and Grayson, 2009).

Electrophysiological studies of freshly isolated bovine coronary artery (Gauthier et al., 2002), mouse carotid artery (Brahler et al., 2009), and rat cerebral parenchymal arteriolar (Hannah et al., 2011) ECs found only sK_{Ca} channel and IK_{Ca} channel currents; no BK_{Ca} channel currents were detected. While it has been reported that ECs in freshly isolated rat cremaster arterioles express protein for BK_{Ca} channels (Ungvari et al., 2002), neither mRNA nor protein for this channel were detected in bovine coronary artery ECs (Gauthier et al., 2002). Murine skeletal muscle resistance artery and arteriolar ECs lack BK_{Ca} channel mRNA (Jackson, 2016). Thus, there may be regional or species heterogeneity in EC expression of BK_{Ca} channels. Additional research appears to be warranted to define if and where EC BK_{Ca} are expressed, how they are regulated and their function in the regulation of myogenic tone.

EC Ca^{2+}-Activated Cl^- Channels and Arteriolar Tone

Electrophysiological studies of bovine pulmonary artery and human umbilical vein ECs demonstrate the functional expression of CaCCs (Nilius et al., 1997; Zhong et al., 2000). Unlike VSMCs (see Section VSMC Ca^{2+}-Activated Cl^- Channels and Arteriolar Tone), initial studies did not report expression of TMEM16A in ECs in lung sections (Huang et al., 2009; Ferrera et al., 2011). However, more recent studies have identified TMEM16A expression and function in human pulmonary artery ECs and have shown that over expression of these channels leads to EC dysfunction (Skofic Maurer et al., 2020). In hypertension, EC TMEM16A also contributes to endothelial dysfunction (Ma et al., 2017). TMEM16A is expressed in murine cerebral capillary ECs where it regulates membrane potential, Ca^{2+} signaling, proliferation, migration, and blood brain barrier permeability (Suzuki et al., 2020). Block of TMEM16A preserves blood brain barrier function after ischemic stroke (Liu et al., 2019). Hypoxia stimulates proliferation of brain capillary ECs *via* increased expression of TMEM16A (Suzuki et al., 2021). Hypoxia also increases expression of TMEM16A in mouse cardiac ECs (Wu et al., 2014).

The function of TMEM16A in arteriolar ECs related to regulation of myogenic tone is not clear. In murine capillary ECs, block of TMEM16A results in membrane hyperpolarization suggesting that in ECs, like in VSMCs (see Section VSMC Ca^{2+}-Activated Cl^- Channels and Arteriolar Tone), activation of these CaCCs leads to membrane depolarization, counter to the effects of activation of EC sK_{Ca} and IK_{Ca} channels which produce robust EC hyperpolarization. Thus, it may be that CaCCs in ECs are part of a negative feedback mechanism to dampen membrane hyperpolarization induced by EC sK_{Ca} and IK_{Ca} channels when intracellular Ca^{2+} is elevated.

EC TRPV4 and Regulation of Arteriolar Tone

Transient receptor potential vanilloid-family member 4 channels are another prominent Ca^{2+}-modulated ion channel expressed in ECs (Sonkusare et al., 2012, 2014; Hong et al., 2018; Chen and Sonkusare, 2020). These channels are formed from a tetramer of six membrane spanning domain subunits, with the pore of the channel formed by a pore-loop between domains 5 and 6 like many other ion channels (**Figure 2G**). They conduct primarily Ca^{2+} and are activated by a diverse array of chemicals including EETs (Nilius et al., 2004). In ECs, TRPV4 channels exist in signaling complexes near MEGJ's along with IK_{Ca} channels, IP_3Rs and other proteins (Sonkusare et al., 2012, 2014; Hong et al., 2018; Chen and Sonkusare, 2020; **Figures 1, 3**). Intracellular Ca^{2+} potentiates the activation of TRPV4 channels through calmodulin that binds to the C-terminal region of this channel (Strotmann et al., 2003).

Endothelial TRPV4 channels mediate agonist-induced, endothelium-dependent vasodilation, particularly in arterioles where activation of these receptors leads to activation of IK_{Ca} channels, EC hyperpolarization and conduction of this hyperpolarization to overlying VSMCs to induce vasodilation (Marrelli et al., 2007; Earley et al., 2009b; Sonkusare et al., 2012, 2014; Zhang et al., 2013; Zheng et al., 2013; Du et al., 2016; Diaz-Otero et al., 2018; **Figure 3**). In addition, TRPV4 channels play a central role in myoendothelial negative-feedback that tempers vascular tone in the absence of an endothelial agonist. Agonist-induced activation of VSMC Gq-coupled receptors leads to a global increase in EC intracellular Ca^{2+}(Dora et al., 1997; Schuster et al., 2001; Tuttle and Falcone, 2001; Jackson et al., 2008; Kansui et al., 2008) that contributes to the negative-feedback regulation of vascular tone (Lemmey et al., 2020). Studies in murine mesenteric resistance arteries have shown that endothelial TRPV4 channels are activated during this process through a mechanism involving Ca^{2+} release through IP_3Rs, resulting in activation of IK_{Ca} channels blunting agonist-induced vasoconstriction (Hong et al., 2018; **Figure 3**). Similarly, studies in rat cremaster arterioles have shown that endothelial TRPV4 channels are activated at low intravascular pressure, leading to TRPV4 Ca^{2+} sparklets (localized $[Ca^{2+}]_{in}$ signals through small groups of TRPV4 channels), activation of IK_{Ca} channels and dampening of myogenic tone (Bagher

et al., 2012). The precise signal that is communicated from VSMCs to ECs to initiate myoendothelial feedback remains in question, with data supporting Ca^{2+} as the signal (Garland et al., 2017) and other findings supporting IP_3 as the signal (Tran et al., 2012; Hong et al., 2018). Additional research will be required to determine whether Ca^{2+} or IP_3 mediates myoendothelial negative-feedback and whether there is heterogeneity among vessels in which signal (Ca^{2+} or IP_3) is used.

EC TRPP1 Channels and Myogenic Tone

Endothelial cells also express TRPP1 channels where they function in shear-stress dependent vasodilation (MacKay et al., 2020). Shear-stress-induced increases in EC $[Ca^{2+}]_{in}$ that activate sK_{Ca} channels, IK_{Ca} channels and EC nitric oxide synthase were shown to be substantially impaired by conditional knockout of EC TRPP1 with no change in Ca^{2+} signals activated by muscarinic receptor activation (MacKay et al., 2020). Calcium-dependent activation of TRPP1 channels was not established in these studies, so $[Ca^{2+}]_{in}$ modulation of these channels in ECs and their role in regulating myogenic tone other than when activated by shear-stress remains to be established.

INTEGRATION OF Ca^{2+}-DEPENDENT ION CHANNELS INTO THE MECHANISMS UNDERLYING PRESSURE-INDUCED MYOGENIC TONE

As outlined in Sections above, Ca^{2+}-dependent ion channels in VSMCs and ECs are involved in the initiation, maintenance and modulation of pressure-induced myogenic tone. **Figure 3** integrates this information into a working model with the function of VSMC and EC Ca^{2+}-dependent ion channels highlighted.

Pressure-Dependent Activation of Mechanosensors Leads to Formation of IP_3 and DAG

Multiple mechano-sensors of wall stress (or strain) initiate the myogenic response culminating in steady-state myogenic tone (**Figure 3**). Putative sensors (in green font in **Figure 3**) include: several G-protein coupled receptors (Brayden et al., 2013; Narayanan et al., 2013; Schleifenbaum et al., 2014; Storch et al., 2015; Kauffenstein et al., 2016; Mederos et al., 2016; Hong et al., 2017; Pires et al., 2017; Chennupati et al., 2019), various cation channels (Welsh et al., 2002; Jernigan and Drummond, 2005; Gannon et al., 2008; VanLandingham et al., 2009; Narayanan et al., 2013; Nemeth et al., 2020), integrins (Davis et al., 2001; Martinez-Lemus et al., 2005; Colinas et al., 2015), matrix metalloproteinases and epidermal growth factor receptors (EGFR; Lucchesi et al., 2004; Amin et al., 2011); and membrane-bound tumor necrosis factor α (mTNF α), TNF α receptor (TNFR) and downstream sphingosine-1-phosphate (S1P) signaling (Kroetsch et al., 2017; **Figure 3**). Pressure-dependent stimulation of these putative mechano-sensors activates phospholipase C (PLC) catalyzing hydrolysis of membrane phosphatidyl inositol 4,5 bisphosphate (PIP_2) to form IP_3 and DAG (**Figure 3**).

Activation of Plasma Membrane Ion Channels Produces Membrane Depolarization

Pressure- and likely DAG-induced activation of plasma membrane TRPC6 channels results in Ca^{2+} influx through these channels (Slish et al., 2002; Welsh et al., 2002). The resultant local $[Ca^{2+}]_{in}$ increase, along with IP_3, activates IP_3Rs to release Ca^{2+} from the ER, amplifying the local $[Ca^{2+}]_{in}$ increase. This subplasmalemmal increase in $[Ca^{2+}]_{in}$ then activates overlying plasma membrane TRPM4 channels. Calcium influx through TRPC6 channels also activates plasma membrane Ca^{2+}-activated Cl^- channels (CaCCs; Bulley et al., 2012; Wang et al., 2016). The cation influx through TRPC6 and TRPM4 channels, and Cl^- efflux through CaCCs causes membrane depolarization (**Figure 3**). As noted in Section Pressure-Dependent Activation of Mechanosensors Leads to Formation of IP_3 and DAG and shown in **Figure 3**, additional cation channels including TRPP1 channels may contribute to the pressure-induced depolarization.

Membrane Depolarization Activates VGCC, Induces Ca^{2+} Influx and Stimulates VSMC Contraction

Membrane depolarization induced by ionic currents through TRPC6 channels, TRPM4 channels, CaCCs and other ion channels activates plasma membrane VGCCs resulting in Ca^{2+} influx. VGCC-mediated Ca^{2+} influx across the plasma membrane, along with IP_3R-mediated Ca^{2+} release from ER Ca^{2+} stores, increases cytoplasmic (global) $[Ca^{2+}]_{in}$ levels, leading to calmodulin-mediated myosin light-chain kinase (MLCK) activation, phosphorylation of the myosin light-chains (MLC), actin-myosin cross-bridge formation, cross bridge cycling and an increase in myogenic tone (vasoconstriction; Cole and Welsh, 2011; **Figure 3**).

K^+ Channels Provide Negative Feedback to Dampen Myogenic Tone

Membrane depolarization-induced activation of VGCCs is inherently a positive-feedback process because the Ca^{2+} influx through these channels will itself lead to depolarization and further activation of VGCCs. This process is limited in VSMCs by activation of at least three negative-feedback processes. Membrane depolarization activates K_V channels, and membrane depolarization along with increased $[Ca^{2+}]_{in}$ activates BK_{Ca} channels. The K^+ efflux through these two K^+ channels (which by themselves would cause membrane hyperpolarization) blunts and limits depolarization-induced activation of VGCC (**Figure 3**; Jackson, 2017, 2020). Additional negative feedback arises from Ca^{2+}-dependent inactivation of VGCCs (Shah et al., 2006; **Figure 3**).

Parallel Activation of Protein Kinase C and Rho-Kinase

In addition to activating TRPC6 channels, the DAG formed from the activity of PLC along with elevated $[Ca^{2+}]_{in}$ activates protein kinase C (PKC) supporting the increase in tone by increasing the activity of TRPM4 channels (supporting depolarization) and VGCCs (promoting Ca^{2+} influx) while blunting the activity of several K^+ channels (also supporting membrane depolarization; Jackson, 2020, 2021; **Figure 3**). The negative feedback involving K_V channels is blunted by Ca^{2+}-dependent inhibition of these channels (Gelband et al., 1993; Ishikawa et al., 1993; Gelband and Hume, 1995; Post et al., 1995; Cox and Petrou, 1999; **Figure 3**). Ca^{2+}-dependent activation of the protein phosphatase, calcineurin, inhibits ATP-sensitive K^+ (K_{ATP}) channels, limiting their activity and promoting depolarization (Wilson et al., 2000; **Figure 3**).

Stimulation of the mechano-sensors in vascular smooth muscle also activates the small G-protein rhoA, which, in turn, activates rho-kinase (Chennupati et al., 2019; **Figure 3**). Rho kinase phosphorylates a number of substrates that also support myogenic tone including inhibition of myosin light chain phosphatase (MLCPPT; Cole and Welsh, 2011), stimulation of actin cytoskeleton remodeling that accompanies activation of the contractile machinery (Loirand et al., 2006; Moreno-Dominguez et al., 2013), inhibition of K_V channels as a consequence of actin remodeling (Luykenaar et al., 2009) and increasing the Ca^{2+} sensitivity of TRPM4 channels (Li and Brayden, 2017; **Figure 3**). Activated PKC also may inhibit MLCPPT through phosphorylation of the inhibitory protein, CPI_{17} (Cole and Welsh, 2011; **Figure 3**).

Endothelial Cells Contribute to the Negative-Feedback Regulation of Myogenic Tone

Endothelial cells lining resistance arteries and arterioles play a negative-feedback role, dampening myogenic tone both through the Ca^{2+}-dependent production of vasodilator autacoids (PGI_2, NO, EETS, etc.) and by conduction of Ca^{2+}-dependent membrane hyperpolarization from the endothelium to overlying VSMCs *via* MEGJs (**Figures 1, 3**). Endothelial cells chemically and electrically converse with VSMCs through MEGJs that may form at myoendothelial projections that penetrate holes in the internal elastic lamina and contact the overlying VSMCs. Heterocellular gap junctions (MEGJs) between ECs and VSMCs form and allow small molecules (like IP_3) and ionic currents (including Ca^{2+}) to move between the cells. Pressure-induced increases in VSMC $[Ca^{2+}]_{in}$ or IP_3 can pass to endothelial cells leading to EC IP_3R-induced Ca^{2+} signals (Ca^{2+} pulsars and wavelets) that can increase the production of Ca^{2+}-dependent EC vasodilator autacoids that feedback to the VSMCs reducing myogenic tone (**Figure 3**). In addition, increased EC $[Ca^{2+}]_{in}$ will activate EC sK_{Ca} and IK_{Ca} channels causing EC membrane hyperpolarization. Myoendothelial gap junctions allow this hyperpolarization to be passed from ECs to VSMCs, producing VSMC hyperpolarization, deactivation of VSMC VGCCs and reduced myogenic tone (**Figure 3**). Thus, the production of EC autacoids and EC membrane potential are both strongly dependent on the activity of Ca^{2+}-dependent ion channels in the endothelium including IP_3Rs, TRPV4 channels, sK_{Ca} channels and IK_{Ca} channels (Lemmey et al., 2020).

FINAL PERSPECTIVE

As outlined in this perspective, Ca^{2+}-activated ion channels in both VSMCs and ECs contribute to the regulation of myogenic tone. However, there appears to be considerable heterogeneity in the specific details of their roles in this process among vessels in different vascular beds around the body. The mechanisms responsible for this heterogeneity remains to be established. It is also clear that there is a paucity of information about the cellular and molecular details surrounding which channels are expressed, their localization and their regulation relative to myogenic tone in arterioles around the body. Mesenteric and cerebral resistance artery ion channel expression and function has been well studied. However, we know relatively little about ion channel expression and function in the downstream arterioles in microcirculation, which is really the business end of the cardiovascular system. Future studies directed specifically at understanding control of ion channel expression and function in the microcirculation and how they vary among vascular beds in different organs is warranted.

AUTHOR CONTRIBUTIONS

WJ conceived, wrote, and edited this manuscript.

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Gating and Regulatory Mechanisms of TMEM16 Ion Channels and Scramblases

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The transmembrane protein 16 (TMEM16) family consists of Ca^{2+}-activated ion channels and Ca^{2+}-activated phospholipid scramblases (CaPLSases) that passively flip-flop phospholipids between the two leaflets of the membrane bilayer. Owing to their diverse functions, TMEM16 proteins have been implicated in various human diseases, including asthma, cancer, bleeding disorders, muscular dystrophy, arthritis, epilepsy, dystonia, ataxia, and viral infection. To understand TMEM16 proteins in health and disease, it is critical to decipher their molecular mechanisms of activation gating and regulation. Structural, biophysical, and computational characterizations over the past decade have greatly advanced the molecular understanding of TMEM16 proteins. In this review, we summarize major structural features of the TMEM16 proteins with a focus on regulatory mechanisms and gating.

Keywords: TMEM16, Anoctamin, CaCC, lipid scramblase, phosphatidylserine, PIP_2, pH, calcium

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INTRODUCTION

Since the elegant experiments that led to the discoveries of TMEM16A/ANO1 and TMEM16B/ANO2 as the long-sought-after Ca^{2+}-activated Cl^- channels (CaCCs) in 2008 (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008), substantial progress has been made to understand the biology of this unique family of transmembrane proteins. Numerous studies confirmed that TMEM16A and TMEM16B are responsible for the endogenous CaCC currents observed in various cell types (Bader et al., 1982; Miledi, 1982; Barish, 1983). More excitingly, new findings uncovered their novel physiological and pathological functions, including smooth muscle contraction, trans-epithelial fluid transport, secretion, tumor progression, sensory transduction, mood control, and motor learning (Hartzell et al., 2005; Duran and Hartzell, 2011; Pedemonte and Galietta, 2014; Oh and Jung, 2016; Whitlock and Hartzell, 2016a; Zhang et al., 2017; Crottes and Jan, 2019).

Among the most striking findings in TMEM16 research is that, unlike initial predictions, the remaining family members are likely not CaCCs. Instead, the majority of the TMEM16 family members characterized thus far are Ca^{2+}-activated phospholipid scramblases (CaPLSases), which can translocate phospholipids down their chemical gradients in a relatively non-selective fashion. As passive phospholipid transporters, TMEM16 CaPLSases can efficiently translocate phospholipids at high speed (4.5×10^4 phospholipids per second for TMEM16F; Watanabe et al., 2018). Therefore, activation of TMEM16 CaPLSases leads to rapid collapse

of membrane phospholipid asymmetry, which can trigger a plethora of cellular responses and physiological functions, such as blood coagulation (Suzuki et al., 2010; Yang et al., 2012), microparticle release (Fujii et al., 2015), membrane repair (Wu et al., 2020), sheddase activation (Sommer et al., 2016; Veit et al., 2018; Bleibaum et al., 2019), endosomal sorting (Petkovic et al., 2020), cell–cell fusion (Griffin et al., 2016; Whitlock et al., 2018; Zhang et al., 2020; Braga et al., 2021), and viral infection (Bevers and Williamson, 2016; Zaitseva et al., 2017; Younan et al., 2018). While the list of new biological functions of TMEM16 CaPLSases and CaCCs keeps growing, their importance in human health and disease has become apparent, as malfunctions in TMEM16 proteins have been implicated in human diseases, including asthma, cancer, bleeding disorders, muscular dystrophy, arthritis, epilepsy, dystonia, and ataxia (Duran and Hartzell, 2011; Pedemonte and Galietta, 2014; Oh and Jung, 2016; Crottes and Jan, 2019). To target TMEM16 proteins and treat TMEM16-related diseases, it is critical to have a comprehensive understanding of these novel proteins at the molecular level.

Structural, functional, and computational characterizations of TMEM16 proteins have provided an in-depth understanding of the mechanisms of permeation, activation, and regulation. Given the space limit of this review, we first briefly summarize the key structural features of TMEM16F CaCCs and CaPLSases and then focus on discussing the molecular mechanism of Ca^{2+}-dependent gating, and how an allosteric Ca^{2+} binding site, phosphatidylinositol-(4,5)-bisphosphate [or $PI(4,5)P_2$], and pH regulate TMEM16 Ca^{2+}-dependent gating. This is by no means a comprehensive review of TMEM16 structure and function. The readers are encouraged to refer to the excellent reviews of the biophysics (Brunner et al., 2016; Whitlock and Hartzell, 2016b; Falzone et al., 2018; Kalienkova et al., 2021; Le and Yang, 2021) and physiology of TMEM16 proteins (Hartzell et al., 2005; Duran and Hartzell, 2011; Pedemonte and Galietta, 2014; Oh and Jung, 2016; Whitlock and Hartzell, 2016a).

OVERALL ARCHITECTURE OF TMEM16 PROTEINS

The first glimpse into the atomic structure of TMEM16 proteins came from the X-ray structures of a fungal TMEM16 homolog from *Nectria haematococca* (or nhTMEM16, **Figure 1A** Left; Brunner et al., 2014), which functions as a CaPLSase and likely also a Ca^{2+}-activated nonselective channel (Lee et al., 2016). Subsequent structural analyses of the fungal afTMEM16, mouse TMEM16A, mouse TMEM16F, and human TMEM16K all revealed their highly conserved architecture (Brunner et al., 2014; Dang et al., 2017; Paulino et al., 2017a,b; Alvadia et al., 2019; Falzone et al., 2019; Feng et al., 2019; Kalienkova et al., 2019). Similar to ClC Cl^- channels and Cl^-/H^+ exchangers (Miller, 2006), a functional TMEM16 protein is a dimer with a double-barreled architecture, in which an independent permeation pore resides in each subunit. The double-barreled architecture was functionally validated by electrophysiological characterizations of TMEM16A concatemers, where each monomer possessed different Ca^{2+} sensitivities or ion selectivities (Jeng et al., 2016; Lim et al., 2016).

Different from the initial prediction of an 8-transmembrane (TM) topology, we now know that each TMEM16 monomer consists of 10 TM segments preceded by a long N-terminal cytosolic domain (NCD) and followed by a short C-terminal extension of TM10 (**Figure 1B**). TM7 and TM8 do not completely traverse the membrane, which, together with TM6, form two highly conserved Ca^{2+} binding sites (**Figures 1B, 2**). The anion permeation pathway of the TMEM16A is shaped like an asymmetric hourglass and is formed by numerous hydrophilic and nonpolar residues from TMs 3–7. The so-called hydrophilic cavity has been shown to form a non-selective permeation pathway for not only ions in the TMEM16 channels, but also phospholipids in the scramblases (Brunner et al., 2014; Dang et al., 2017; Jiang et al., 2017; Paulino et al., 2017a,b; Lee et al., 2018; Bushell et al., 2019; Falzone et al., 2019; Le et al., 2019b). Notably, in the fungal nhTMEM16 and afTMEM16 as well as the human TMEM16K structures, the hydrophilic cavity has been captured in an "open" conformation in which the peripheral TM4 and TM6 are physically separated, exposing the hydrophilic cavity to the lipid environment (**Figure 1A** Right; Falzone et al., 2018; Kalienkova et al., 2021). This putative "open" lipid-conducting state supports the notion that TMEM16 scramblases catalyze lipid translocation *via* a "credit card" model previously proposed for phospholipid flippases (Pomorski and Menon, 2006). This model implies that the headgroups of permeating phospholipids may slide along the hydrophilic groove of TMEM16 scramblases, while their acyl tails remain in the hydrophobic lipid environment, a hypothesis that has been supported by extensive structural, functional, and molecular dynamics (MD) studies (Brunner et al., 2014; Bethel and Grabe, 2016; Jiang et al., 2017; Lee et al., 2018; Bushell et al., 2019; Kalienkova et al., 2019; Le et al., 2019b). For dual function ion channel/scramblases, ions may permeate adjacent to lipid headgroups through a proteolipid pore (Whitlock and Hartzell, 2016b). In support of this idea, a recent computational study suggested that the ion permeation pathway in the fungal nhTMEM16 and human TMEM16K is partially lined by ordered lipid headgroups (Kostritskii and Machtens, 2021). The lipid headgroup identity, pore-lining residues, and membrane voltage all exert appreciable effects on ion permeation and selectivity (Kostritskii and Machtens, 2021). By contrast, all current Ca^{2+}-bound structures of the TMEM16A CaCC and the dual function TMEM16F ion channel/scramblase paradoxically adopt tightly closed permeation pathways that are too narrow to allow the passage of ions or lipids (Dang et al., 2017; Paulino et al., 2017a; Alvadia et al., 2019; Feng et al., 2019; **Figures 2A,B**). The reason for these structural observations remains elusive and requires future investigation.

Based on structural, functional, and computational evidence of Ca^{2+}-dependent activation and $PI(4,5)P_2$-dependent regulation (see in the next sections), we recently proposed a modular model of TMEM16 proteins to simplify the complex TMEM16 architecture (**Figures 1B–D**; Le et al., 2019a).

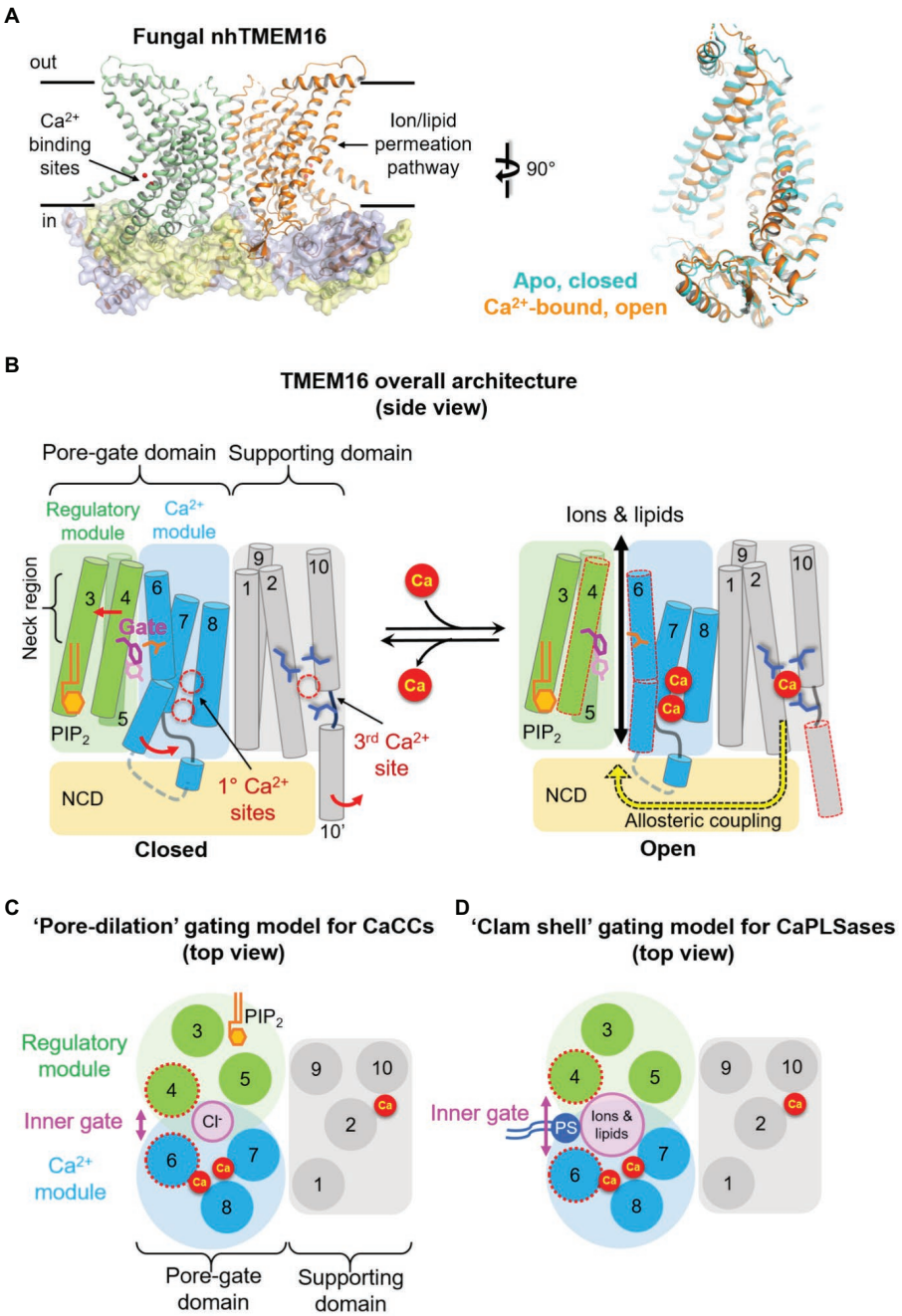

FIGURE 1 | Architecture of TMEM16 proteins. **(A)** Left: X-ray structure of the fungal nhTMEM16 bound to Ca^{2+} (PDB: 4WIS). Right: comparisons of the permeation pathway from cryo-EM structures of nhTMEM16 in an apo, closed state (cyan, PDB: 6QM4) and Ca^{2+}-bound, open state (orange, PDB: 6QM9). **(B)** A simplified cartoon showing the overall architecture (side view) and the "modular design" model of TMEM16 proteins. Three sidechains in the middle of the pore represent the inner activation gate residues (F518, Y563, and I612) of TMEM16F CaPLSase. The putative conformational changes induced by Ca^{2+} binding and subsequent activation gate opening are shown on the right. The neck region refers to the narrowest region of the permeation pathway. NCD, N-terminal cytosolic domain; PIP_2, $PI(4,5)P_2$. **(C)** A top view at the level of the inner activation gate showing the "pore-dilation" gating model for TMEM16 CaCCs. According to this model, Ca^{2+}-induced conformational changes dilate the permeation pore without separating the TM4/TM6 interface. In this way, only Cl^- ions but not phospholipids permeate through the protein-enclosed activation gate. **(D)** A top view at the level of the inner activation gate showing the "clam-shell" gating model for TMEM16 CaPLSases. According to this model, Ca^{2+}-induced conformational changes lead to the separation of TM4 and TM6 at the neck region, resulting in a semi-open pore that faces the lipid core of the membrane. This clam shell-like opening enables phospholipid headgroups to access and subsequently permeate through the pore.

According to this model, a TMEM16 monomer can be divided into several structurally and functionally distinct domains and modules. Besides the NCD, the transmembrane region can be divided into two domains: the pore-gate domain (PGD) and the supporting domain. Consisting of TMs 3–8, the PGD not only forms the permeation pathway for ions

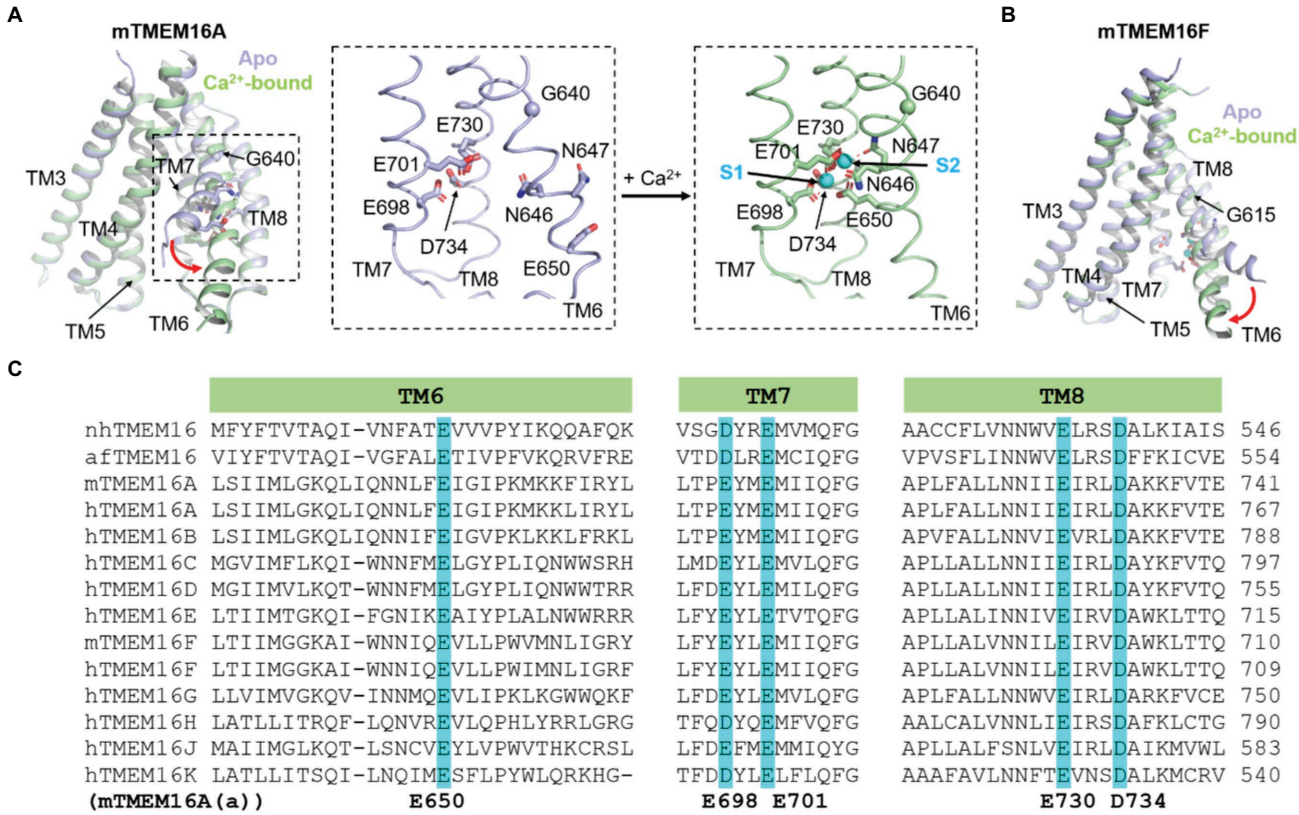

FIGURE 2 | Ca^{2+}-dependent activation of TMEM16A CaCC and TMEM16F CaPLSase. **(A)** Ca^{2+}-induced conformational changes of TMEM16A. Insets show close-up views of the Ca^{2+} binding sites of TMEM16A. S1 and S2 refer the lower and upper bound Ca^{2+} ions, respectively. **(B)** Ca^{2+}-induced conformational changes of TMEM16F. The Ca^{2+}-free (apo) structures are shown in light blue, and the Ca^{2+}-bound structures are in light green. PDB codes are 5OYG and 5OYB for apo and Ca^{2+}-bound mouse TMEM16A, respectively, and are 6QPB and 6QP6 for apo and Ca^{2+}-bound mouse TMEM16F, respectively. Only TMs 3–8 are shown for clarity. **(C)** Protein sequence alignment of the fungal nhTMEM16 and afTMEM16 and human (h) and murine (m) TMEM16A-K showing the highly conserved Ca^{2+} binding residues in TM6, 7 and 8 (highlighted in cyan). Numbering of TMEM16A's Ca^{2+} binding residues is based on the (a) isoform.

and phospholipids, but also harbors the highly conserved primary Ca^{2+} binding sites (**Figures 2A,C**) and, in the case of TMEM16A, a putative $PI(4,5)P_2$ binding site (**Figures 1B,C**). As Ca^{2+} binding and $PI(4,5)P_2$ binding are structurally and functionally segregated, the PGD of TMEM16A can be further divided into two modules (Le et al., 2019a). The Ca^{2+} module consists of TMs 6–8 and is responsible for binding Ca^{2+} and initiating Ca^{2+}-dependent activation. The regulatory module (TMs 3–5) forms the other half of the PGD, which works synergistically with the Ca^{2+} module to facilitate TMEM16 gating and permeation. In TMEM16A, $PI(4,5)P_2$ binding to the regulatory module stabilizes the open state and prevents the PGD from collapsing and entering the desensitized state. TMs 1, 2, 9, and 10, on the other hand, form the supporting domain. As will be discussed below, the supporting domain contains a conserved third Ca^{2+} binding site that is allosterically coupled to the PGD (**Figure 1B**). The supporting domain also helps establish the dimer interface within the membrane through inter-subunit interactions between the extracellular regions of TM10. The dimer interface creates two large hydrophobic cavities, or dimer cavities, along the central axis of all TMEM16 proteins. In addition to the TM10 interactions, the fungal nhTMEM16 and afTMEM16 as well as the human TMEM16K adopt a "domain-swapped" organization in which their C-terminal ends have extensive inter-subunit interactions with the NCD of the adjacent protomer. Interestingly, "domain-swapping" is not observed in the TMEM16A and TMEM16F structures. Beyond facilitating dimer formation, supporting domain interactions potentially serve to stabilize the PGD.

It is worth noting that four conserved disulfide bonds constrain the relatively long extracellular loops connecting TM1-2, 3–4, 5–6, and 9–10 in most mammalian TMEM16 proteins, including TMEM16A and TMEM16F. Disruption of these disulfide bonds leads to dysfunctional channels (Yu et al., 2012), suggesting that the stability of the extracellular loops is important for TMEM16A and TMEM16F activity. Interestingly, the extracellular loops of the fungal nhTMEM16 and afTMEM16 and the endoplasmic reticulum (ER)-resident human TMEM16K scramblase are significantly shorter and lack disulfide bonds (Brunner et al., 2014; Bushell et al., 2019; Falzone et al., 2019). Since these TMEM16 proteins still function as CaPLSases and Ca^{2+}-activated nonselective ion channels, the long extracellular loops seem dispensable

for ion and lipid transport functions. The precise functions of the extracellular loops are unclear and require future investigation.

In summary, structure/function studies have elucidated many of the defining elements of the TMEM16 family. These elements may be conserved in the evolutionarily related osmo- and mechano-sensing OSCA/TMEM63A (Murthy et al., 2018) and TMC channels (Hahn et al., 2009; Ballesteros et al., 2018; Medrano-Soto et al., 2018). To simplify the growing complexity, we propose a modular design of the TMEM16 proteins (**Figures 1B,C**), which may also facilitate the understanding of the molecular mechanisms of related proteins.

Ca^{2+}-DEPENDENT ACTIVATION OF TMEM16 PROTEINS

All mammalian TMEM16 ion channels and lipid scramblases require the binding of intracellular Ca^{2+} for activation, albeit at different levels of potency. The TMEM16A and TMEM16B CaCCs are synergistically gated by intracellular Ca^{2+} and membrane voltages. TMEM16A is highly sensitive to Ca^{2+} with an estimated EC_{50} of 0.4 to 1 μM at positive membrane potentials or 0.7 to 6 μM at negative membrane potentials (Yang et al., 2008; Ferrera et al., 2009; Xiao et al., 2011; Yu et al., 2012; Brunner et al., 2014; Ni et al., 2014; Tien et al., 2014; Lim et al., 2016; Cruz-Rangel et al., 2017; Le et al., 2019a). Despite their similarity, TMEM16B displays a lower Ca^{2+} sensitivity with an estimated EC_{50} of around 1.2 to 3.3 μM at positive membrane potentials and 1.8 to 4.9 μM at negative potentials (Pifferi et al., 2009; Stephan et al., 2009; Cenedese et al., 2012; Adomaviciene et al., 2013; Pifferi, 2017). One notable feature is that TMEM16A- and TMEM16B-mediated currents are outward rectifying under the low open probability and display time-dependent activation and deactivation kinetics (Caputo et al., 2008; Yang et al., 2008; Pifferi et al., 2009; Stephan et al., 2009). However, these channels are no longer time- and voltage-dependent when they are fully opened by saturating Ca^{2+}.

The dual function TMEM16F ion channel and phospholipid scramblase is less sensitive to Ca^{2+}. The estimated EC_{50} values range from 3.4 to 105 μM, depending on the configuration and ionic conditions of the patch clamp recording (Yang et al., 2012; Grubb et al., 2013; Shimizu et al., 2013; Scudieri et al., 2015; Feng et al., 2019; Le et al., 2019b; Nguyen et al., 2019; Ye et al., 2019). The Ca^{2+} sensitivity for TMEM16F scrambling activity has not been accurately measured. However, based on the co-occurrence of TMEM16F current and scramblase activity recorded using patch clamp-lipid scramblase fluorometry (PCLSF) assay (Yu et al., 2015; Liang and Yang, 2021), it is expected that the Ca^{2+} sensitivity for TMEM16F CaPLSase activity is comparable to the Ca^{2+} sensitivity for channel activity. TMEM16F-mediated ionic conductance is elicited by the synergistic activation of membrane depolarization and Ca^{2+} binding (Yang et al., 2012). Unlike TMEM16A and TMEM16B, the TMEM16F channel always requires membrane depolarization for activation and its current remains strongly outward rectifying even at high Ca^{2+} concentrations. It is yet unknown whether membrane voltage can promote CaPLSase activity.

Mutagenesis studies on TMEM16A CaCC successfully identified five highly conserved acidic residues as putative Ca^{2+} binding residues, including E650 on TM6, E698 and E701 on TM7, E730 and D734 on TM8 (Yu et al., 2012; Tien et al., 2014; **Figures 2A,C**, numbering based on the TMEM16A(a) isoform lacking the EAVK segment). Neutralizing mutations (to alanine or glutamine) strongly reduce the Ca^{2+} sensitivity of TMEM16A from the sub-micromolar range to the millimolar range. Subsequent structural and functional studies not only validated these electrophysiological findings but also revealed three additional asparagine residues (N646 and N647 of TM6 and N726 of TM8) as additional Ca^{2+} coordinates (Brunner et al., 2014; Dang et al., 2017; Paulino et al., 2017a). Within each TMEM16 monomer, the Ca^{2+} binding residues cluster together and form two highly conserved Ca^{2+} binding sites, herein referred to as the primary Ca^{2+} sites (**Figures 1B, 2**). The highly conserved primary Ca^{2+} binding sites among different TMEM16 homologs suggest that these evolutionarily conserved proteins maintain a similar activation mechanism.

The primary TMEM16 Ca^{2+} binding sites have several unique features (**Figures 1B, 2**). First, the Ca^{2+} binding residues reside within the membrane electrical field, which is in excellent agreement with a previous prediction (Arreola et al., 1996). The membrane location of the Ca^{2+} binding sites in TMEM16 proteins may partially contribute to their weak voltage-dependent Ca^{2+} activation (Hartzell et al., 2005; Pifferi et al., 2009; Xiao et al., 2011; Yang et al., 2012), as Ca^{2+} ions need to travel within the membrane electric field to reach the binding sites. Second, the primary Ca^{2+} binding sites are located near the ion/lipid permeation pathway. Such proximity between the Ca^{2+} binding sites and the activation gates implies that TMEM16 proteins can efficiently transmit Ca^{2+} binding energy to operate their activation gates.

Structural and functional studies have shown that Ca^{2+}-induced TM6 conformational changes are critical for Ca^{2+}-dependent activation of both TMEM16 ion channels and scramblases (**Figures 1, 2**; Dang et al., 2017; Paulino et al., 2017a; Peters et al., 2018; Alvadia et al., 2019; Feng et al., 2019). Structural studies of the TMEM16A CaCC showed that in the absence of Ca^{2+}, TM6 adopts an alpha-helical conformation with a kink at G640 (**Figures 2A,C**; Dang et al., 2017; Paulino et al., 2017a). This kink causes the C-terminal segment of TM6 to swing away from TM7 and TM8, thereby rendering the negatively charged Ca^{2+} binding residues accessible to the cytosol. The highly electronegative environment created by the apo Ca^{2+} binding sites also serves to impede Cl^- entry from the intracellular side (Paulino et al., 2017b; Lam and Dutzler, 2018). It was suggested that Ca^{2+} ions first bind to and neutralize the four highly acidic residues from TM7 and TM8, providing an attractive environment that allows TM6 to move toward TM7 and TM8 by interacting with the bound Ca^{2+} ions *via* N647 and E650. During this process, TM6 rotates around the G640 hinge because of the interactions between N647, E650, and the two bound Ca^{2+}, subsequently leading to the formation of a π-helix (**Figure 2A**). Superimposing the Ca^{2+}-bound and

Ca^{2+}-free structures reveals that Ca^{2+} binding leads to partial widening of the central constriction site in TMEM16A, though, paradoxically, the permeation pathway is still too narrow for anion passage. Supporting the functional importance of TM6 in TMEM16A gating, several mutations on TM6 such as I637A, I637K, G640A/P, Q645A, and P654A were shown to alter the channel's Ca^{2+} sensitivity (Dang et al., 2017; Paulino et al., 2017a; Lam and Dutzler, 2018; Peters et al., 2018; Le et al., 2019b). These mutations likely shift the equilibrium of TM6 to favor either the open conductive state (G640A/P, I637A/K, and Q645A) or the closed non-conductive state (P654A). A recent computational study further supports the importance of TM6 conformational changes in Ca^{2+}-dependent gating of TMEM16A (Shi et al., 2021). Based on MD simulations, the authors concluded that separation of TM6 and TM4 may lead to expansion of the ion permeation pore and consequently the opening of the channel. This is consistent with the "pore-dilation" model (**Figure 1C**) derived from functional tests (Le et al., 2019b).

Conformational changes of TM6 also seem critical for the gating of the TMEM16F ion channel/scramblase, albeit *via* an opposite movement of the cytosolic end of TM6 compared to TMEM16A TM6 (**Figure 2**). However, analogous to TMEM16A, binding of two Ca^{2+} ions to N620, N621, and E624 of TM6, E667 and E670 of TM7, and E699 and D703 of TM8 neutralizes the Ca^{2+} binding sites and allows TM6 to approach TM7 and TM8 *via* a rigid body movement around G615, equivalent to TMEM16A's G640 (**Figure 2**). Because of a missing residue near the G615 hinge (**Figure 2C**), Ca^{2+} binding does not result in partial unwinding of TM6 and hence the π-helix does not form in TMEM16F (Alvadia et al., 2019; Feng et al., 2019). A similar transition from a bent to straight conformation of TM6 was also observed in the structures of TMEM16F with zero or one Ca^{2+} bound, respectively (Feng et al., 2019). It is worth noting that while the fungal afTMEM16 and nhTMEM16 homologs lack a glycine hinge, TM6 also undergoes a similar swinging movement around the equivalent region upon Ca^{2+} binding (Falzone et al., 2019; Kalienkova et al., 2019). These observations further illuminate the conserved gating mechanism shared among TMEM16 ion channels and scramblases.

While Ca^{2+}-induced conformational changes in TM6 were unambiguously shown to be critical for the gating of TMEM16 ion channels and scramblases, recent studies on TMEM16A (Tak et al., 2019) and TMEM16F (Roh et al., 2021) proposed another interesting Ca^{2+}-dependent gating. Tak et al. (2019) suggested that the TMEM16A CaCC harbors an EF-hand-like domain consisting of a cluster of acidic residues (TMEM16A D285 to D297) that could serve as a reservoir for Ca^{2+} binding before being transferred to the primary sites in TMs 6–8 for subsequent activation. Neutralization of these acidic residues reduces both TMEM16A's Ca^{2+} and voltage sensitivity. While TMEM16F does not appear to have such an EF-hand-like domain, Roh et al. (2021) showed that neutralizing acidic residues in the equivalent N-terminal domain of TMEM16F reduces its Ca^{2+} sensitivity, consistent with the importance of this acidic Ca^{2+} reservoir in channel gating. Furthermore, the N-terminal Ca^{2+} reservoir in TMEM16F has less acidic residues compared to that of TMEM16A and contains additional basic residues. Replacing the N-terminal Ca^{2+} reservoir of TMEM16F with the equivalent EF-hand-like N-terminal domain of TMEM16A markedly enhances TMEM16F's Ca^{2+} sensitivity, suggesting that the differences in electronegativity at this region may contribute to determining Ca^{2+}-dependent gating in TMEM16 proteins (Tak et al., 2019; Roh et al., 2021).

Another intriguing phenomenon about TMEM16F Ca^{2+}-dependent activation is the long (~5–10 min) delay after establishing the whole-cell patch clamp configuration (Grubb et al., 2013; Shimizu et al., 2013; Scudieri et al., 2015; Yu et al., 2015; Lin et al., 2018; Liang and Yang, 2021; Stabilini et al., 2021). This delay persists even when the pipette solution contains 100–200 μM Ca^{2+}. Therefore, the delay cannot be simply explained by the relatively low Ca^{2+} sensitivity of TMEM16F, which may require prolonged diffusion time for intracellular Ca^{2+} to reach the threshold concentration to activate TMEM16F. Paradoxically, TMEM16F current can be instantaneously activated without delay under inside-out configuration (Yang et al., 2012; Lin et al., 2018; Liang and Yang, 2021). It seems apparent that some intracellular factors might be responsible for the patch configuration-dependent discrepancy on TMEM16F activation. Although the detailed mechanisms are still unclear, a recent study provided important clues (Lin et al., 2018). The authors found that disrupting the actin cytoskeleton with cytochalasin-D (cytoD) significantly shortens the delay and accelerates TMEM16F activation. Analogously, the actin filament-stabilizing agents phalloidin and jasplakinolide inhibit TMEM16F current activation. These results suggest that the actin cytoskeleton may negatively regulate TMEM16F ion channel activity under the whole-cell configuration. Interesting, the authors also showed that intracellular magnesium ATP but not sodium ATP further prolongs the delay for TMEM16F current activation. How these intracellular factors affect TMEM16F current activation and if they also affect TMEM16F lipid scrambling activity warrant further investigations.

TMEM16 INNER ACTIVATION GATE

Structural, functional, and computational studies have demonstrated a crucial role for pore-lining TM6 residues in gating of both TMEM16 channels and TMEM16 scramblases. However, a comprehensive understanding of their gating mechanisms requires the identification of the physical activation gate that opens and closes to control ion and phospholipid permeation in response to Ca^{2+} binding. Such activation gates have been proposed for both TMEM16A and TMEM16F (Le et al., 2019b; Lam et al., 2021). Using MD simulations and an optimized lipid scrambling assay, three bulky and hydrophobic residues–F518 in TM4, Y563 in TM5, and I612 in TM6, were identified as the major constituents of the scramblase inner steric activation gate in TMEM16F (**Figure 1B**; Le et al., 2019b). Removing steric hindrance *via* alanine substitutions of these residues leads to constitutively active TMEM16F scramblases, whereas substitution with leucine or a bulky tryptophan strongly impairs TMEM16F scrambling activity following Ca^{2+} stimulation.

On the other hand, mutating the inner gate with polar or charged residues greatly enhances TMEM16F lipid scrambling and ion channel activities. Most of these mutations require culturing the transfected cells in Ca^{2+}-free media to suppress TMEM16F gain-of-function (GOF)-induced cytotoxicity, suggesting that basal Ca^{2+} activity is sufficient to open the inner activation gate. Remarkably, F518K and Y563K result in constitutively active TMEM16F scramblases even when the primary Ca^{2+} binding sites are destroyed. More strikingly, the TMEM16A L543K mutation, equivalent to TMEM16F F518K, converts the TMEM16A CaCC into a GOF phospholipid scramblase (Le et al., 2019b). Based on these functional observations and various TMEM16 scramblases captured in different conformations (Alvadia et al., 2019; Falzone et al., 2019; Feng et al., 2019; Kalienkova et al., 2019), a "clam-shell" model was proposed to describe the Ca^{2+}-dependent gating of the TMEM16 phospholipid permeation pathway (Le et al., 2019b; **Figure 1D**). According to this model, Ca^{2+}-induced conformational changes at the primary Ca^{2+} binding sites interrupt the interactions between TM4 and TM6 in the neck region, leading to the separation of TM4 and TM6. This clam-shell-type opening exposes the hydrophilic interior of the permeation pathway to the hydrophobic phase of the membrane, thereby allowing phospholipid headgroups to gain access and scramble (**Figures 1B,D**). Clam-shell opening also enables ion permeation through the proteolipid pore. Replacing the bulky, hydrophobic residues at the inner activation gate with smaller, polar, or charged amino acids weakens the interactions between TM4 and TM6, leading to enhanced permeation or a constitutively open permeation pathway for both lipids and ions.

As Cl^- permeation through CaCC requires an enclosed protein environment, it is conceivable that TMEM16A gating may not follow the "clam-shell" gating model of the TMEM16 scramblases. Instead, Ca^{2+}-induced conformational changes only appear to dilate the central pore of TMEM16A, allowing tight control of Cl^- permeation (Dang et al., 2017; Paulino et al., 2017a; Le et al., 2019b; Shi et al., 2021; **Figure 1C**). The hydrophobic residues L543, I546, I547, and I637 (L547, I550, I551, and I641 in the (ac) isoform) at the equivalent locations to the TMEM16F inner gate residues likely form the hydrophobic gate to control TMEM16A Cl^- permeation (Le et al., 2019b; Lam et al., 2021) as evidenced by alanine and lysine mutations promoting TMEM16A activation. Interestingly, L543K enables TMEM16A activation in the absence of Ca^{2+} and reduces its anion selectivity, in addition to converting TMEM16A into a phospholipid scramblase as mentioned above (Le et al., 2019b). Interestingly, a previous discovery showed that substitution of a 35 amino acid segment spanning TM4 and TM5 of TMEM16A with the corresponding segment in TMEM16F rendered TMEM16A capable of scrambling phospholipids (Yu et al., 2015). Inspired by the MD simulations of fungal nhTMEM16, a follow-up study identified three additional mutations (V543S, V543T, K588N, numbering based on the TMEM16A(ac) isoform) on two pore lining residues, which can also convert TMEM16A CaCC into lipid scramblases (Jiang et al., 2017). These functional studies thus imply that TMEM16A CaCC may preserve an evolutionary potential to permeate phospholipids. The width of TM4/TM6 separation during gating is likely the key structural determinant for a TMEM16 protein to serve as a pure ion channel or a phospholipid scramblase (**Figures 1C,D**). For a TMEM16 CaPLSase, Ca^{2+} binding induces wide opening of the TM4/TM6 interface, thereby allowing phospholipid headgroups to gain access and scramble. On the other hand, TM4/TM6 of TMEM16 CaCCs clash with each other in the neck region of the permeation pathway, which prevents them from separating. Therefore, Ca^{2+} binding only allows ion flux without phospholipid permeation. When a charged mutation at the inner gate weakens the interactions between TM4 and TM6, the interface between the two helices may be forced to open widely so that phospholipids can permeate. Future structural, functional, and computational studies are needed to test this hypothesis. It is worth noting that endogenous CaPLSases are ubiquitously expressed in various cell lines (Kunzelmann et al., 2009). Therefore, a cell line without endogenous CaPLSase activity (Le et al., 2019b,c; Liang and Yang, 2021) is essential to experimentally examine the mutational effects on scrambling activities.

REGULATORY MECHANISMS OF TMEM16 ION CHANNELS AND LIPID SCRAMBLASES

Allosteric Regulation of TMEM16 by a Third Ca^{2+} Binding Site

In addition to the extensively studied primary Ca^{2+} binding sites in TMs 6–8 (**Figure 2A**), recent structural studies of the mouse TMEM16F and the human ER-localized TMEM16K CaPLSases revealed an additional Ca^{2+} site located in the supporting domain (Alvadia et al., 2019; Bushell et al., 2019; **Figure 1B**). This third Ca^{2+} site is formed by several charged residues from TM2 and TM10 of the same subunit. In both proteins, the bound third Ca^{2+} ion is coordinated by the carboxylate groups of two highly conserved acidic residues (E395 and D859 in mTMEM16F, E259 and D615 in hTMEM16K) and the main-chain carbonyl group of an isoleucine (I857 in mTMEM16F and I613 in hTMEM16K). The main-chain carbonyl of S854 in TMEM16F (A610 in hTMEM6K) also appears to provide a coordination for the bound Ca^{2+}. Interestingly, there is a conserved lysine (K398 in mTMEM16F, K262 in hTMEM16K), which apparently forms a stabilizing electrostatic interaction with the aspartate in TM10.

Recently, using the TMEM16A CaCC as a model protein, a comprehensive functional characterization of the third Ca^{2+} binding site in TM2 and TM10 was conducted (Le and Yang, 2020). First, by studying both a WT and the GOF Q645A mutant background, the authors revealed that mutation of the third Ca^{2+} site residues, including E425A, K428A, D879A, and D884A (**Figure 3**), paradoxically alters channel activation even in the absence of Ca^{2+} binding. Also, because the primary Ca^{2+} sites confound accurate assessment of the third Ca^{2+} site's function, two charge-reversing mutations, E701K and D734R, both of which eliminate Ca^{2+} binding to the primary Ca^{2+}

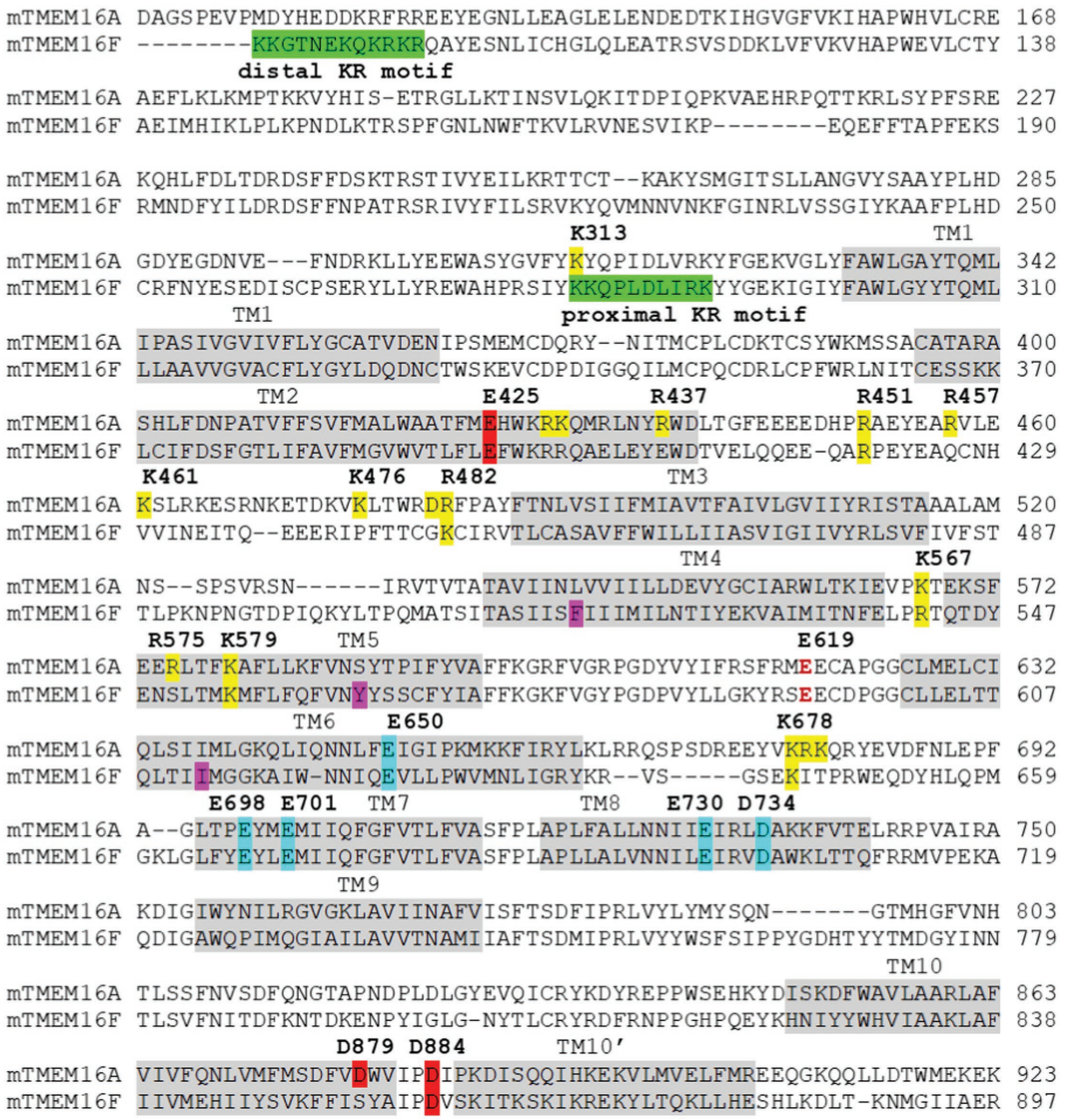

FIGURE 3 | Sequence alignment of the murine (m) TMEM16A (the "a" isoform) and TMEM16F. The transmembrane domains (TM) are highlighted in light gray. The distal and proximal motifs (Aoun et al., 2016; Ye et al., 2018) important for PI(4,5)P_2 binding in TMEM16F are highlighted in green. Residues that are important for PI(4,5)P_2 binding in TMEM16A (Le et al., 2019a; Yu et al., 2019; Ko et al., 2020) are highlighted in yellow. Residues at the third Ca^{2+} site (Le and Yang, 2020) are highlighted in red, and residues forming the primary Ca^{2+} sites are highlighted in cyan. Residues that form the inner gate (F518, Y563, and I612) in TMEM16F (Le et al., 2019b) are highlighted in magenta. Intracellular pH affects the primary Ca^{2+} binding sites (cyan highlight; Chun et al., 2015; Liang and Yang, 2021) and extracellular pH works on a conserved glutamate residue (E619 in TMEM16A, dark red text; Cruz-Rangel et al., 2017).

sites in TMs 6–8, were introduced. The GOF Q645A was included to establish basal channel activity which the authors used to measure the Ca^{2+} sensing capacity of the third site. By eliminating the contribution of the primary Ca^{2+} sites, the authors showed that the third site has a high affinity for Ca^{2+} with an estimated apparent K_D of ~320 nM, and that Ca^{2+} binding markedly enhances channel activation (Le and Yang, 2020). This hypothesis was bolstered by the observation that single alanine mutations of the three acidic E425, D879, and D884 residues strongly reduce Ca^{2+} sensing of the third site, whereas that of the basic K428 displays a less pronounced reduction. Double alanine mutations of the acidic residues at the third site completely abolish Ca^{2+} sensing, further confirming that the third Ca^{2+} site is solely responsible for the Ca^{2+}-dependent activity of the triple mutant background. Strikingly, conformational perturbation of the third site *via* cadmium (Cd^{2+})-mediated bridging of substituted cysteines at E425 in TM2 and D879 in TM10 strongly inhibits channel activation in a manner independent of the primary Ca^{2+} sites. These results could also explain previous studies implicating the functional importance of TM10', the extended alpha helix following TM10. In fact, replacing or truncating the C-terminal region following TM10 markedly altered the Ca^{2+} sensitivity of TMEM16A (Scudieri et al., 2016; Dang et al., 2017). Chemical

crosslinking experiments also suggested that TM10' may form inter-subunit interactions with the TM2-3 loop (Scudieri et al., 2016), a region that is important for voltage-dependent channel activation (Ferrera et al., 2009; Xiao et al., 2011). Furthermore, the TMEM16K structures also revealed that TM10' forms inter-subunit interactions with the TM2-TM3 loop and undergoes a pronounced conformational transition during activation of the scrambling pathway (Bushell et al., 2019). Thus, it is tempting to speculate that Ca^{2+} binding to the third site allosterically controls TMEM16A activation, likely by influencing the inter-subunit coupling between TM10' of one subunit and TM2-TM3 loop of the second subunit (**Figure 1B**). Future studies are required to fully delineate the functional role and mechanistic underpinnings of the third Ca^{2+} site.

Finally, it is worth noting that several mutations at or near the third Ca^{2+} site have been implicated in several human diseases. A missense mutation of a third Ca^{2+}-coordinating residue, D615N, in TMEM16K was identified in a spinocerebellar ataxia type 10 (SCAR10) patient with unknown pathophysiology (Balreira et al., 2014). The equivalent mutation in TMEM16A, D884N, was shown to also reduce channel activation (Le and Yang, 2020). Linkage analysis with exome-sequencing identified 6 pathogenic mutations in TMEM16C that are associated with autosomal-dominant craniocervical dystonia, most notably two missense mutations R494W and W490C (Charlesworth et al., 2012). The W490 and R494 residues are located within TM2 at the putative third Ca^{2+} site flanking the highly conserved K491, which is equivalent to murine TMEM16A K428, murine TMEM16F K398, or TMEM16K K262 (Alvadia et al., 2019; Bushell et al., 2019). A more complete understanding of the third Ca^{2+} site could provide further insight into the human pathophysiological role of these clinically relevant mutations.

PI(4,5)P_2-Dependent Regulation of TMEM16 Proteins

Despite constituting only a minor part in the inner leaflet of the plasma membrane, phosphatidylinositol-(4,5)-bisphosphate [or PI(4,5)P_2] is known to regulate a large number of ion channels and transporters (Suh and Hille, 2008; Hille et al., 2015). PI(4,5)P_2 was initially suggested to play an inhibitory role in regulating endogenous TMEM16A channels in rat pulmonary artery cells (Pritchard et al., 2014). Reducing PI(4,5)P_2 levels *via* PLC activation or PI4K inhibition potentiates Ca^{2+}-dependent currents of TMEM16A in pulmonary artery smooth muscle cells, whereas addition of PI(4,5)P_2 markedly reduces its activity. However, it is worth noting that several approaches used to alter PI(4,5)P_2, namely PLC activation or inhibition of PI4K, could also affect other intracellular signaling events that may lead to changes in intracellular Ca^{2+}. One possibility is activation of PLC, while reducing PI(4,5)P_2 also leads to additional Ca^{2+} release from internal stores, thereby enhancing TMEM16A activation. In fact, numerous subsequent studies from several laboratories all suggested that PI(4,5)P_2 serves as a positive regulator of TMEM16A (Ta et al., 2017; De Jesus-Perez et al., 2018; Le et al., 2019a; Tembo et al., 2019; Yu et al., 2019; Ko et al., 2020) and paradoxically a negative regulator of TMEM16B CaCC (Ta et al., 2017). Depletion of membrane PI(4,5)P_2 rapidly desensitizes TMEM16A's channel activity elicited by sub-micromolar Ca^{2+} both in whole-cell and excised patch recordings. This desensitization under sub-micromolar Ca^{2+} can be rapidly recovered by exogenous application of PI(4,5)P_2 (Le et al., 2019a; Yu et al., 2019). A hallmark feature of TMEM16 CaCCs is their prominent rundown during prolonged Ca^{2+}-dependent activation (Wang and Kotlikoff, 1997; Kuruma and Hartzell, 2000; Ayon et al., 2019; Tembo et al., 2019); exogenous PI(4,5)P_2 application largely attenuates TMEM16A's rundown under saturating Ca^{2+} in excised membrane patches (Reisert et al., 2003; De Jesus-Perez et al., 2018; Le et al., 2019a; Tembo et al., 2019).

To gain further insight into the molecular basis of PI(4,5)P_2-dependent regulation of TMEM16A, unbiased mutagenesis screens were conducted to identify basic residues that play important roles in desensitization in TMEM16A (Le et al., 2019a; Yu et al., 2019). On one hand, Le et al. identified a cluster of basic residues located on the cytosolic sides of TM3, 4, 5, and the TM2-3 loop as the potential binding site for PI(4,5)P_2 (**Figure 3**). MD simulations further support spontaneous and favorable PI(4,5)P_2 binding to this putative site in TMEM16A. Supporting the modular design proposed above, mutating the basic residues in TM3-5 elicits no discernible effects on Ca^{2+}-dependent channel gating, despite pronouncedly enhancing current rundown under saturating Ca^{2+} (**Figures 1B,C**). On the other hand, Yu et al. reported that TMEM16A may harbor a network of PI(4,5)P_2 binding sites, most notably sites A/1, B/2, and C/4 (Yu et al., 2019). Site A/1 is located near the dimer interface and formed by R429, K430, and R437 of TM2 and K313 of pre-TM1 (**Figure 3**). Site B/2 is located at the cytosolic C-terminal end of the gating TM6 and mainly consists of K682 (K678 in the (a) isoform), R683 (R679), and K684 (K680; **Figure 3**). As TM6 and TM7 are both involved in Ca^{2+} binding, PI(4,5)P_2 binding could directly affect Ca^{2+}-dependent channel gating. Finally, site C/4 is situated on TM2-3 loop and is defined by R461 (R457), K480 (K476), and R484 (R480; **Figure 3**). This site spatially overlaps with the PI(4,5)P_2 binding site proposed by Le et al., which comprises the TM2-3 linker as well as cytosolic segments of TM3-5 (Le et al., 2019a). MD simulations by Yu et al. also revealed that binding of PI(4,5)P_2 alters the conformation of the gating TM6 helix, increasing Cl^- accessibility, and that occupancy of multiple PI(4,5)P_2 binding sites led to further dilation of the permeation pathway (Yu et al., 2019).

More recently, Ko et al. reported that TMEM16A exhibits isoform-specific PI(4,5)P_2 sensitivity (Ko et al., 2020). By co-expressing TMEM16A with the voltage-sensitive lipid phosphatase DrVSP and using whole-cell configuration with 115 or 445 nM intracellular Ca^{2+}, the authors showed that PI(4,5)P_2 hydrolysis following membrane depolarization-induced activation of DrVSP led to reduced TMEM16A activity. Interestingly, the TMEM16A(ac) isoform is more sensitive toward PI(4,5)P_2 depletion than the TMEM16A(a) isoform, which lacks the EAVK segment in the TM2-3 loop. Consistent with the proposed PI(4,5)P_2 binding site reported by Le et al.,

Ko et al. also identified R482 (R486 in the TMEM16A(ac) isoform) in TM2-3 loop as the most critical residue for PI(4,5)P_2 binding (**Figure 3**). Mutation of R482 to alanine abolishes TMEM16A's PI(4,5)P_2 sensitivity, as evidenced by the lack of inhibitory effects on mutant channel activity following PI(4,5)P_2 degradation by DrVSP. Pharmacological inhibition of CaMKII promotes TMEM16A opening due to augmented single channel conductance. Notably, S669 (S673) at the cytosolic end of TM6 is likely the substrate for CaMKII-mediated phosphorylation, as the phosphomimetic mutation S669D reduces, whereas the S669A mutation enhances the PI(4,5)P_2 sensitivity of TMEM16A. These results hint at an allosteric mechanism involving PI(4,5)P_2 binding and CaMKII-dependent phosphorylation in controlling TMEM16A channel activity.

A recent study using multi-microsecond atomistic simulations in explicit solvent and membrane found that specific binding of PI(4,5)P_2 to the proposed binding site in TM3-5 consistently leads to spontaneous pore opening, which is wide enough to allow Cl^- permeation (Jia and Chen, 2021). This pore opening is mediated by the separation of TM4 and TM6 as well as by increased hydration at the central constriction site. It was suggested that upon PI(4,5)P_2 binding, the cytosolic end of TM4 moves toward PI(4,5)P_2, whereas its N-terminus (towards the outer leaflet) moves in the opposite direction, thereby separating from TM6 and widening the central constriction site. The "pivot" movement of TM4 is endowed by the helix–helix packing between TM4 and TM5 on the intracellular side. This proposed PI(4,5)P_2-dependent gating in TMEM16A is reminiscent of the TMEM16 scramblases in which disruption of the TM4 and TM6 interaction leads to opening of the lipid pathway (**Figure 1D**; Falzone et al., 2019; Kalienkova et al., 2019).

Ion channel activity of TMEM16F also exhibits a reduced Ca^{2+} sensitivity and pronounced current rundown during prolonged Ca^{2+} stimulation, both of which were shown to be a result of the rapid dissociation and/or hydrolysis of endogenous membrane-bound PI(4,5)P_2 (Ye et al., 2018). Interestingly, an early study on the role of TMEM16F in accessory cholera enterotoxin-stimulated Cl^- secretion also suggested that inhibition of PI(4,5)P_2 synthesis or depletion of PI(4,5)P_2 markedly attenuated TMEM16F-mediated Cl^- current in Caco-2 cells (Aoun et al., 2016). It was suggested that PI(4,5)P_2 may interact with TMEM16F at two adjacent sites (or KR motifs) at the N-terminus formed by two clusters of basic residues: one proximal site formed by K281-K290 and one distal site formed by K87-R98 (numbering based on the mouse TMEM16F; Aoun et al., 2016; **Figure 3**). However, whereas mutation or deletion of the distal KR motif did not affect PI(4,5)P_2 binding, mutation of the basic residues at the proximal KR motif markedly reduced PI(4,5)P_2 binding, underscoring the functional importance of the proximal KR motif in PI(4,5)P_2 binding. Paradoxically, electrophysiological studies by Ye et al. (2018) suggested that neutralization of the distal KR motif, including K87, K88, K95, R96, K97, and R98, reduced TMEM16F Ca^{2+} sensitivity as well as the ability of exogenous PI(4,5)P_2 to rescue TMEM16F current after rundown. By contrast, neutralization of the basic residues in the proximal KR motif (K281, K282, R289, and K290) had no effect on TMEM16F Ca^{2+} sensitivity (**Figure 3**). While the reason for this discrepancy remains unknown, it could be attributed to their different functional studies—co-IP and electrophysiology–of TMEM16F in addition to the complexity of mutational analyses. Nevertheless, it is worth noting that K313 residue of TMEM16A, which belongs to the equivalent proximal KR motif (K313–K322), could be important for PI(4,5)P_2 binding, as its mutation significantly reduced the stimulatory effect of PI(4,5)P_2 on TMEM16A (Yu et al., 2019; **Figure 3**). So far, no basic residues in TMs 3–5 of TMEM16F, which are equivalent to the proposed regulatory module in TMEM16A (Le et al., 2019a; Yu et al., 2019; Ko et al., 2020), have been implicated in PI(4,5)P_2 binding. This implies that TMEM16A and TMEM16F may maintain distinct PI(4,5)P_2-dependent regulation.

A recent structural study revealed the potential structural role of PI(4,5)P_2 in regulating TMEM16F scrambling (Feng et al., 2019). In the absence of PI(4,5)P_2, TM6 adopts a straight conformation and PI(4,5)P_2 supplementation allows it to undergo a pronounced upward movement toward the membrane to widen the intracellular vestibule without changing the ion permeation pore, especially the upper constriction region (Feng et al., 2019). The resulting kinked conformation of TM6 at P628 causes distortion and thinning of the membrane, which is believed to be an important factor for lipid scrambling in TMEM16F (Bethel and Grabe, 2016; Falzone et al., 2019; Kalienkova et al., 2019). Future functional and structural studies are needed to examine if PI(4,5)P_2 indeed plays a regulatory role in TMEM16F scrambling and whether such PI(4,5)P_2-dependent conformational changes affect TMEM16F channel activity.

Intracellular pH Regulation of TMEM16 Proteins

Previous studies showed that low intracellular pH (pH_i) suppresses endogenous Ca^{2+}-activated Cl^- channels (CaCCs) from the human colon carcinoma cell line T84 and lacrimal gland acinar cells (Arreola et al., 1995; Park and Brown, 1995). Consistent with these observations, low pH_i was shown to strongly inhibit channel activation of heterologously expressed TMEM16A, TMEM16B, and TMEM16F ion channel activity (Chun et al., 2015). Low pH_i causes a rightward shift in the Ca^{2+} EC_{50} curves of TMEM16A and TMEM16B without affecting the voltage-dependent, heat-dependent, or E_{act}-mediated (E_{act} is a putative activator of TMEM16A) activation of TMEM16A. The authors further demonstrated that double mutation of Ca^{2+} binding residues in TM6-8, including N650A/E654Q (TM6, numbering based on the TMEM16A(ac) isoform), E702Q/E705Q (TM7), and E734Q/D738N (TM8) abolished this proton-mediated inhibition. Based on this evidence, the authors proposed that protons may inhibit TMEM16A channel activation by competing with Ca^{2+} binding to Ca^{2+} binding sites in TM6-8.

A recent comprehensive investigation of pH_i regulation on TMEM16 proteins, including TMEM16A ion channel activity and TMEM16F ion channel and lipid scrambling activities,

was conducted using a patch clamp-lipid scrambling fluorometry (PCLSF) assay (Liang and Yang, 2021). Consistent with previous results in HEK293 cells (Chun et al., 2015) and in native cells (Arreola et al., 1995; Park and Brown, 1995), low pH_i was found to significantly attenuate TMEM16A and TMEM16F ion channel activities and TMEM16F lipid scrambling activity. In addition, high pH_i largely potentiates TMEM16A and TMEM16F ion channel activities and TMEM16F-lipid scrambling activity. Mechanistically, pH_i exerts its effect specifically on the two primary Ca^{2+} binding sites, as evidenced by the following results. First, the binding site point mutation E667Q significantly suppresses intracellular pH sensitivity of TMEM16F ion channel activity, consistent with previous results (Chun et al., 2015). Second, pH_i exerts negligible effects on the pore-lining residue, Q559K, and the third Ca^{2+} binding site, D859A and E395A. Third, pH_i exerts no effect in the absence of intracellular Ca^{2+} on GOF mutations, namely TMEM16A L543Q and Q645A and TMEM16F Y563K and F518K. Based on these observations, pH_i regulatory effects were proposed to stem from protonation or deprotonation of the Ca^{2+} binding sites, which in turn reduces or enhances Ca^{2+} binding affinity, respectively. Identifying the molecular underpinning of pH_i regulation of TMEM16 ion channel and scrambling activities will help contextualize their physiological and pathological roles, such as in platelet activation, tumor progression, and sperm–egg fusion (Whitlock, 2021).

Extracellular pH Regulation of TMEM16 Proteins

In contrast to the effects by pH_i on TMEM16A, low extracellular pH enhances TMEM16A channel opening without altering the apparent Ca^{2+} sensitivity (Cruz-Rangel et al., 2017). This suggests that extracellular pH does not exert its effect through the Ca^{2+} binding sites like pH_i. Using mutagenesis screening of the extracellular acidic residues, the authors found that one residue, E623, located at the extracellular end of TM6, largely suppresses the effect of extracellular pH on TMEM16A when mutated to alanine. They suggested that protons likely function by promoting protonation of E623, which reduces the energy barrier for Cl^- entry. It should be noted that E623 (E619 in the (a) isoform) of TM6 and R515 (R511) together constitute the equivalent SE site proposed by Bethel and Grabe (2016). As this residue is highly conserved in all the TMEM16 family proteins, it is likely that extracellular pH also influences other TMEM16 members, including TMEM16F. Future investigations will be needed to assess the effects of extracellular pH on other TMEM16 members.

FUTURE PERSPECTIVES

Structural, functional, and computational studies in the past decade have greatly advanced our understanding of TMEM16 proteins at the molecular level. In the next phase, the answers to the following questions will further advance our understanding of these enigmatic proteins. First, it will be important to demonstrate how the third Ca^{2+} site is allosterically coupled to the PGD and how all three Ca^{2+} bindings sites synergistically control TMEM16 activation under physiological conditions. Second, future investigations are needed to dissect how Ca^{2+} and voltage synergistically operate TMEM16 gating. The answer to this question is critical to uncover the physiological functions of TMEM16 proteins in excitable cells such as neurons and muscles. Together, we have started to understand the molecular mechanisms of TMEM16 ion and lipid permeation and identified several molecular determinants that define whether a TMEM16 protein is a sole ion channel or a dual function scramblase/ion channel. Comprehensive studies are needed to demonstrate how ion and phospholipid permeation are dynamically controlled by Ca^{2+}- and voltage-induced conformational changes in the PGD. Substantial progress has been made on deciphering how pH and $PI(4,5)P_2$ regulate TMEM16 proteins. Identifying other physiological regulatory factors, such as post-translational modifications, are needed to further reveal how TMEM16 protein activities are fine-tuned under physiological conditions. Additionally, the Ca^{2+}-bound TMEM16A and TMEM16F structures were captured in non-conductive states. Future structural studies are needed to capture the open conformations, which will enhance our understanding of TMEM16 gating transitions in response to Ca^{2+} and voltage stimulation. Apart from the ER-resident TMEM16K, the other mammalian TMEM16 proteins expressed in intracellular organelles are largely uncharacterized. Functional and structural characterization of these TMEM16 proteins will help us better evaluate their biological functions in health and disease. Finally, the evolutionary relationships between TMEM16, OSCA/TMEM63, and TMC proteins within the transmembrane channel-scramblase (TCS) superfamily are intriguing. A combination of structural, functional, and computational approaches is needed to unveil the molecular underpinnings of how this superfamily of membrane ion channels and scramblases posses different permeation, activation, and gating properties. In this review, we summarize the collective efforts from the TMEM16 field over the past decade. We propose a "modular design" model for TMEM16 assembly, and the "clam-shell" and "pore-dilation" gating/permeation models for TMEM16 scramblases and channels, respectively. We hope these simplified models serve as a steppingstone for answering the aforementioned questions, driving the field forward.

AUTHOR CONTRIBUTIONS

SCL, PL, AJL, and HY wrote the manuscript. SCL plotted all the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Hypoxic Regulation of the Large-Conductance, Calcium and Voltage-Activated Potassium Channel, BK

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Hypoxia is a condition characterized by a reduction of cellular oxygen levels derived from alterations in oxygen balance. Hypoxic events trigger changes in cell-signaling cascades, oxidative stress, activation of pro-inflammatory molecules, and growth factors, influencing the activity of various ion channel families and leading to diverse cardiovascular diseases such as myocardial infarction, ischemic stroke, and hypertension. The large-conductance, calcium and voltage-activated potassium channel (BK) has a central role in the mechanism of oxygen (O_2) sensing and its activity has been related to the hypoxic response. BK channels are ubiquitously expressed, and they are composed by the pore-forming α subunit and the regulatory subunits β (β1–β4), γ (γ1–γ4), and LINGO1. The modification of biophysical properties of BK channels by β subunits underly a myriad of physiological function of these proteins. Hypoxia induces tissue-specific modifications of BK channel α and β subunits expression. Moreover, hypoxia modifies channel activation kinetics and voltage and/or calcium dependence. The reported effects on the BK channel properties are associated with events such as the increase of reactive oxygen species (ROS) production, increases of intracellular Calcium ($[Ca^{2+}]_i$), the regulation by Hypoxia-inducible factor 1α (HIF-1α), and the interaction with hemeproteins. Bronchial asthma, chronic obstructive pulmonary diseases (COPD), and obstructive sleep apnea (OSA), among others, can provoke hypoxia. Untreated OSA patients showed a decrease in BK-β1 subunit mRNA levels and high arterial tension. Treatment with continuous positive airway pressure (CPAP) upregulated β1 subunit mRNA level, decreased arterial pressures, and improved endothelial function coupled with a reduction in morbidity and mortality associated with OSA. These reports suggest that the BK channel has a role in the response involved in hypoxia-associated hypertension derived from OSA. Thus, this review aims to describe

the mechanisms involved in the BK channel activation after a hypoxic stimulus and their relationship with disorders like OSA. A deep understanding of the molecular mechanism involved in hypoxic response may help in the therapeutic approaches to treat the pathological processes associated with diseases involving cellular hypoxia.

Keywords: obstructive sleep apnea, BK channel, hypoxia, cardiovascular disease, MaxiK channel

INTRODUCTION

Calcium and voltage-activated potassium channels (BK channels) are ubiquitously expressed potassium (K^+) channels involved in diverse physiological processes like the regulation of smooth muscle arterial tone (Latorre et al., 2017). The BK channel activity is modulated by different stimulus like membrane depolarization and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Functional variety of BK channels is associated with posttranscriptional and posttranslational modifications along with the association with accessories subunits called β (β1–β4), γ (γ1–γ4) and LINGO1 (Torres et al., 2007; Contreras et al., 2013; Dudem et al., 2020). BK channel activation causes membrane hyperpolarization, leading to a decrease in vasoconstriction and relaxation of the blood vessels. Modifications in channel activity increase the risk of pathologies associated with the vascular tone as hypertension (Amberg and Santana, 2003). Hypoxia, a condition where the level of oxygen is decreased, promotes diverse cellular responses as regulation of gene transcription, membrane depolarization, and changes in $[Ca^{2+}]_i$ (Prabhakar et al., 2001). Hypoxia modifies the function of diverse ion channels, including BK channels, through changes in the α/β subunits expression or changes in channel activity (McCartney et al., 2005). Diverse pathophysiological clinical conditions, including chronic obstructive pulmonary disease (COPD), asthma, and obstructive sleep apnea (OSA), are associated to hypoxia (Prabhakar et al., 2001). OSA is a disorder that induces chronic intermittent hypoxia by the predisposition of upper airway to fail during sleep (Sforza and Roche, 2016). Patients with OSA develop alterations including cognitive and cardiovascular diseases (Bradley and Floras, 2009; Benjafield et al., 2019). A myriad of mechanisms are involved in the effects of OSA in disease commencement and/or progression, and the function of BK channel has been proposed to play a critical role in the development of these pathologies (Navarro-Antolín et al., 2009; Caballero-Eraso et al., 2019). This review describes the diverse mechanisms involved in the modulation of the BK channel activity by hypoxia. In addition, we will discuss the role of the BK channel modulation in the progress of cardiovascular diseases in patients with OSA.

CELLULAR AND MOLECULAR MECHANISMS ASSOCIATED WITH HYPOXIA RESPONSES

Oxygen (O_2) is the central molecule for cellular respiration in aerobes. The level of O_2 is controlled by specialized cellular sensors that are fundamental for maintaining its homeostasis. The fine regulation of O_2 balance underlies cellular and overall physiological processes (Vjotosh, 2020). In humans, the rate of breathing regulates the availability of O_2, which diffuses into the blood, binds to hemoglobin, and is distributed to all tissues (Schödel and Ratcliffe, 2019). In hypoxia, the level of O_2 is insufficient for the maintenance of normal cellular function (Ziello et al., 2007). However, hypoxia can also be induced in normal physiological conditions during physical activity. The carotid body is the main peripheral organ sensing fluctuations in arterial O_2, which is measured by changes in O_2 tension. These variations trigger the reflex responses that aim to increase the pulmonary gas exchange, suppressing the hypoxia effects (Jonz et al., 2016). In addition, every cell needs mechanisms that ensure an adequate cell performance under diverse O_2 concentrations and hypoxia (Schödel and Ratcliffe, 2019). For instance, in hypoxic episodes, different signals promote the enhancing of respiration to increase O_2 levels in the lung by the constriction of lung vascular smooth muscle to improve blood flow and promote O_2 delivery to different tissues. Moreover, metabolic pathways are modified to reduce oxygen consumption (Seta et al., 2004; Nakayama and Kataoka, 2019).

Hypoxia induces the activation of various mechanisms, including the modulation of ion channels, and the regulation of transcription factors that mediate the expression of several genes involved in the hypoxic response. Inhibition of voltage-gated K^+ channels prompt membrane depolarization in excitable O_2-sensing cells as glomus cells in the carotid body. Hypoxia also induces changes in $[Ca^{2+}]_i$ that conduces to the activation of protein kinases and phosphatases along with Ca^{2+}-dependent signaling pathways involved in hypoxia gene expression (Seta et al., 2004). Therefore, $[Ca^{2+}]_i$ variations are the primary response of many cell types to hypoxia and have a significant role in the modulation of signaling pathways and gene expression.

Hypoxia-inducible factor (HIF) is a heterodimer comprising an oxygen-regulated α subunit and a constitutively expressed β subunit (Wang et al., 1995; Semenza, 2007). HIFs are transcription regulators that bind to specific DNA regions called hypoxia-responsive element (HRE), and are modulated by O_2 level (West, 2017). In normoxia, HIF-1α is hydroxylated and binds the von Hippel-Lindau (VHL), a protein that targets HIF-1α for degradation via ubiquitin-proteasome (Tanimoto, 2000; Shimoda and Semenza, 2011). Diverse signaling pathways regulate HIF. Phosphorylation induced by PI3K/Protein kinase B (Akt) and protein kinase A (PKA) inhibits proteasomal degradation of HIF-1α. Additionally, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) promotes HIF-1α nuclear accumulation (Kietzmann et al., 2016; Xiao et al., 2017). In hypoxia, HIF-1α hydroxylation is inhibited and it is not targeted for degradation. Then, it translocates to the nucleus, where it dimerizes with HIF-1β. The dimer binds to

specific HRE and regulates different target genes involved in the adaptation to hypoxia. These events increase the non-oxidative synthesis of ATP and prevent the excess of mitochondrial ROS generation, improving the O_2-carrying capacity of blood and increasing the number of vessels irrigating the hypoxic tissues (Chandel et al., 1998; López-Barneo et al., 2001; Semenza, 2012). HIF regulates the expression of several hypoxic-associated genes, such as vascular endothelial growth factor (*VEGF),* endothelial nitric oxide synthase *(eNOS),* leptin *(LEP),* LDL-receptor-related protein 1 *(LRP1),* adrenomedullin *(ADM),* epidermal growth factor *(EGF)*, metabolism [glucose transporter *(GLUT 1/3),* hexokinase *(HK1/2),* pyruvate dehydrogenase kinase 1 *(PDK1),* pyruvate kinase M *(PKM),* lactate dehydrogenase *(LDHA)*], cell proliferation [myelocytomatosis virus oncogene cellular homolog (c-*MYC),* insulin-like growth factor 2 *(IGF2),* DNA-binding protein inhibitor *(ID2),* and inducible nitric oxide synthase *(iNOS)],* transforming growth factor α *(TGFα)* and BCL2 Interacting Protein 3 *(BNIP3)* (Kelly et al., 2003; Manalo et al., 2005; Mole et al., 2009; Rey and Semenza, 2010; Shimoda and Semenza, 2011; West, 2017; Schödel and Ratcliffe, 2019; Sousa Fialho et al., 2019). HIF-1α also modulates redox signaling pathways in the heart. Particularly, during acute or chronic hypoxia where ROS is increased. The associated mechanism involves the up-regulation of the transcription levels of pro-oxidant molecules (Karar et al., 2007). Although some studies suggest that hypoxia decrease oxidative stress by increasing antioxidant activity, most of the studies demonstrate that hypoxia-induced ROS has a harmful effect (Aguilar et al., 2018).

Diverse cellular consequences of hypoxia have been reported. In addition to regulation of expression of genes by transcription factors, ion channel families have been described as direct effectors of the hypoxic response. A given ionic conductance, depending of the cells where they are expressed, can be inhibited or activated in response to hypoxia (López-Barneo et al., 2001). The increase in resistance of pulmonary arterioles, produced by the hypoxia-induced rise in arterial smooth muscle cell tone, is associated with the modulation of different ion channels activity. Transient Receptor Potential (TRP) channels, L-type Ca^{2+} channels, K_v channels as BK, and TWIK-related tandem pore domain acid-sensitive K^+ channel (TASK)-type are important to set the resting membrane potential and modulate membrane depolarization (Wang J. et al., 2006; Weissmann et al., 2006; Sommer et al., 2008; Whitman et al., 2008; Jonz et al., 2016). In the next sections, we will describe the direct effect of hypoxia on the activity of the large conductance, Ca^{2+} -activated K^+ (BK), and their association with pathological conditions underpinning cardiovascular diseases.

HYPOXIA AND CARDIOVASCULAR DISEASES

Hypoxia can relieve or intensify the severity of risk factors associated with cardiovascular diseases (CVD) (Ullah and Wu, 2021). Chronic hypoxia is associated with the generation of pathological conditions, such as ischemic ventricular arrhythmias, cardiomyocyte and cardiac death, pulmonary hypertension, and liver fibrosis (Madjdpour et al., 2003; Mesarwi et al., 2016; Moczydlowska et al., 2017; Morand et al., 2018; Corrado and Fontana, 2020). On the contrary, acute hypoxia has been demonstrated to have a protecting role in acute liver and kidney diseases, and in myocardial ischemia (Schellinger et al., 2017; Shu et al., 2019; Gao et al., 2020; Ullah and Wu, 2021).

Ischemic heart disease, heart failure, or OSA, among other diseases, can induce hypoxic conditions. Hypoxia induces activation of HIF and their associated signaling pathways (Corrado and Fontana, 2020). OSA is a disorder where recurrent pauses of ventilation during the sleep conduce to upper airway obstruction, intermittent hypoxemia, and the increase in respiratory effort (Chen et al., 2021; Diamond and Ismail, 2021; Mochol et al., 2021). OSA has a high prevalence depending on age and ethnic group, and it is a major public health issue due to its effect on work performance and productivity (De Luca Canto et al., 2015). Benjafield et al. (2019) estimated that OSA affects almost 1 billion people in the world (Benjafield et al., 2019). There are reports about the modification of the equilibrium pro/antioxidant as patients with OSA show a rise in the superoxide anion level, lipid peroxidation, and diminished antioxidant mechanism (Arnaud et al., 2020). The latter is associated with recurrences of hypoxia-reoxygenation cycles and production of superoxide anion, which promotes the stabilization of HIF-1α through the activation of phospholipase C pathway (Belaidi et al., 2016; Farías et al., 2016). OSA adversely alters the cardiovascular structure, inducing changes such as endothelial dysfunction. Additionally, OSA is reported as an independent risk factor for initiation or progression of cardiovascular diseases like hypertension, heart failure, ischemic heart disease, and stroke (Marin et al., 2005; Bradley and Floras, 2009; Wang et al., 2013; Orrù et al., 2020; Chen et al., 2021; O'Donnell et al., 2021). The treatment of OSA includes continuous positive airway pressure (CPAP) and mandibular advancement devices (MAD), but there is no effective drug therapy for OSA. CPAP and MAD keep the upper airway open during sleep. It has been suggested that CPAP and MAD therapies attenuate some cardiovascular diseases including atherosclerosis, hypertension, heart failure, and cardiac arrhythmias (Barnes et al., 2004; Gotsopoulos et al., 2004; Bradley and Floras, 2009; Marklund et al., 2019; Yamamoto et al., 2019; Chen et al., 2021; O'Donnell et al., 2021).

BK CHANNEL STRUCTURE, LOCALIZATION, AND MODULATION

Large conductance, Ca^{2+} -activated K^+ (BK) channels are K^+ channels ubiquitously expressed in mammalian cells. They are involved in different physiological processes like the regulation of smooth muscle arterial tone and neurotransmitter release, among others (Latorre et al., 2017). BK channels are tetramers where each subunit is constituted by the pore-forming α subunit formed by seven transmembrane domains (S0-S6), being the NH_2 termini exposed to the extracellular side, and the COOH termini facing the intracellular. In that region, there are two regulators of conductance of K^+ domains named RCK1 and RCK2 (Meera et al., 1997; Jiang et al., 2001; Torres et al., 2007; Wu et al., 2010;

Pantazis and Olcese, 2016). BK α subunit has a modular organization with the pore region comprised between the S5-S6 region and the voltage sensor located in S0-S4 transmembrane domains (Meera et al., 1997; Díaz et al., 1998). The presence of multiple Ca^{2+} -binding sites confers to the channel sensitivity to a vast Ca^{2+} concentration range (0.1–100 μM) (Bao et al., 2004; Latorre and Brauchi, 2006). The Ca^{2+} -binding site called Ca^{2+} bowl, located in the RCK2, acts as a high affinity Ca^{2+} binding site (Wu et al., 2010). Functional diversity of the channel is generated by different BKα splice variants, posttranslational modifications, and the association with auxiliary β, γ, and LINGO1 subunits (Contreras et al., 2013; Pantazis and Olcese, 2016; Dudem et al., 2020).

Alternative splicing at the C terminus region produces diverse splice variants of the BK channel including ZERO (channels without the STREX insert) and Stress-Regulated Exon (STREX), which have cellular differential expression and tissue distribution (Zarei et al., 2001; Chen et al., 2005; Contreras et al., 2013). STREX variant is a highly conserved motif within an alternatively spliced cysteine-rich insert. This feature confers to the channel sensitivity to high Ca^{2+} and hypoxia (Shipston et al., 1999). Hypoxia induces a reduction in the BK channel open probability (Po) by a Ca^{2+} -dependent shift to the right in the voltage activation. Residues C23, S24, and C25 are critical for the hypoxic response in the STREX variant (McCartney et al., 2005). ZERO is a cAMP-sensitive splice variant that induces a lower Ca^{2+} sensitivity, compared with the STREX variant (Shipston et al., 1999; Friis et al., 2003).

The physiological function of BK channels varies depending on both cell type and cellular localization. For example, in sinoatrial node (SAN) cells and vascular smooth muscle, BK channels are expressed in the plasma membrane (Leblanc et al., 1994; Gollasch et al., 1996; Lai et al., 2014), whereas in cardiomyocytes, BK channels are localized at the inner mitochondrial membrane (IMM) (Singh et al., 2013; Goswami et al., 2019).

Sinoatrial node (SAN) cells act as a pacemaker of the cardiac conduction system, and thus, determine the heart rate (Mangoni and Nargeot, 2008). The functional expression of BK channels in SAN cells have been demonstrated using paxilline, a BK channel inhibitor. Paxilline administration in mice reduces the heart rate in wild-type (WT) but not in BK channel α subunit knock-out animals (*kcnma1*$^{-/-}$). Specifically, paxilline decreases action potential firing in SAN cells. Immunocytochemistry revealed that BK channels are expressed in the plasma membrane and partially overlapped with the hyperpolarization activated cyclic nucleotide gated K^+ channel 4 (HCN4) (Lai et al., 2014). BK channels are also expressed in human coronary artery vascular smooth muscle cells (Gollasch et al., 1996) and in rabbits' coronary myocytes. In these cells, their role is to maintain the resting membrane potential (Leblanc et al., 1994).

Calcium and voltage-activated potassium channels (BK) are also expressed in the IMM but not in the plasma membrane in mice cardiomyocytes. In this cell type, a splice variant from the plasma membrane *Kcnma1* gene is expressed in the IMM. This variant targets the mitochondria through its C-termini (Singh et al., 2013), which differs from other proteins that target mitochondria through its N-termini (Chacinska et al., 2009; Li M. et al., 2010). In mitochondria, the BK channels play an essential role in protecting the heart from ischemic insult (see section "Mitochondrial BK Channel Participate in Hypoxic Protection"). Moreover, in cardiomyocytes of infant rabbits, mitochondrial BK channels also protect the heart against ischemia (Shi et al., 2007).

The regulatory β1-subunit of BK channels is also expressed in cardiomyocytes, and it colocalizes with BK channels in the IMM. Furthermore, β1-subunit functionally interacts with BK channels increasing the Po. Additionally, β1-subunit increases the localization of BK channels at the IMM (Balderas et al., 2019).

Auxiliary Subunits Modulate the BK Channel Properties

Differential expression of BK auxiliary subunits confers to the channel functional diversity (**Figure 1**). In the smooth muscle, the α subunit is co-expressed with the β1 subunit, which prompts membrane hyperpolarization and vasorelaxation, decreasing the risk of pathologies associated with vascular tone regulation (Amberg and Santana, 2003). The β1-induced effects are associated with the improvement of apparent sensitivity to Ca^{2+} and slowing down the activation and deactivation kinetics. These effects are elicited by changes of the allosteric coupling between gating and the Ca^{2+} binding sites along with the reduction of the voltage dependence of the voltage sensor activation process (Nimigean and Magleby, 2000; Orio and Latorre, 2005; Contreras et al., 2012). β2 subunits also increase the apparent Ca^{2+} sensitivity and slowed the gating kinetics, associated with an increase in the allosteric coupling factors (Orio and Latorre, 2005). The β3 subunit has four members (β3a, β3b, β3c, and β3d). The most studied members are β3a and β3b, which induce BK channel inactivation (Brenner et al., 2000a; Uebele et al., 2000; Gonzalez-Perez and Lingle, 2019). It has been reported that β4 subunit slows the gating kinetics, reduces the apparent voltage-sensitive of the channel activation, and modifies the apparent Ca^{2+} sensitivity in two ways: by the inhibition of the channel activity in low $[Ca^{2+}]_i$ and by the increase of the channel activity in high $[Ca^{2+}]_i$. These effects are associated with the stabilization of the active conformation of the voltage sensor, and a reduction in the number of the gating charges per sensor (Brenner et al., 2000a; Wang B. et al., 2006; Contreras et al., 2012).

γ1 (LRRC26), γ2 (LRRC52), γ3 (LRRC55), and γ4 (LRRC38) subunits display tissue-specific expression and function (Yan and Aldrich, 2012; Evanson et al., 2014). They produce a significant modification in the voltage dependence of BK channel activation, recognized as a shift to negative potentials of ∼140, 100, 50, and 20 mV in the presence of γ1 (LRRC26), γ2 (LRRC52), γ3 (LRRC55), and γ4 (LRRC38), respectively (Yan and Aldrich, 2012; Zhang and Yan, 2014). The increase in voltage sensitivity induced by γ1 has been associated with vasodilation observed in arterial smooth muscle cells (Evanson et al., 2014). Moreover, knocking down the expression of the γ1 subunit contributes to vasoconstriction (Yan and Aldrich, 2012). γ1 subunit also induces the acceleration of the activation kinetics, while the deactivation kinetics become slower. It has been proposed that γ2, γ3, and γ4 subunits can modify the apparent Ca^{2+} sensitivity

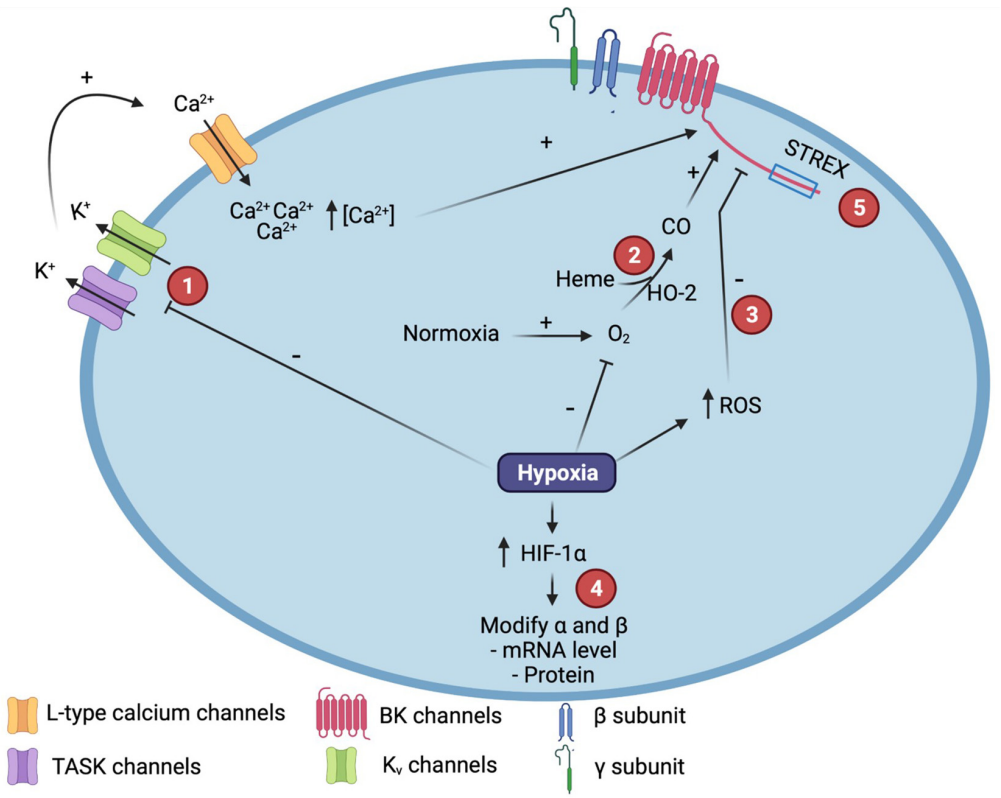

FIGURE 1 | Molecular mechanisms involved in the modulation of Calcium and voltage-activated potassium (BK) channel function by hypoxia. (1) Hypoxia promotes TWIK-related tandem pore domain acid-sensitive K^+ channel (TASK) or Kv channels inhibition that induces membrane depolarization, activation of L-type voltage-dependent calcium channels and the increase in intracelular calcium concentration, underpining BK channel activation. (2) In normoxia, hemeoxigenase (HO-2) catalyzes Heme degradation to produce carbon monoxide (CO), increasing the open probability (Po) of the BK channel. (3) ROS production increased after hypoxia induces the decreasing of BK channel Po. (4) After hypoxia, HIF-1α is increased and modulates BK subunits expression through the interaction with hypoxia-response elements (HRE). (5) STREX variant confers to BK channel sensitivity to hypoxia. ROS, Reactive Oxygen Species; HIF, Hypoxia Inducible Factor. Created with BioRender.com.

(Latorre et al., 2017). LINGO1 is an accessory subunit of BK channels that induces a rapid inactivation of the channel, slowing down the deactivation process and shifting their activation to negative potentials (Dudem et al., 2020).

Posttranslational Regulation of BK Channel

Palmitoylation is a post-translational modification associated with regulation of the BK channel plasma membrane localization without variations in single-channel conductance or the Ca^{2+} and voltage sensitivity. BK channel palmitoylation occurs in the STREX insert and in the residues C53, C54, and C56, located in the intracellular loop S0-S1 (Jeffries et al., 2010; Zhou et al., 2012).

On the other hand, BK α or β subunits undergo reversible posttranslational modifications as protein phosphorylation. These control BK channel activity. Protein kinase A (PKA), Ca^{2+}—and diacylglycerol-activated protein kinase C (PKC), cyclic guanosine monophosphate (cGMP) -activated protein kinase G (PKG), and adenosine monophosphate (AMP)-activated protein kinase (AMPK) have been reported to phosphorylate BK channel α subunit in serine, threonine, and tyrosine residues. The phosphorylation in the BK channel activity is depending on the channel variant. For instance, PKA stimulates ZERO variant activation but induces STREX splice variant inhibition. The final effects of phosphorylation are tissue-depending, like inducing BK channel activation in smooth muscle but inhibit the channel activity in pituitary cells (Contreras et al., 2013; Kyle and Braun, 2014; Shipston and Tian, 2016).

Modulation of BK Channel Activity by Endogenous Molecules

Diverse endogenous molecules, including heme, carbon monoxide (CO), and reactive oxygen/nitrogen species, have been reported to activate BK channels (Hou et al., 2009; Kyle and Braun, 2014). The free intracellular heme binds to a region in the RCK1 and RCK2 segments and decrease or increase the BK channel Po at positive or negative voltages, respectively. These effects are modulated by the action of heme oxygenase enzyme (HO) by regulating the heme group degradation (Horrigan et al., 2005; Muñoz-Sánchez and Chánez-Cárdenas, 2014). Electrophysiological assays demonstrated that CO increase the Po of the channel in cell-free membrane patches, suggesting

channel modulation by CO direct binding in RCK1 domain (Jaggar et al., 2002; Hou et al., 2008; Williams et al., 2008).

Calcium and voltage-activated potassium (BK) channels redox modulation has been widely described (Hou et al., 2009). H_2O_2 decreases the Po through amino acid modifications. These are reversed by the addition of reducing agents as glutathione (GSH) or dithiothreitol (DTT). C433 and C911, located in the RCK1 and COOH terminus, respectively, have been reported to contribute to the sensitivity of the BK channel to reactive species (DiChiara and Reinhart, 1997; Tang et al., 2001, 2004; Soto et al., 2002; Brakemeier et al., 2003). STREX variant has additional cysteine residues that increase the inhibitory effect of oxidation in the channel (Erxleben et al., 2002). O_2^-, NO, and peroxynitrite are reactive molecules also affecting the BK channel activity (Hou et al., 2009). Finally, fatty acids, phospholipids, steroids, and other lipid metabolites can also modulate BK channel properties (Hou et al., 2009). In another way, it has been reported that decreases in intracellular pH increase BK channel activation by inducing a shift to the left in the voltage-dependence (Avdonin et al., 2003; Hou et al., 2009; Kyle and Braun, 2014).

As was described, the structural properties of the BK channels play different important physiological roles. In addition, both BK channel expression and its activity are regulated by various mechanisms which confer functional diversity to the channel. In the next section, we will describe the reported mechanism involved in the modulation of the BK channel by hypoxia and the possible physiological impact of that modulation.

BK CHANNEL MODULATION IN HYPOXIA

Calcium and voltage-activated potassium (BK) channel has been described as an oxygen-sensor, being one of the most important intermediaries in the hypoxic response of different tissues (McCartney et al., 2005). Hypoxic-mediated BK channel response is diverse, i.e., it is increased in some tissues while reduced in others. The modifications in the BK channel activity induced by hypoxia can be related to maintain the hypoxia response or decrease the hypoxic effects that induce cell damage such as changes in $[Ca^{2+}]_i$. These effects are mediated by diverse mechanisms including oxidative stress (Liu et al., 1999), heme protein interaction (Hoshi and Lahiri, 2004), the gene transcription regulation mediated by HIF-1α (Resnik et al., 2006; Ahn et al., 2012; Tao et al., 2015), and splice-variant-specific pathways (McCartney et al., 2005). In this section, we will describe the principal mechanisms involved in the BK channel response to hypoxia. Further, we revise the effects in both channel activity and BK subunits expression induced by decreased in O_2 level (**Figure 1**).

Hypoxic-Induced Modulation of the BK Channel Activity

It has been previously reported that hypoxia promotes a rise in the BK channel activity through membrane depolarization, and increases in $[Ca^{2+}]_i$. Hypoxia promotes membrane depolarization by different mechanisms including the inhibition of K^+ channels as $K_v1.2$ and $K_v1.5$ (Seta et al., 2004). Blocking K_v channels with the antagonist tetraethylammonium (TEA) evoked a similar response as hypoxia in glomus cells (Pardal et al., 2000; López-Barneo et al., 2001; Wang and Kim, 2018). TASK channels expressed in glomus cells are also hypoxic-inhibited channels. It has been reported that a decrease in cytosolic Mg-ATP or phosphorylation via AMP-activated kinases, among others, are events that promote TASK inhibition leading to membrane depolarization, consequent activation of L-type voltage-dependent Ca^{2+} channels, and rising in $[Ca^{2+}]_i$, and consequently increasing BK channel Po (Buckler, 2015; O'Donohoe et al., 2018).

Hippocampal neurons exposed to hypoxia for 6 h exhibited a significant rise in BK channel Po and unitary conductance after 6 h of reoxygenation. The normal parameters in channel activity were recovered after 24 h of reoxygenation. These results were associated with an increase in $[Ca^{2+}]_i$ without changes in the BK α subunit expression (Chen et al., 2013; Wang and Kim, 2018). Some authors have proposed that BK channels are active at rest, and that hypoxia inhibits their activity (Peers and Wyatt, 2007). On the contrary, some reports suggest that BK channels are closed in normoxia while hypoxia induces the channel activation to diminish the hypoxia-induced cell damage (Pardal et al., 2000; Gomez-Niño et al., 2009). The overall effect of hypoxia-induced BK channel activity on cell survival depends on specific factors present in a given cell type or tissue. For instance, the activation of the BK channel after hypoxia/reoxygenation induces hippocampal neuronal apoptosis. Meanwhile the hyperpolarization induced in glomus cells by BK channel activation constraints the rise in $[Ca^{2+}]_i$, and, therefore, limits the hypoxic response (Chen et al., 2013; Wang and Kim, 2018).

Changes in the Expression of the BK Channel Subunits Induced by Hypoxia

It is known that BK β subunits expression contributes to the molecular diversity of BK channels (Torres et al., 2014). An important role for BK β1 subunit in regulating vascular tone was demonstrated by systemic hypertension in mice carrying a deletion of the *Kcnmb1*, the gene encoding for β1 subunit (Brenner et al., 2000b; Chang et al., 2006). Additionally, the association between severity of asthma and a loss-of-function polymorphism in *Kcnmb1*, suggests a role of this subunit in modulating smooth muscle cells tone of airway in humans (Seibold et al., 2008). Different reports show that hypoxic response can also be modulated by differential expression of β subunits. In ovine pulmonary artery smooth muscle cells (PASMC), primary cultures, and ovine cerebral artery smooth muscle, hypoxia induced a rise in mRNA and protein expression level of both BKα and β1 subunits. Similar results were observed in rats that have been maintained in hypobaric hypoxic chambers for 3 weeks where mRNA levels of BKα and β1 subunits increased approximately threefold and twofold, respectively (Resnik et al., 2006; Tao et al., 2015).

The rise in the β1 subunit expression enhances the BK channel apparent sensitivity to $[Ca^{2+}]_i$ and the current density, leading to hyperpolarization, subsequent closing of voltage-gated Ca^{2+} channels, and decreasing the $[Ca^{2+}]_i$ (Torres et al., 2007; Castillo et al., 2015). The increase in BK channel activity may offer an adequate brain O_2 level when there is a lower arterial O_2 concentration, as it was reported in ovine artery smooth muscle (Tao et al., 2015).

Reported changes in the BK subunits expression are suggested to occur via post-translational modification by phosphorylation/dephosphorylation and by transcriptional regulation through the interaction with HIF-1α (Resnik et al., 2006; Ahn et al., 2012; Tao et al., 2015). After hypoxic treatment of hPASMC, it was observed an increase in the expression of both HIF-1α and KCNMB1 and knocking down HIF-1α avoided the hypoxia-induced KCNMB1 expression. In addition, it was demonstrated that human KCNMB1 promoter has HREs that are critical for HIF-1α binding and hypoxic-modulation of KCNMB1 expression (Ahn et al., 2012). *Kcnmb1*$^{-/-}$ mice showed higher right ventricular systolic pressure after hypoxic stimulus, compared with WT mice. These experiments demonstrated the importance of the BKβ1 subunit in the modulation of the pulmonary vascular response to chronic and acute hypoxia, and suggests a connection between BKβ1 expression and HIF-1α activity in the regulation of the tone in the microcirculation (Barnes et al., 2018).

Besides the increase in the BKβ subunit expression, it was reported that chronic hypoxia enhanced α/β colocalization at the plasma membrane without changes in mRNA expression, suggesting post-transcriptional regulation of the BK β subunit (Hartness et al., 2003). From these results, it has been proposed that hypoxic events originated from diverse cardiorespiratory diseases, conduct to adaptative cellular responses, including changes in the BK subunit expression or increase in functional α/β complex at the plasma membrane (Hartness et al., 2003).

In some cells/tissues a decrease in β subunit expression has also been reported after hypoxic stimuli. Rat myocytes exposed to prenatal hypoxia showed a reduction in the channel voltage and Ca^{2+} -sensitivity, associated with a drop in the BK β1 subunit mRNA and protein expression. These changes were without variations in the BKα subunit expression (Liu et al., 2018a,b). All these findings suggest that BK channel modulation induced by hypoxia is cell specific. BK channels expressed in rat vascular smooth muscle cells are functionally less active, decreasing the vasorelaxant effect of the channel and leading to high blood pressure, vascular dysfunction, and cardiovascular alterations (Lewis et al., 2002; Navarro-Antolín et al., 2005; Liu et al., 2018a). However, in ovine PASMC primary cultures and ovine cerebral smooth muscle, hypoxia induced a rise in mRNA and protein expression level of both BKα and β1 subunits.

The BKβ4 subunit expression is also modulated by hypoxia. In podocytes, an increase in BK β4 mRNA and protein levels were observed after chronic hypoxia without modifications in the expression of the BK α subunit. The higher expression of the β4 subunit decreases the BK channel activity by a shift in the voltage-dependent activation toward depolarized voltages, and a significant increase in the time constant for channel activation. In conclusion, Zhang et al. (2012), suggested that reducing BK channel activity promotes podocyte depolarization, leading to a decrease in Ca^{2+} influx through TRPC6 channels. The consequent change in $[Ca^{2+}]_i$ modifies Ca^{2+}-dependent signaling pathways associated with hypoxic response (Zhang et al., 2012).

In addition to increases in BK β subunit expression, a rise in mRNA from BK channel α subunit, and changes in the protein distribution were positively associated with the extent of the hypoxic response in pulmonary arteria. That response correlates with structural changes as alterations in the intimal thickening, suggesting an adaptative response in patients with the COPD to attenuate hypoxic pulmonary vasoconstriction (Peinado et al., 2008).

Mechanism Mediating BK Channel Response to Hypoxia

Calcium and voltage-activated potassium (BK) channel activity is modulated by additional mechanisms, including heme oxygenase-2 (HO-2), ROS regulation, and STREX-associated interaction (Hoshi and Lahiri, 2004; Navarro-Antolín et al., 2005; **Figure 1**). In mammalian cells, the enzyme HO-2 is implicated in the degradation of the heme group to produce biliverdin, iron, and carbon monoxide (CO). HO-2 function has been associated to O_2 sensing and hypoxic response through the regulation of the BK channel activity (Hoshi and Lahiri, 2004; Ortega-Sáenz et al., 2006; Muñoz-Sánchez and Chánez-Cárdenas, 2014). The mechanism involves the BK channel activation by CO and the physical interaction of HO-2 with the channel. It has been reported that knocking down of HO-2 induces a decrease in the expression of BK α subunit. Hypoxia can induce a redox dysregulation that prompts the HO-2 deficiency, decreases the CO levels, and reduces the channel activity (Naik and Walker, 2003; Williams et al., 2004; Ortega-Sáenz et al., 2006). Co-immunoprecipitation with HO-2 in carotid body cells was only observed when channels were expressing both α and β subunit (Riesco-Fagundo et al., 2001; Williams et al., 2004; Ortega-Sáenz et al., 2006). On the contrary, a weak interaction between HO-2 and BK channel was observed in pulmonary arterial smooth muscle. Furthermore, in mouse lines deficient in either HO-2 or BKα subunit it was not observed any role of these proteins in the hypoxic effects (Roth et al., 2009), suggesting that hypoxic-modulated interaction and function of HO-2 and BK channels could be tissue-specific and the interaction HO-2/BK channel could not be considered as a universal O_2 sensor. Currently, there are not reports related to the role of γ or LINGO1 subunits in the modulation of BK channel function by hypoxia.

Another mechanism involved in the BK channel modulation is associated with redox regulation of BK channel binding of the heme group. In normoxia, the heme group has a low affinity to the channel in contrast to the high affinity that shows for HO-2. That event induces heme degradation and an increase in CO, promoting channel activation. On the contrary, in hypoxia, HO-2 has a lower affinity for the heme group, generating an increase in heme concentration and decreasing CO production. Both stimuli

reduce the channel activity (Tang et al., 2003; Yi and Ragsdale, 2007; Ragsdale and Yi, 2011) (**Figure 1**).

BK channel is also modulated through cAMP and GMP-dependent protein kinase (PKG) by specific phosphorylation of multiples Ser residues in the C terminus of the α subunit. PKC-dependent phosphorylation shifts the voltage-dependence to more negative potentials, increasing BK channel voltage-sensitivity (Zhou et al., 2001; Kyle et al., 2013). In middle cerebral arteries, long-term hypoxia affected the PKG-modulation of the BK channel activity. It was found that hypoxia reduces the BKα subunit expression and decreases the association of PKG with the BK channel, inhibiting the channel activation and the consequent vasorelaxation (Thorpe et al., 2017).

On the other hand, hypoxia and endogenous H_2S induce similar inhibition of the BK channels in the carotid body. The inhibition mediated by H_2S endogenous production demonstrated the involvement of that molecule in the hypoxic-modulated BK channel activity, and suggest it as sensor for hypoxia in vasculature (Li Q. et al., 2010). A contrary effect was reported in vascular smooth muscle, where H_2S has a regulating function of myogenic tone through BK channel activation (Jackson-Weaver et al., 2011). These results demonstrated that BK channel modulation depends on the cell type, however, more studies are necessary to establish the effect of H_2S in hypoxic modulated BK channel activity.

Calcium and voltage-activated potassium (BK) channel inhibition, induced by low O_2 in neocortical neurons, is mediated by variations in the cellular redox potential, and depends on cytosolic factors, demonstrating the channel regulation by oxidative stress (Liu et al., 1999). Similarly, in rat CA1 hippocampal neurons, hypoxia induced a decrease in the BK channel Po due to a reduced mean channel open time and an increased closed time. Channel activity was restored after treatment with oxidizing agents, suggesting that hypoxia decreases cellular oxidation potential in CA1 neurons (Gao and Fung, 2002). Furthermore, hypoxia stimulates the production of reactive species ROS and reactive nitrogen species (RNS), and increases oxidative stress (Fresquet et al., 2006; Hu et al., 2016). In smooth muscle from sheep, uterine artery was reported a significant effect of stress oxidative in the inhibition of BK channel activity by hypoxia. These changes were showed to be mediated by downregulation of the BK β1 subunit (Zhu et al., 2014; Hu et al., 2016). The authors reported that the treatment with the antioxidant N-acetylcysteine (NAC) eliminates the hypoxic-mediated inhibition of the BK channel's current density, reestablished β1 expression, and restored the arterial tone. After NAC treatment, there were no differences in relaxation induced by the BK channel agonist NS1619 in normoxic and hypoxic animals. In addition, the antioxidant treatment also rescues the hormonal steroid effect induced on the BK channel activity. The proposed mechanism involves post-translational modifications induced by ROS and KCNMB1 gene repression (Zhu et al., 2014; Hu et al., 2016).

Reactive oxygen species (ROS) effect has been reported in cardiovascular diseases. Moreover, it has been proposed that oxidative stress induced by ROS impairs the BK channel activation by changing the cysteine 911 (C911), located in RCK2 near to the Ca^{2+} bowl. It was shown that adding H_2O_2 to the intracellular side induced the BKα + β1 channel inhibition through a decrease in Po and Ca^{2+} sensitivity without modification of the unitary conductance. Authors suggest that observed functional properties of the BK channel after oxidative stress are comparable to the found in absence of β1 subunit (Soto et al., 2002; Tang et al., 2004; Tano and Gollasch, 2014).

Hypoxia promotes a decrease in the modulatory effect that tamoxifen and steroid hormone induces on the BK channel activity, which was associated with the diminished expression of the BK β1 subunit (Navarro-Antolín et al., 2005; Liu et al., 2018a). Recently, we reported that changes in cholesterol concentration reduces the effects of 17β-Estradiol (E2) in the BK channel activity. However, that effect was not induced by changes in the expression of the BK β1 subunit (Granados et al., 2021). Considering that hypoxia reduces the membrane cholesterol concentration, we suggested that reported changes in the modulation of Tamoxifen and E2 after hypoxia could also be related to changes in membrane cholesterol concentration. However, it is necessary to carry on more experiments to unveil the complete mechanisms associated with that effect (Zhang et al., 2018).

McCartney et al. (2005) reported that BK channel sensitivity to hypoxia is splice-variant-specific, conferred by the stress-regulated exon (STREX) (**Figure 1**). The hypoxic inhibition induced in the STREX variant is Ca^{2+}-sensitive with no effect on single-channel conductance nor the voltage sensitivity. The hypoxic inhibition required Serine S24 and Cysteines C23 and C24, suggesting an important role of these residues in the hypoxic response. The ZERO variant was non-sensitive to hypoxia (McCartney et al., 2005; Tano and Gollasch, 2014).

Mitochondrial BK Channel Participate in Hypoxic Protection

In addition to plasma membrane BK channels, there are diverse reports about the involvement of mitochondrial BK channels ($mitoBK_{Ca}$) in the hypoxic response and protection against ischemic injury (Xu et al., 2002; Shi et al., 2007; Goswami et al., 2019). $mitoBK_{Ca}$ is expressed in adult cardiomyocytes and it is encoded by the splice variant of the *Kcnma1* gene DEC, which has an insert of 50 amino acids in the C-terminal region that is responsible for targeting the channel to mitochondria (Singh et al., 2013; Szteyn and Singh, 2020). $mitoBK_{Ca}$ has been reported to have similar properties to BK channels expressed in the plasma membrane (Xu et al., 2002; Balderas et al., 2019). These channels induce a cardioprotective mechanism that involves the decrease of ROS production and the depolarization of the mitochondria matrix by K^+ flux and the reduction of Ca^{2+} overloading during ischemia and reperfusion (Xu et al., 2002; Stowe et al., 2006; Shi et al., 2007; Borchert et al., 2011; Goswami et al., 2019). Interestingly, the reported effect was observed only in the normoxic hearts but not in chronic hypoxic hearts. Considering that BKα subunit expression was not changed, it is suggested that the effect was associated with a significant reduction in $mitoBK_{Ca}$ channel activity, probably via decreasing in Ca^{2+} sensitivity (Riesco-Fagundo et al., 2001; Shi et al., 2007). In addition to BKα

subunit, it has been demonstrated the mitochondrial expression of β1 subunit in cardiac tissue and myocytes, where it is critical to mediate cardioprotective response (Wang et al., 2008; Borchert et al., 2011; Balderas et al., 2019). These effects are not related to changes in the β1 subunit expression but are associated with activation of the channel possibly induced by modifications in glycosylation level of β1 subunit. However, more assays are necessary to prove the suggested hypothesis (Wang et al., 2008; Borchert et al., 2011).

Experiments using BK β1 KO mice demonstrated that mitoBK$_{Ca}$ channel activity in mitochondria was negligible when BK β1 subunit was absent, suggesting a reduction in the number of channels in mitochondria. Moreover, it was proposed a role of β1 in targeting BK channel to mitochondria. Activity of mitoBK$_{Ca}$ was associated with Ca^{2+} homeostasis in cardiac cells (Balderas et al., 2019). The presence of a second population of mitoBK$_{Ca}$ that activates at more depolarizing potentials suggests that BK can be associated with other regulatory subunits to produce channels with different voltage-sensitivity (Balderas et al., 2019). In addition, Frankenreiter et al. (2017), reported a positive regulation of mitoBK$_{Ca}$ by cGMP, which regulates ROS homeostasis in oxidatively stressed cardiomyocyte mitochondria and induces a significant increase in channel Po. That effect is associated with cardioprotective properties in models of ischemia/reperfusion injury possible through cGMP/cGKI (cGMP-dependent protein kinase type I) pathway (Frankenreiter et al., 2017).

Hypoxic modulation of the BK channel depends on cells or tissue. In some cells, hypoxic stimuli inhibit the BK channel activity. Meanwhile, in other cells, hypoxia promotes channel activation. Modulation of the channel activity has an important role in the hypoxic response, as it has been proposed to act as an "emergency brake." BK channel response to hypoxia has been associated with the development of diverse pathologies such as preeclampsia (Hu et al., 2012; Zhu et al., 2013) and neuronal injury (Tjong et al., 2008). In addition, there are several reports about the role of BK channel in hypoxic-modulated cardiovascular diseases, like pulmonary artery hypertension derived from COPD, chronic inhalation of CO, or OSA (Bonnet et al., 2003; Dubuis et al., 2005; Peinado et al., 2008; Navarro-Antolín et al., 2009; Roth et al., 2009; Ahn et al., 2012; Barnes et al., 2018; Liu et al., 2018b; Li et al., 2019). Moreover, it has been proposed that BK channel openers might be used in stroke, epilepsy, asthma, and hypertension (Kirby et al., 2013).

BK CHANNEL ACTIVITY IN THE HYPOXIA RESPONSE DERIVED FROM OBSTRUCTIVE SLEEP APNEA

Intermittent hypoxia and enhanced sympathetic activity increase the risk of cardiovascular disease and cognitive impairment in individuals with OSA (Brożyna-Tkaczyk et al., 2021). The mechanism by which OSA prompts cardiovascular diseases includes the increase in oxidative stress and activation of HIF-1α, both implicated in the modulation of the BK channel activity (Gabryelska et al., 2020; Orrù et al., 2020; Chen et al., 2021). CPAP, the most frequent treatment for OSA, improves the quality of life, decreased blood pressure to normal levels, and caused a significant reduction in oxidative stress (Zhao and Mehra, 2017; Baran et al., 2021).

Leukocytes from patients with obstructive sleep apnea-hypopnea syndrome (OSAHS) showed a decrease in the mRNA expression of the BK channel. When patients were exposed to CPAP therapy, it was observed a significant increase in the expression of the BK β1 subunit, which was paralleled with the adjustment of blood O_2 tension. The authors reported a relation between oxygenation level, arterial tension, and BK β1 subunit expression. Considering the effect of CPAP in the BK subunit expression, it is suggested that BK channels contribute to vascular dysregulation in OSAHS (Navarro-Antolín et al., 2009; Caballero-Eraso et al., 2019). Endothelial dysfunction is an early clinical marker of atherosclerosis, and a risk marker for cardiovascular diseases. Patients with OSA showed endothelial dysfunction, correlated with a decrease in the expression of the BK β1 subunit, which was recovered after CPAP. An improvement in endothelial function was also observed after CPAP treatment, diminishing the cardiovascular risk (Caballero-Eraso et al., 2019). However, the CPAP treatment has not been demonstrated to provoke a significant blood pressure lowering effect in patients with OSA and nocturnal hypertension (Chen et al., 2020). These findings suggest that it is necessary to investigate the exact pathophysiological mechanisms involved in hypertension and cardiovascular risk associated with OSA.

CONCLUSION AND PERSPECTIVE

Hypoxia, mediated by OSA, promotes a reduction in the BK β1 subunit expression that could induce a decrease in the BK channel Ca^{2+} sensitivity, maintaining membrane depolarization, and triggering the activation of L-type Ca^{2+} channels. The opening of these channels will produce a rise in $[Ca^{2+}]_i$. The induced response promotes the development of pathologies like hypertension, heart attack, and stroke, all of which are associated with hypoxia. Considering the described processes associated with hypoxic response, the mechanisms involved in OSA-derived hypoxia may be related to the increase in oxidative stress. However, there is no evidence to confirm that hypothesis, and further studies are necessary. In addition, there is no information about the effect of OSA in the expression of α or other β subunits. It could be interesting to carry on a deep study of the effect of OSA in the expression of the different BK subunits, as well as the possible role of other ion channels in the modulation of the BK channel activity. Moreover, exploration of other processes induced by hypoxia as HO-2 or HIF-1α regulation are important to unveil the complete mechanism involved in BK channel regulation by OSA, which could be considered a therapeutic approach to treat diseases derived from that disorder.

AUTHOR CONTRIBUTIONS

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REVIEW
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The Molecular Basis for the Calcium-Dependent Slow Afterhyperpolarization in CA1 Hippocampal Pyramidal Neurons

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Neuronal signal transmission depends on the frequency, pattern, and timing of spike output, each of which are shaped by spike afterhyperpolarizations (AHPs). There are classically three post-spike AHPs of increasing duration categorized as fast, medium and slow AHPs that hyperpolarize a cell over a range of 10 ms to 30 s. Intensive early work on CA1 hippocampal pyramidal cells revealed that all three AHPs incorporate activation of calcium-gated potassium channels. The ionic basis for a fAHP was rapidly attributed to the actions of big conductance (BK) and the mAHP to small conductance (SK) or Kv7 potassium channels. In stark contrast, the ionic basis for a prominent slow AHP of up to 30 s duration remained an enigma for over 30 years. Recent advances in pharmacological, molecular, and imaging tools have uncovered the expression of a calcium-gated intermediate conductance potassium channel (IK, KCa3.1) in central neurons that proves to contribute to the slow AHP in CA1 hippocampal pyramidal cells. Together the data show that the sAHP arises in part from a core tripartite complex between Cav1.3 (L-type) calcium channels, ryanodine receptors, and IK channels at endoplasmic reticulum-plasma membrane junctions. Work on the sAHP in CA1 pyramidal neurons has again quickened pace, with identified contributions by both IK channels and the Na-K pump providing answers to several mysteries in the pharmacological properties of the sAHP.

Keywords: sAHP, slow AHP, hippocampus, pyramidal cell, KCa3.1, IK, CaV1.3, ryanodine receptor

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INTRODUCTION

Hippocampal CA1 pyramidal cells were one of the first central neurons to draw attention as a model for understanding the factors that control neuronal membrane excitability. The existence of spike-evoked AHPs of different durations were among the first responses reported with intracellular recordings using the *in vitro* slice preparation in the early 1980's (Alger and Nicoll, 1980; Hotson and Prince, 1980; Gustafsson and Wigström, 1981; Wong and Prince, 1981; Lanthorn et al., 1984; Madison and Nicoll, 1984; Lancaster and Adams, 1986; Lancaster and Nicoll, 1987). Three post-spike AHPs of increasing duration were identified as incorporating calcium-dependent potassium channels: a fast AHP (fAHP, ∼10 ms), medium AHP (mAHP, 50–100 ms), and slow AHP (sAHP, ∼3–20 s) (**Figures 1A,B**) (for reviews see Storm, 1990; Sah and Davies, 2000; Vogalis et al., 2003b; Stocker, 2004; Adelman et al., 2012; Andrade et al., 2012). Recordings with microelectrodes rapidly established a primary contribution of high voltage-activated calcium currents that activate big conductance (BK, KCa1.1) potassium channels in driving the fAHP and spike repolarization

(Lancaster and Nicoll, 1987; Storm, 1987; Shao et al., 1999; Vogalis et al., 2003b; Gu et al., 2007). The mAHP includes contributions by small conductance calcium-dependent potassium channels (SK, KCNN.x) and Kv7 (KCNQ) potassium channels that can influence spike output and synaptic transmission (Storm, 1987, 1989; Gu et al., 2005; Lawrence et al., 2006; Buchanan et al., 2010; Adelman et al., 2012; Chen et al., 2014; Wang et al., 2014; Church et al., 2015). Through years of work the sAHP became recognized as one of the most significant factors controlling spike output in pyramidal cells, and a response that can be realistically considered one of the largest inhibitory responses in the brain. The sAHP was thus shown to be important in controlling synaptic and intrinsic plasticity (Borde et al., 1995, 1999; Sah and Bekkers, 1996; Lancaster et al., 2001; Kumar and Foster, 2004; Le Ray et al., 2004; Fuenzalida et al., 2007; Sametsky et al., 2009; Kaczorowski, 2011; Tedoldi et al., 2020), circuit function with age (Landfield and Pitler, 1984; Campbell et al., 1996; Power et al., 2002; Disterhoft et al., 2004; Tombaugh et al., 2005; Thibault et al., 2007; Matthews et al., 2009; Moore and Murphy, 2020), and if disrupted, leads to repetitive spike output and epileptiform discharge (Alger and Nicoll, 1980; Fernandez de Sevilla et al., 2006; Skov et al., 2009; Tiwari et al., 2019). The sAHP was further distinguished as being under regulatory control by multiple transmitters and second messengers (Madison and Nicoll, 1982, 1986; Lancaster and Nicoll, 1987; Sah and Isaacson, 1995; Pedarzani and Storm, 1996; Zhang et al., 1996; Pedarzani et al., 1998; Haug and Storm, 2000; Lancaster et al., 2001; Melyan et al., 2002; Wong and Schlichter, 2014; Mohan et al., 2019; Tiwari et al., 2019).

Despite identifying several functional roles for the sAHP, defining its underlying molecular basis has been a subject of intense study for over 30 years (Andrade et al., 2012). Recent developments in the pharmacology of potassium channels, super-resolution microscopy, and even a return to microelectrode recordings have renewed the field with multiple findings on the basis for the sAHP. In particular, it has come to light that the sAHP in CA1 pyramidal cells is comprised of one component mediated by calcium-gated potassium channels, and a second component produced by the Na-K ATPase (Na-K pump) that overlaps and extends the calcium-dependent sAHP (Thompson and Prince, 1986; Fukuda and Prince, 1992; Gulledge et al., 2013; Tiwari et al., 2018; Mohan et al., 2019, 2021). Depending on the preceding spike train the calcium-dependent sAHP can extend from ∼3–5 s (10 spikes) up to 20 s (150 spikes), with even more growth of the Na-K phase up to 25–30 s (150 spikes) (Tiwari et al., 2018).

This review will focus on the history of work on two closely related factors: (i) the potassium channels that underlie the calcium-dependent component of the sAHP, and (ii) the calcium sources that drive this response in CA1 pyramidal cells. We thus use the term "slow AHP" primarily in reference to the calcium-dependent component. In this we recognize cell-specific differences in ion channels that can modify or contribute to a slow AHP (i.e., Kv7, Slack, Kir6, HCN, and the Na-K pump), and refer readers to other papers of interest (Schwindt et al., 1989; Maccaferri et al., 1993; Joiner et al., 1998; Sah and Davies, 2000; Faber and Sah, 2003; Wallen et al., 2007; Tzingounis and Nicoll, 2008; Tzingounis et al., 2010; Villalobos and Andrade, 2010; Kaczorowski, 2011; Tanner et al., 2011; Andrade et al., 2012; Gulledge et al., 2013; Chen et al., 2014; Kim et al., 2016; Tiwari et al., 2018; Laker et al., 2021). Given cell-to-cell variability, we largely distinguish between data obtained in CA1 hippocampal pyramidal cells compared to either CA3 pyramidal cells or neocortical pyramidal cells even though there is valuable overlap in some of the findings. To restrict recordings as much as possible to calcium-dependent potassium channels distinct from the mAHP and Na-K pump we focus on the IsAHP typically evoked by a step command or by suprathreshold repetitive spike trains of 5–10 pulses. Using these parameters the typical duration of the calcium-dependent slow AHP and IsAHP is 1–5 s.

The extent of extent of efforts to resolve the molecular basis for the sAHP make it impossible to be all-inclusive in citing examples of key findings in previous work. Indeed, a literature search using the terms "slow AHP OR sAHP AND hippocampus" since 1980 returns over 9,000 results. For brevity we do not review the extensive data involving transmitter and second messenger regulation of the sAHP, or the mechanisms that underlie an increase in sAHP amplitude with age. Rather, we recognize the sum contribution of many labs and thousands of studies that contributed to resolving the factors that produce the sAHP in CA1 pyramidal cells, and choose examples that are representative or can illustrate the path that led to our current understanding.

THE MOLECULAR IDENTITY OF SLOW AFTERHYPERPOLARIZATION CHANNELS

The sAHP can be evoked synaptically (Lancaster and Wheal, 1984; Lancaster and Nicoll, 1987; Zhang et al., 1996; Lancaster et al., 2001), during repetitive spike discharge (Madison and Nicoll, 1982, 1984), and following the end of a long spike train (**Figures 1C–H**; Alger and Nicoll, 1980; Hotson and Prince, 1980; Gustafsson and Wigström, 1981; Wong and Prince, 1981). When examined during repetitive discharge evoked by current injection the sAHP grows with successive spikes in the train to promote spike accommodation (**Figure 1B**; Madison and Nicoll, 1982, 1984). Under voltage clamp the IsAHP can be evoked and distinguished from that of the ImAHP following a step command as an unclamped outward current (**Figure 1H**; Lancaster and Adams, 1986; Madison et al., 1987). Unlike the fAHP and mAHP, the sAHP was not affected by classical blockers of potassium channels available in earlier years, including apamin, TEA or 4-AP (Lancaster and Nicoll, 1987; Lancaster et al., 1991). Key factors reported in early studies were a block of the sAHP by the scorpion toxin charybdotoxin (ChTx), β adrenoreceptor agonists, and several neurotransmitter modulators (Madison and Nicoll, 1982, 1986; Haas and Greene, 1984; Madison et al., 1987; Sah and Isaacson, 1995; Pedarzani and Storm, 1996; Zhang et al., 1996; Haug and Storm, 2000). However, pinpointing the isoform(s) of calcium-gated potassium channel responsible for the sAHP proved challenging.

Kv7 Channels

The voltage-gated Kv7 channels (KCNQ family) first known to generate M current also bind calmodulin (CaM) that can confer sensitivity to internal calcium concentration (Marrion et al., 1991; Wen and Levitan, 2002; Gamper and Shapiro, 2003; Gamper et al., 2005; Chang et al., 2018; Zhuang and Yan, 2020). The effects of calcium-CaM interactions on Kv7 channels are complex and often result in a decrease in channel current, with the exact effects depending on the specific combination of co-expressed isoforms (Marrion et al., 1991; Gamper and Shapiro, 2003; Gamper et al., 2005; Chang et al., 2018; Zhuang and Yan, 2020). Each of the Kv7.2, Kv7.3, and Kv7.5 isoforms are expressed in hippocampus, although have differential contributions to the sAHP in principle output neurons of CA1, CA3, and dentate gyrus (Shah et al., 2002; Pan et al., 2006; Tzingounis and Nicoll, 2008; Tzingounis et al., 2010; Kim et al., 2012, 2016). Kv7 channels were thus shown to contribute to the sAHP in CA3 pyramidal (Tzingounis and Nicoll, 2008; Tzingounis et al., 2010; Kim et al., 2012, 2016) and dentate granule cells (Tzingounis and Nicoll, 2008; Laker et al., 2021). In at least one case the Kv7 channel blocker XE-991 was reported to block up to 33% of the sAHP of CA1 pyramidal cells (Tzingounis and Nicoll, 2008) while other studies reported little to no role for Kv7 channels on the sAHP (Aiken et al., 1995; Gerlach et al., 2004; Gu et al., 2005; Tzingounis et al., 2010). This could reflect the understanding that Kv7 channels have a voltage range for activation outside that required to contribute to the sAHP unless subject to modulation by phosphatidylinositol 4,5-bisphosphate (PiP2) (Zhang et al., 2013; Greene and Hoshi, 2016; Kim et al., 2016). It has also been shown that whole-cell recording conditions can wash out factors needed for normal Kv7 function, requiring the use of perforated patch recordings (Loussouarn et al., 2003; Gamper et al., 2005). Given that calcium-CaM interactions often lead to inhibition of specific Kv7 isoforms that are expressed in hippocampus (Gamper and Shapiro, 2003; Gamper et al., 2005; Zhang et al., 2016), and the need for modulatory factors to detect Kv7 function, this review will focus on potassium channels directly activated by calcium.

SK1 (KCa2.1) Channels

Several lines of evidence came to suggest a role for SK channels in the sAHP, and particularly that of the SK1 isoform through activation by L-type calcium channels. Supporting data came from fluctuation noise analysis and single channel recordings that returned evidence for a small conductance (2–5 pS) potassium channel (Sah and Isaacson, 1995; Selyanko et al., 1998) that was within the range of SK channel isoforms (Kohler et al., 1996; Hirschberg et al., 1998; Marrion and Tavalin, 1998). A set of eloquent recordings revealed a functional coupling between L-type channels and presumed SK channels within single on-cell patch recordings in pyramidal cells (Marrion and Tavalin, 1998). Immunolabels for the SK1 isoform and L-type calcium channels were colocalized in acutely dissociated pyramidal cells (Bowden et al., 2001). Finally, ensemble averages of evoked L-type calcium channels or SK-like potassium channels created macro currents that recapitulated the time course of the sAHP (Cloues et al., 1997; Bowden et al., 2001; Lima and Marrion, 2007). As a result, findings were interpreted to reflect the activity of SK1 channels triggered by L-type calcium influx with properties that would appear to fit the onset, peak, and duration of the sAHP (Tanabe et al., 1998; Lima and Marrion, 2007). However, the introduction of an SK1 knockout mouse that did not affect the sAHP appeared to set aside the possible role for SK1 channels (Bond et al., 2004).

IK (KCa3.1) Channels

Recordings to assess the role of calcium-gated potassium channels in generating sAHPs were not just restricted to the hippocampus. This was particularly the case for cells in the enteric nervous system of the gastrointestinal tract that generate an sAHP with remarkably similar properties to that of CA1 pyramidal cells (Kunze et al., 1994; Vogalis et al., 2002a,b, 2003a; Furness et al., 2004; Neylon et al., 2004; Nguyen et al., 2007). Work there identified the role of another member of the KCCN family that generates an intermediate conductance calcium-gated potassium channel (KCNN4, SK4, KCa3.1, IK). These channels are from the same gene family as SK1-3 channels with ∼45% homology in sequence (Ishii et al., 1997; Logsdon et al., 1997; Joiner et al., 2001; Kaczmarek et al., 2017), and were often referred to as an SK4 isoform. As for other members of their family, IK channels are voltage-independent and bind CaM to sense intracellular calcium concentration (Khanna et al., 1999; Joiner et al., 2001; Wong and Schlichter, 2014; Lee and MacKinnon, 2018). However, IK channels exhibit a higher conductance in the range of 20–90 pS compared to ∼10 pS for SK channels, and a unique pharmacological profile that includes apamin insensitivity and specific sites for binding of the blockers TRAM-34, Senicapoc, NS-6180, ChTx and maurotoxin (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997; Jensen et al., 1998; Wulff et al., 2001, 2007; Visan et al., 2004; Ataga et al., 2008; Strobaek et al., 2013; Kaczmarek et al., 2017; Alexander et al., 2019; Brown et al., 2020).

The advances made for cells in the enteric nervous system were almost transferred to hippocampal neurons when the antimycotic drug clotrimazole that blocked both the sAHP in enteric neurons and expressed IK channels (Ishii et al., 1997; Logsdon et al., 1997; Jensen et al., 1998; Neylon et al., 2004) also blocked the sAHP recorded in dissociated hippocampal cultures (Shah et al., 2001). But the best *in situ* hybridization techniques of the day that first identified IK channels did not detect its expression in the brain (Ishii et al., 1997; Logsdon et al., 1997; Jensen et al., 1998; Joiner et al., 2001). The reason for this is unknown as IK channels are expressed in endothelial and smooth muscle cells of the cerebrovasculature and in microglia (Van Renterghem et al., 1995; Neylon et al., 1999; McNeish et al., 2006; Kaushal et al., 2007; Hannah et al., 2011). Added to this were findings that clotrimazole was relatively non-specific in also blocking calcium current and the SK-mediated mAHP (Shah et al., 2001). Finally, since fluctuation noise analysis suggested that the sAHP was produced by a channel with a conductance of ∼5 pS, there was little reason to suspect an intermediate conductance channel as a contributing factor. Together the data came to support a long-held impression that IK channels (KCNN4) were simply not expressed in central neurons and thus not responsible for generating the sAHP in CA1 pyramidal cells

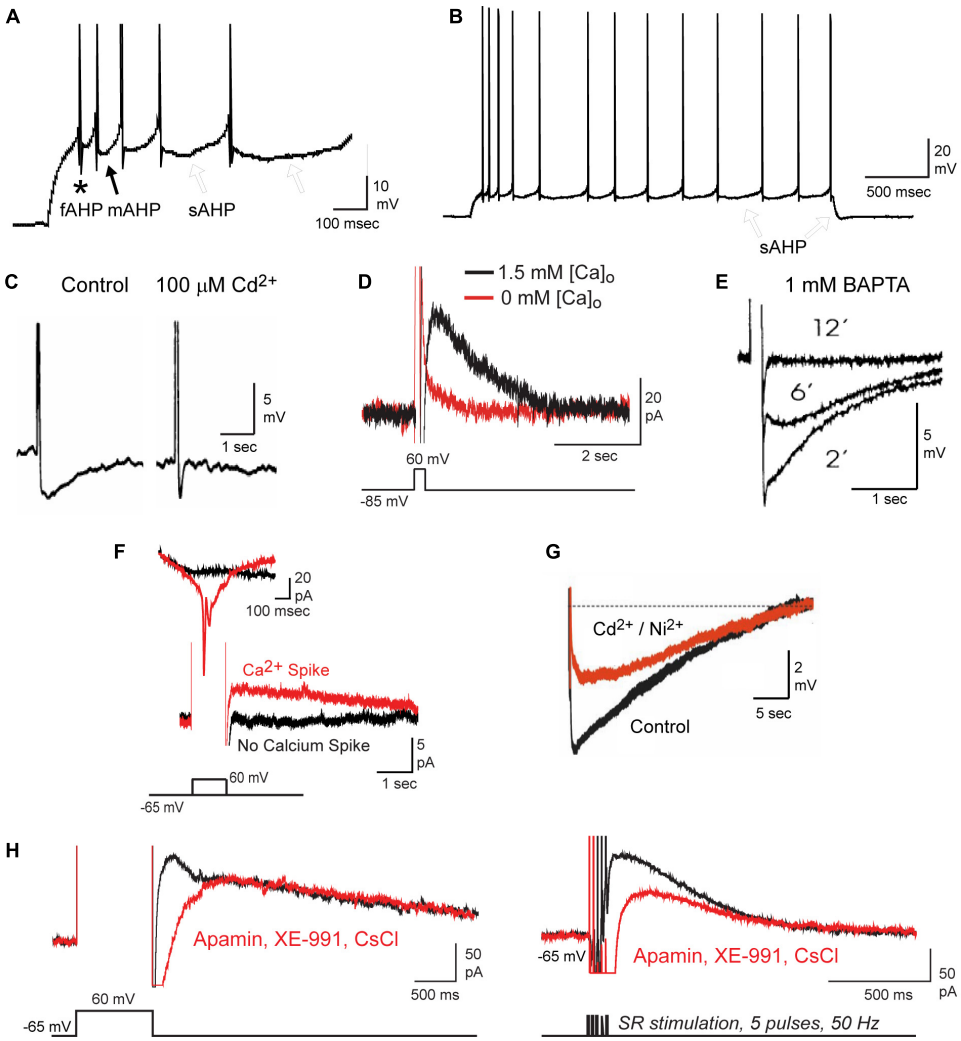

FIGURE 1 | Repetitive spike discharge activates a calcium-dependent sAHP in CA1 pyramidal cells. **(A,B)** Current-evoked spike firing in a CA1 pyramidal cell evokes a sequential series of fAHP (*asterisk*), mAHP (*filled arrow*), and sAHP *(open arrows*) **(A)**. A progressive increase in the sAHP promotes spike accommodation and a post burst sAHP (**B**, *open arrows*). Spikes are truncated in **(A)** for display purposes. **(C–E)** The sAHP is blocked by Cd^{2+} **(C)**, 0 mM calcium medium **(D)**, or over time through internal perfusion of BAPTA **(E)**. **(F)** The IsAHP evoked under voltage clamp by a step depolarization requires activation of membrane calcium conductance, detected here as an unclamped calcium spike. **(G)** The calcium-dependent component of the sAHP evoked by a preceding 50 Hz 150 spike train can be distinguished from an overlapping contribution by the Na-K pump upon perfusion of 200 μM Cd^{2+} and Ni^{2+}. **(H)** The IsAHP can be evoked by either a voltage command step or by repetitive synaptic stimulation and recorded in the absence of any contribution by SK (100 nM apamin), Kv7 (10 μM XE-991) or HCN (2 mM CsCl) channels. Figures are modified from King et al. (2015) **(A,B,D,F,H)**, Lancaster and Nicoll (1987) **(C)**, Zhang et al. (1995) **(E)**, and Tiwari et al. (2018) **(G)**. The baseline temperature for each data set was **(A,B,D,F,H)** 32–34°C (King et al., 2015), **(C)** 29–31°C (Lancaster and Nicoll, 1987), **(E)** 34°C (Zhang et al., 1995), and **(G)** 35°C (Tiwari et al., 2018).

(Sah and Faber, 2002; Vogalis et al., 2003b; Stocker et al., 2004; Adelman et al., 2012).

Yet there were growing reports of at least IK immunolabel in primary sensory neurons (Boettger et al., 2002; Mongan et al., 2005), spinal cord motor neurons (Mongan et al., 2005; Bouhy et al., 2011), and rod photoreceptors (Pelucchi et al., 2008). Engbers et al. (2012) directly tested the expression and function of IK channels in rat cerebellar Purkinje cells. Here parallel fiber input activated an sAHP of ∼400 ms that proved to be insensitive to all classic potassium channel blockers including apamin, TEA, 4-AP, or iberiotoxin, but was blocked by ChTx (Engbers et al., 2012). By this time the chemistry of blockers for IK channels had advanced significantly with the introduction of TRAM-34, a triarylmethane drug derived from clotrimazole that blocks the channel at internal sites with an IC_{50} ∼25 nM in expression systems (Wulff et al., 2000, 2001; Jenkins et al., 2013). Bath applied TRAM-34 (100 nM) rapidly blocked the Purkinje cell sAHP in the slice preparation, with complementary tests identifying the presence of KCa3.1 mRNA, and expression of a calcium-gated potassium channel of ∼36 pS that had a direct association with Cav3.2 (T-type) calcium channels (Engbers et al., 2012). It was subsequently shown that this Cav3-IK

interaction also provides a repolarizing conductance in Purkinje cell nodes of Ranvier that secures axonal spike propagation (Gründemann and Clark, 2015).

IK CHANNELS AS A CONTRIBUTING FACTOR IN THE CA1 PYRAMIDAL CELL SLOW AFTERHYPERPOLARIZATION

IK Expression

Given the evidence for IK expression in Purkinje cells, other brain regions were tested for IK expression using an IK-specific monoclonal antibody and a transgenic mouse line in which GFP expression was tied to promoter activity of the KCNN4 gene (Turner et al., 2015). Control tests confirmed that the antibody labeled a single band on western blots of mouse or rat brain (**Figure 2A**) and had no cross-reaction with SK channel isoforms. In hippocampus IK immunolabel was detected primarily in the somatic region of neurons with intermediate labeling intensity in CA1 pyramidal cells and even higher intensities in CA3 (**Figures 2B,C**) and CA4 regions (Turner et al., 2015). IK immunolabel was detected in both pyramidal and GABAergic cells, with notably high levels in dentate hilar interneurons (**Figure 2D**). These patterns were matched by the pattern of GFP expressed in cells exhibiting KCNN4 promoter activity (**Figure 2E**). Finally, direct verification of IK mRNA and protein sequence was obtained through single cell RT-PCR from CA1 cells in the rat slice preparation (Turner et al., 2016). Here it was found that cells with spike firing patterns characteristic of pyramidal cells or interneurons exhibited a PCR product size between 550 and 650 bp by using KCNN4-specific primers. This revealed the predicted sequence for IK channel protein surrounding the region of the pore and the presence of binding sites for TRAM-34, NS-6180, ChTx, and MTx. However, the binding site for apamin, the specific blocker for SK channels, was absent from the pore sequence (**Figures 2F,G**) (Turner et al., 2016).

IK Channels and Slow Afterhyperpolarization Pharmacology

The collective advances made in defining the expression pattern and pharmacology of IK channels allowed a reexamination of the possibility for IK channels to represent sAHP channels in CA1 pyramidal cells. A series of patch clamp recordings primarily in rat *in vitro* hippocampal slices revealed that the sAHP in CA1 pyramidal cells exhibited the complement of pharmacological properties that define IK channels (King et al., 2015). For these tests all recordings were conducted in the presence of apamin, XE-991, and CsCl to remove any contamination by SK, Kv7 or HCN channel isoforms. The IK channel blocker TRAM-34 was applied at a concentration no higher than 1 μM, a level previously recommended to reduce off target effects (Jenkins et al., 2013). Complementary work also established that this level of TRAM-34 had no effects on BK, Kv7.3, or TMEM16B (Ano2) channels expressed in isolation (King et al., 2015). TRAM-34 was effective in reducing the sAHP with bath application under these conditions. Yet to speed the actions of TRAM-34 in postsynaptic cells while preserving synaptic inputs the majority of recordings were conducted using internal electrode perfusion (King et al., 2015; Turner et al., 2016). This process also enabled the important ability to collect recordings with control electrolyte before adding TRAM-34 to the electrode to achieve a rapid block at its internal binding sites (Wulff et al., 2001; Turner et al., 2016).

TRAM-34 was found to block the IsAHP, spike accommodation, and the prominent sAHP that followed repetitive spike trains within 2 min of switching the contents of the electrode from control electrolyte to one containing 1 μM TRAM-34 (**Figures 3A,B**). Similar tests in mice revealed that TRAM-34 blocked the post burst sAHP evoked following a train of stratum radiatum (SR) inputs in wild type (wt) mice, but had no effect on a low amplitude hyperpolarization (presumably Na-K pump-mediated) in a line of KCa3.1 knockout (KO) mice (**Figure 3C**; King et al., 2015). To further test specific modulators of IK channels they confirmed that 100 nM ChTx blocked the IsAHP evoked by a brief 50 Hz SR stimulus train (**Figure 3D**). A block of the SR-evoked post-train sAHP was further obtained in whole-cell recordings with the selective IK channel blocker Senicapoc (100 nM) (**Figure 3E**; Maezawa et al., 2012). Conversely, the SR-evoked post-train sAHP was increased in amplitude by applying 1-EBIO (100 μM) or SKA-31 (1 mM), two agonists that increase the sensitivity of IK channels to [Ca]i (**Figure 3F**; Wulff et al., 2007). All together these results built a strong case that sAHP channels in CA1 pyramidal cells exhibit the unique pharmacological profile that defines IK channels (King et al., 2015; Turner et al., 2016).

Single Channel Recordings

If the sAHP is generated by IK channels then channel properties should be different from those of SK channels, and be evoked in a manner that could explain the long duration sAHP after a preceding spike train. Three studies have used single channel recordings of potassium channels that contribute to the sAHP that have many properties consistent with IK channels.

Lancaster et al. (1991) used inside-out patch recordings in dissociated hippocampal cultured neurons to record calcium-dependent potassium channels that were linear in conductance at hyperpolarized potentials, but exhibited a Mg^{2+}-dependent inward rectification for high voltage steps (Lancaster et al., 1991). Thus, under conditions of physiological internal levels of Mg^{2+}, a measured value of ∼20 pS for channel conductance at hyperpolarized potentials dropped to ∼10 pS for depolarizing steps. Interestingly, these authors noted that in several cases these "small conductance" channels persisted even in the presence of apamin. Marrion and colleagues used on-cell patch recordings in CA1 pyramidal cells in the rat slice preparation to further identify single potassium channels underlying the sAHP (Marrion and Tavalin, 1998; Bowden et al., 2001; Lima and Marrion, 2007). At the time of their recordings no steps were taken to test for apamin sensitivity. These authors reported a calcium-dependent, voltage-independent channel of 10 pS (Marrion and Tavalin, 1998; Bowden et al., 2001) or ∼19 pS (Lima and Marrion, 2007). They also uncovered an important property where a brief high frequency train (i.e., 10 pulses, 50 Hz) of spike-like command

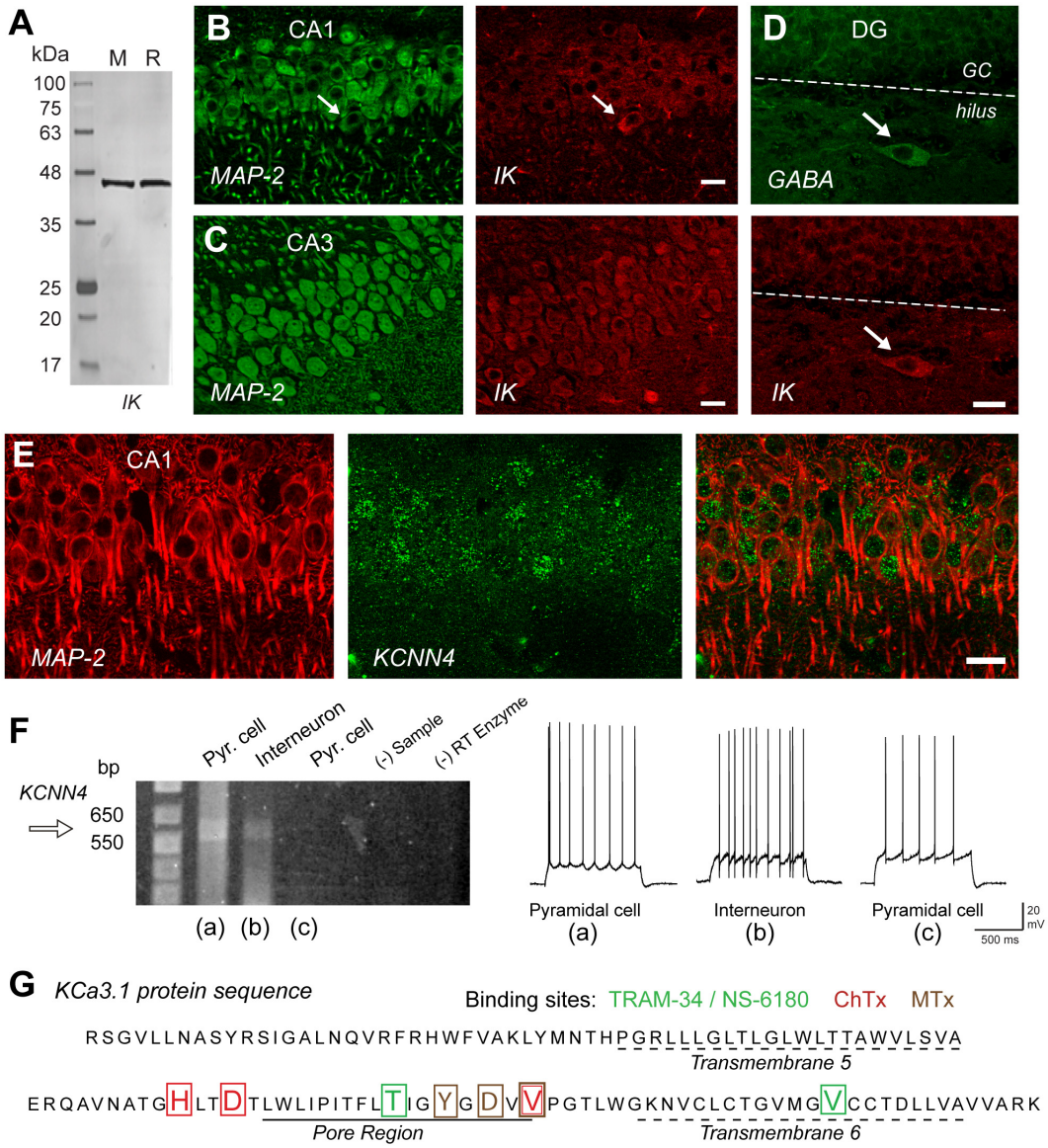

FIGURE 2 | IK channels are expressed in excitatory and inhibitory neurons in the hippocampus. **(A)** Western blot testing the specificity of an IK channel antibody (Santa Cruz D-5, sc-365265) that reports a single band of the correct molecular weight from rat (R) or mouse (M) brain homogenates. **(B,C)** Dual labeling for MAP-2 (*green*) as a structural indicator and anti-IK reveals IK immunolabel (*red*) in pyramidal cell bodies of rat hippocampus in both the CA1 and even higher intensity in CA3. **(D)** Dual immunolabel for GABA and IK in dentate gyrus reveals light IK immunolabel in granule cells and more intense label in a GABAergic hilar interneuron. *Arrows* in **(B,D)** indicate presumed inhibitory cell types with prominent IK immunolabel. **(E)** Dual labeling in CA1 hippocampus for MAP-2 (*red*) and an anti-GFP antibody (*green*) to identify cells expressing GFP in relation to KCa3.1 promoter activity in a transgenic mouse line. **(F)** Single cell RT-PCR of rat KCNN4 mRNA from cells identified electrophysiologically as exhibiting pyramidal cell or interneuron spike patterns before establishing an outside-out seal formation to retain electrode contents. One pyramidal cell and interneuron show a detectable band for KCNN4 cDNA at the predicted band PCR product size. Control lanes lacking sample (- Sample) or reverse transcriptase (RT) enzyme are negative. **(G)** The protein sequence of KCa3.1 translated from pooled samples in **(F)** for single cell RT-PCR cDNA product. The sequence includes transmembrane segments 5 and 6 and the intervening pore region of IK channels, complete with binding sites for four different IK antagonists coded by color to the blocker listed above. ChTx, charybdotoxin; MTx, maurotoxin. Scale bars **(B–E)**, 20 μm. Figures are modified from Turner et al. (2015) **(A–E)** and Turner et al. (2016) **(F,G)**. The baseline temperature for recordings in **(F)** was 32–34°C (King et al., 2015).

pulses immediately recruited strong bouts of channel openings that could persist for at least 5 s (**Figure 4A**; Bowden et al., 2001; Lima and Marrion, 2007). Moreover, calculating an ensemble average of these channel openings revealed a current that peaked within 500 ms of the end of the pulse train and decayed with a τ ∼1.3–1.6 s, values very similar to the IsAHP evoked under whole-cell conditions with the same form of spike train (Lima and Marrion, 2007).

King et al. (2015) also applied on-cell patch recordings at the soma of rat CA1 pyramidal cells in the slice preparation but under conditions in which IK channel activity was pharmacologically isolated. Using an electrode solution containing 3.25 mM

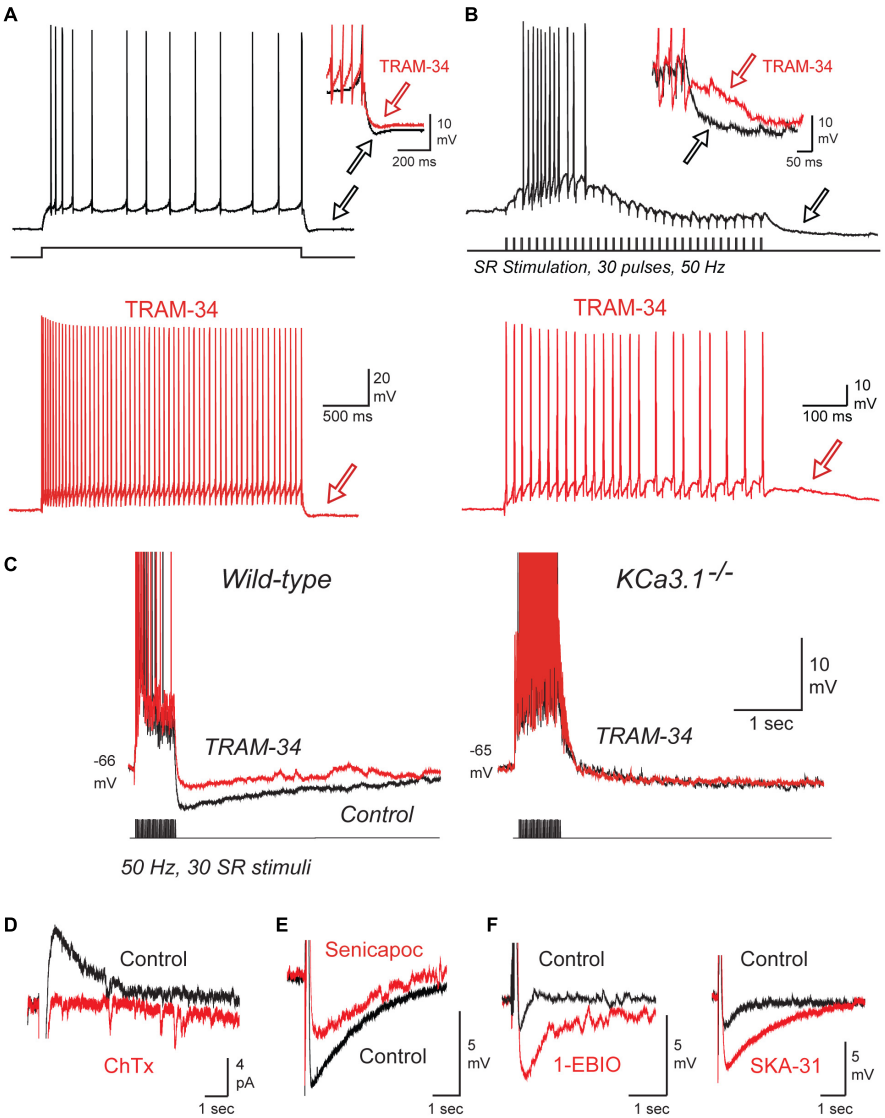

FIGURE 3 | The sAHP and spike accommodation exhibit the unique pharmacological profile of IK. **(A,B)** Repetitive spike discharge in rat CA1 pyramidal cells in tissue slices *in vitro* evoked in response to a square wave current pulse **(A)** or repetitive SR synaptic stimulation **(B)**. Spike accommodation and the post train sAHP (*arrows*) are reduced by 1 μM TRAM-34. **(C)** A current-evoked spike train and afterpotential from CA1 pyramidal cells showing a prominent sAHP in wild type but not KCa3.1$^{-/-}$ mice. TRAM-34 blocks the sAHP in *wt* mice but has no effect on the post-train response in KCa3.1$^{-/-}$ mice. **(D)** The IsAHP evoked by a five pulse 50 Hz SR stimulus train in a perforated patch recording is blocked by local pressure ejection of 100 nM ChTx. **(E,F)** In whole-cell recordings the sAHP evoked after a five pulse 50 Hz SR stimulus train is reduced by the IK blocker Senicapoc (100 nM) **(E)** and enhanced by the IK agonists 1-EBIO (100 μM) or SKA-31 (1 mM) **(F)**. All recordings were conducted in the presence of 100 nM apamin, 10 μM XE-991, and 50 μM picrotoxin to block SK and Kv7 channels and GABA receptors, respectively. Tests on ChTx in **(D)** included 5 mM TEA in the bath to block BK channels. TRAM-34 was internally infused through the electrode in **(A–C)**. Figures are modified from King et al. (2015). The baseline temperature for all recordings was 32–34°C (King et al., 2015).

potassium and 1.5 mM calcium at ∼34°C to simulate physiological conditions, recordings revealed a channel of ∼30 pS. These authors also noted an apparent reduction in current amplitude and flickering at high levels of membrane polarization, as previously reported by both Lancaster et al. (1991) and in studies of expressed IK channels (Ishii et al., 1997; Logsdon et al., 1997; Jensen et al., 1998). Again they reported that channel activity was relatively difficult to detect at rest but became immediately evident after a brief, high frequency train of spike-like commands or SR synaptic stimulation (5–20 pulses, 50 Hz). Currents recorded in on-cell or outside-out patch recordings were enhanced by DC-EBIO and blocked by BAPTA-AM or 8-bromo-cAMP (King et al., 2015). Interestingly, SR stimulation uncovered enough channels to evoke a macropatch outward current of 1–5 s duration that exhibited a sharp onset and offset of activity (**Figure 4B**). Isolated single channels showed the same immediate and long-duration bouts of opening in response to a five pulse, 50 Hz SR stimulus train (**Figure 4C**).

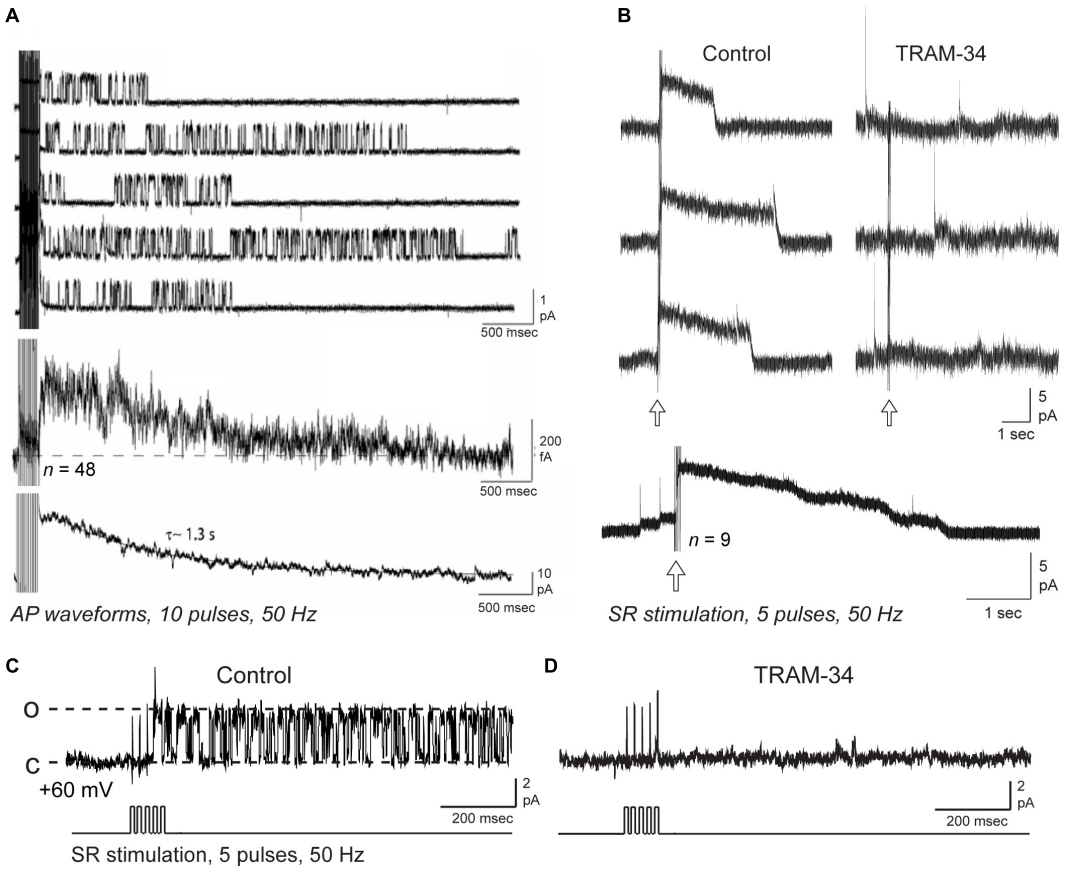

FIGURE 4 | Repetitive synaptic or spike trains trigger IK channel openings over a time course that recapitulates the IsAHP. Shown are on-cell patch recordings of potassium channels from rat CA1 pyramidal cell somata in the slice preparation, with channel current illustrated with respect to the cell interior and open states shown as upward [i.e., *dashed lines* in **(C)** for open (o) and closed (c) states]. **(A)** Somatic on-cell patch recordings of potassium channels using 2.5 mM in the electrode exhibit bouts of channel opening following a train of 10 action potential waveforms. Lowest trace shows the ensemble current generated from 48 null-subtracted sweeps, and for comparison the IsAHP recorded in whole-cell mode from another pyramidal cell. **(B–D)** On-cell patch recordings from CA1 pyramidal cell somata using 3.25 mM potassium in the electrode and a net 60 mV holding potential and pharmacological isolation of IK channels. An outward macroscopic current **(B)** is recorded with a rapid onset and offset following five pulse SR stimulus trains (*arrows*) is blocked by bath applied 1 μM TRAM-34. Lowest trace shows an ensemble average from separate SR stimulus trains. **(C,D)** A single channel (~36 pS) recorded at the soma shows prolonged bursts of openings following an SR stimulus train **(D)**. Both the macropatch current in **(B)** and SR-evoked single channels **(C,D)** are blocked by bath perfusion of 1 μM TRAM-34. The recordings in **(B–D)** isolated IK channels using 1 μM TTX, 100 nM apamin, 10 μM XE-991, 5 mM TEA, 5 mM 4-AP, and 2 mM CsCl to allow normal activation of synaptic inputs (see King et al., 2015, Supplementary Table S1). Transients in **(B)** reflect capacitive transients from spontaneous spike discharge in the cell. Figures are modified from Lima and Marrion (2007) (Copyright [1997] Society for Neuroscience) **(A)** and King et al. (2015) **(B–D)**. The baseline temperature was **(A)** was 19–24°C (Lima and Marrion, 2007), and **(B,D)** 32–34°C (King et al., 2015).

Creating an ensemble average of SR-evoked IK channel activity effectively recapitulated the sAHP as a long duration outward current that decayed over 5 s time (King et al., 2015). Importantly, both single channels and macropatch currents evoked by SR stimulation were blocked by bath application of the IK channel blocker TRAM-34 (**Figures 4B,D**).

The similarities between the single channel data of Lancaster et al. (1991), Marrion and Tavalin (1998), Bowden et al. (2001), Lima and Marrion (2007), and King et al. (2015) along with those for expressed IK channels (Ishii et al., 1997; Logsdon et al., 1997; Jensen et al., 1998) are striking. While a lower conductance of 10–19 pS was found in first recordings (Lancaster et al., 1991; Marrion and Tavalin, 1998; Bowden et al., 2001; Lima and Marrion, 2007) compared to 30 pS in King et al. (2015), there were differences in recording conditions that could account for this (ion gradients, charge carrier, temperature, Mg^{2+} block, and inward rectification). It is thus very likely that at least some of the first channel recordings of SK-like channel activity and those found under conditions when IK channels are isolated are one and the same.

IK Channels and the Slow Afterhyperpolarization – Conflicting Results

Work published 1 year after the King et al. (2015) study raised questions as to the role of IK channels given apparent difficulties in detecting IK current or minimal blockade by TRAM-34

applied in the bath or internally through the electrode (Wang et al., 2016). The results appear to contrast entirely with those of King et al. (2015) in reporting no significant effect of TRAM-34 beyond what was attributed to general rundown of current over time. Yet King et al. (2015) had also considered and ruled out the potential influence of run-down of IsAHP currents over 30 min time (see King et al., 2015; Supplementary Figure S3). Some differences between these studies were that King et al. (2015) recorded the IsAHP at near-physiological temperatures vs. room temperature in Wang et al. (2016), although the influence of this on TRAM-34 sensitivity has not been directly addressed. King et al. (2015) also recorded all data in the presence of apamin, XE-991 and CsCl to block SK, Kv7 and HCN channels, whereas these were included in only a subset of recordings in Wang et al. (2016). The potential for this set of blockers to affect recordings remains to be determined. King et al. (2015) further used IK KO mice and recorded a minimal sAHP while Wang et al. (2016) reported a seemingly normal sAHP in IK KO mice that was no different than in *wt* mice. It should be noted that these differences could reflect compensatory mechanisms during development that could include the Na-K pump that have not been fully assessed. Yet both studies agreed in finding that TRAM-34 had no effect on the IsAHP in IK KO mice, a result further supported by a lack of the IK agonist DC-EBIO in IK KO mice (King et al., 2015). Wang et al. (2016) tested TRAM-34 through bath application or in some recordings by including TRAM-34 in the electrode from the outset before obtaining a whole-cell patch configuration. Turner et al. (2016) subsequently clarified the need to use an electrode perfusion system if TRAM-34 is introduced internally given a remarkably fast block of IK channels (1–2 min) if TRAM-34 is present in the electrode upon breaking into whole-cell recording mode. King et al. (2015) used PKAcat or 8-bromo-cAMP to focus on the effects of increasing PKA on the sAHP, recently shown to induce downregulation of IK channels (Tiwari et al., 2019). Wang et al. (2016) used the cholinergic agonist carbachol to increase kinases as a standard test to block and verify the presence of an sAHP with expected properties. The results here might be expected to be different given that carbachol will activate PKA, PKC, and PKG – NO pathways that are now found to also block the Na-K pump-mediated sAHP (Chen et al., 2017; Mohan et al., 2019, 2021; Tiwari et al., 2019). Given the reliance by Wang et al. (2016) on the effects of carbachol to confirm recordings of the sAHP, it is uncertain how much of the response blocked in that study might be attributed to the Na-K pump.

The differences between data sets of King et al. (2015) and Wang et al. (2016) were thus substantial, and revealed at least important effects of the method of applying TRAM-34 (Turner et al., 2016) and the time frame of its effects when applied in the bath or externally. The latter findings are also emphasized by differences between CA1 pyramidal and cerebellar Purkinje cells, in that bath application of even 100 nM TRAM-34 rapidly blocked an IK-mediated sAHP in Purkinje cells (Engbers et al., 2012) compared to a relatively slower block of the CA1 cell sAHP by bath applied 1 μM TRAM-34 (King et al., 2015). Importantly, a block of the sAHP by TRAM-34 has now been repeated in CA1 pyramidal cells and neocortical pyramidal cells (Sahu et al., 2017, 2019; Tiwari et al., 2018, 2019; Roshchin et al., 2020), lending support for the effects of TRAM-34. A specific role for IK channels in pilocarpine-induced epileptic discharge was also reported (Tiwari et al., 2019). The reason for such dramatic differences in the results of the Wang et al. (2016) and King et al. (2015) studies are thus unknown at this time, but suggest some unknown factor(s) that can affect drug sensitivities that remain to be identified.

IK-SK1 Heteromeric Channel Formation

An explanation for some of the difficulties defining sAHP pharmacology may now have been provided in a study of heteromeric channel formation by IK and SK1 channel isoforms (Higham et al., 2019). This study arose from extensive work on SK channel isoforms showing a species-specific ability to assemble as heteromeric channels, and that heteromerization of potassium channel subunits can change channel properties (Manganas and Trimmer, 2000; Akhtar et al., 2002; Benton et al., 2003; Etxeberria et al., 2004; Monaghan et al., 2004; Sokolov et al., 2007; Al-Sabi et al., 2010; Brueggemann et al., 2011; Church et al., 2015; Autuori et al., 2019). Indeed, it was previously shown that rat SK1 and SK2 subunits can form heteromeric channels in CA1 pyramidal cells that alters apamin sensitivity, while human SK1 and SK2 subunits do not share this property (Church et al., 2015). Marrion and colleagues tested the potential for human SK1 and IK channel isoforms to heteromerize, and any effects this could have on channel activity (Higham et al., 2019). The spatial proximity between these proteins was tested in the tsA-201 cell system to detect fluorescence resonance energy transfer (FRET) between donor and acceptor fluorophores as an indicator of molecules positioned < 10 nm distance. Here FRET was detected between an eGFP donor and mKate acceptor pair for expression of either SK1 or IK subunits, as predicted for subunits that form homomeric channels (**Figure 5A**). However, FRET was also detected when SK1-eGFP was coexpressed with IK-mKate, revealing a proximity of SK1 and IK subunits that would be expected for a heteromeric channel assembly (**Figure 5B**). SK1 and IK immunofluorescent labels in tsA-201 cells were further found in close proximity to one another in the membrane through the use of stochastic optical reconstruction microscopy (STORM) (**Figures 5C,D**). Morphological cluster analysis to quantify nearest neighbor distances between labels revealed a Poisson-like distribution for clusters of a given expressed isoform (i.e., IK-IK, SK1-SK1). However, the histogram for minimal nearest neighbor distances between IK and SK1 clusters was right-skewed, indicating a preferential close association between these subunits (**Figure 5E**). Together these imaging measures of spatial proximity argue for a prominent level of heteromeric assembly of IK and SK1 subunits when coexpressed.

IK-SK1 Heteromeric Assembly Alters Channel Properties

Outside-out patch recordings from transfected tsA-201 cells revealed that when expressed in isolation SK1 channels exhibited the expected block by 100 nM apamin, no sensitivity to TRAM-34, and a single channel conductance of ∼20 pS (**Figure 5F**). IK channels expressed in isolation were apamin-insensitive, fully blocked by TRAM-34 perfusion, and had a single channel

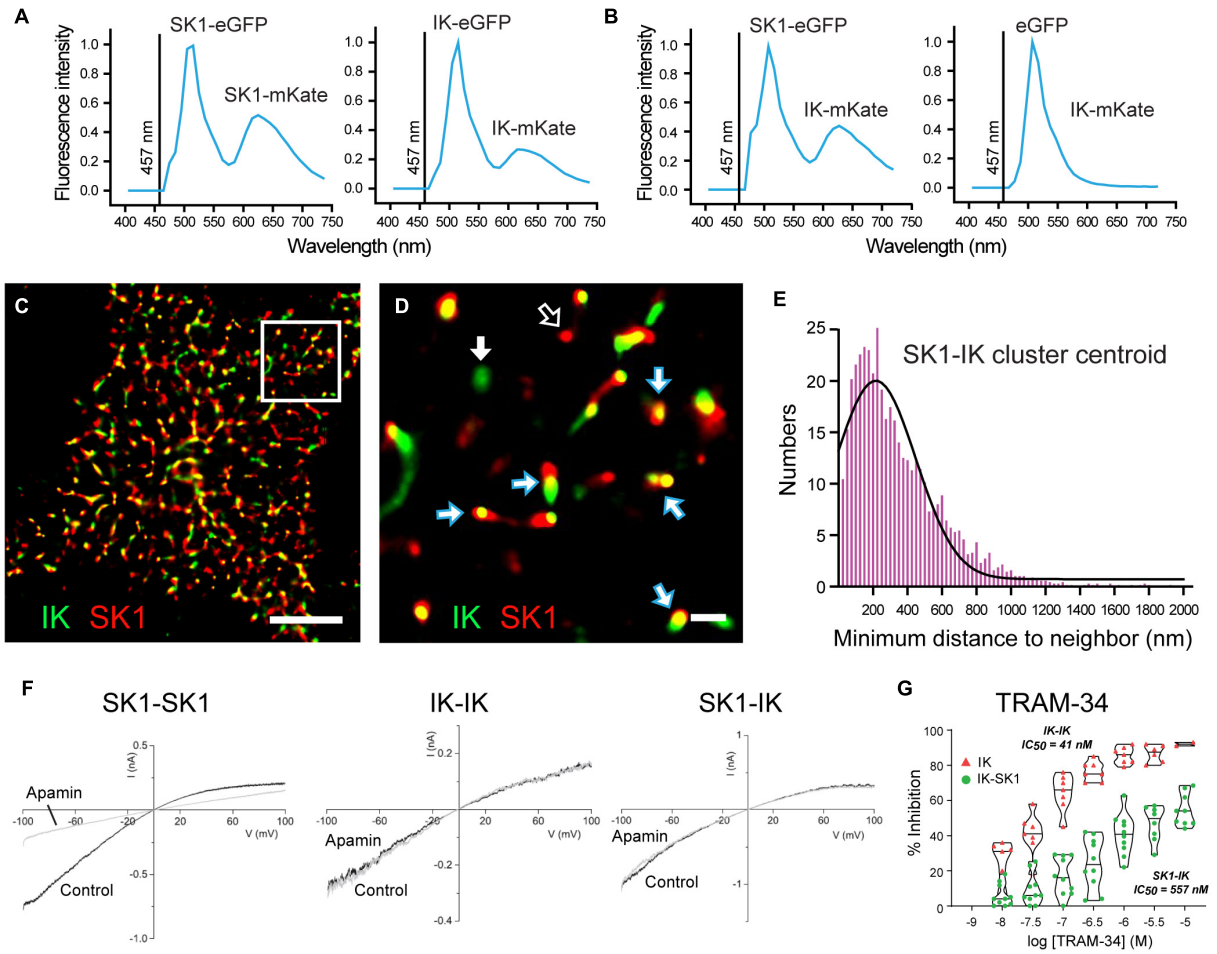

FIGURE 5 | Heteromerization of IK and SK1 channel subunits changes channel properties. **(A,B)** FRET imaging in live tsA-201 cells expressing human SK1 and IK channel subunits as fluorescent constructs to allow eGFP to act as FRET donor and mKate as acceptor molecule upon activation at 457 nm. **(A)** Positive controls confirming that FRET can be used to detect subunit assembly of SK1- or IK-labeled constructs as homomeric channels. FRET is detected as a dual emission for eGFP activated directly by 457 nm excitation, and a second peak for mKate excited by the eGFP emission. **(B)** FRET is elicited if SK1-eGFP and IK-mKate are coexpressed, but not if eGFP alone is coexpressed with IK-mKate. **(C,D)** Super-resolution images using STORM imaging of fluorescent-tagged SK1 (*red*) and IK (*green*) channels expressed in tsA-201 cells. A low resolution image of detected clusters is presented in **(C)** and a magnified view of the Region of Interest in **(D)**. Clusters of fluorescent tags for SK1 and IK are most often overlapping (*blue/white arrows*), or in some cases, present as isolated clusters for IK (GFP, green) (*solid white arrow*) or SK1 (*mKate, red*) (*open white arrow*). **(E)** A right-skewed histogram plot of the nearest-neighbor distances between IK and SK1 clusters reveals a preferential co-association of labels consistent with heteromeric channel formation (bin width, 25 nm). **(F,G)** Membrane currents evoked in outside-out patches from tsA-201 cells expressing human SK1 or IK cDNA in isolation (homomeric assembly) or coexpressing SK1 and IK subunits (heteromeric assembly). Application of 100 nM apamin blocks SK1 homomeric channels but not homomeric IK channels or channels recorded when SK1 and IK subunits are coexpressed **(F)**. Violin plots of the effects of TRAM-34 in tsA-201 cells in **(G)** indicate that bath applied TRAM- 34 blocks homomeric IK channels with IC_{50} = 41 nM but heteromeric SK1-IK channels with an IC_{50} = 557 nM. Scale bars: 5 μm **(C)** and 500 nm **(D)**. Figures are modified from Higham et al. (2019). The baseline temperature for recordings in **(A,B,F,G)** was 22°C (King et al., 2015; Higham et al., 2019).

conductance of 53 pS (**Figure 5F**). Yet when IK and SK1 subunits were coexpressed the channel properties changed to being apamin-*insensitive* and with a reduced TRAM-34 sensitivity, shifting from IC_{50} = 21 nm for IK channels expressed in isolation to IC_{50} = 557 nm for coexpressed IK-SK1 subunits (**Figures 5F,G**). Single channel conductance of IK-SK1 channels also changed to 36 pS, a value midway between the two channels in isolation but in the range expected for an intermediate conductance potassium channel. Finally, a similar decrease in sensitivity was found for ChTx (100 nM) that blocked 95% of homomeric IK channel current but only 30% of IK-SK1 channel current (Higham et al., 2019). Some of the apparent discrepancies in the reported effects of TRAM-34 may then reflect the use of 1 μM TRAM-34 that is closer to the IC_{50} value for heteromeric IK-SK1 channels compared to application at 5 μM (Tiwari et al., 2018). The reduced sensitivity of heteromeric IK-SK1 channels to ChTx might also account for reported difficulties of detecting a block of the sAHP by 10–100 nM ChTx in earlier studies (Lancaster and Nicoll, 1987; Shah and Haylett, 2000, 2002). However, at this time it is not known if these properties apply

to native CA1 pyramidal cells in rodents or humans, or if IK channels interact in the same way with SK2 or SK3 isoforms.

Calcium Sensors

The activation and kinetics of calcium-gated potassium current is also a reflection of the sensitivity of the calcium sensor. One explanation for the slow onset of the sAHP was the potential involvement of an intermediate molecule for its activation. IK channels are known to be gated by CaM that binds to a pocket on the C terminus (Joiner et al., 2001; Wong and Schlichter, 2014) but there is no *a priori* reason to suspect a slow CaM interaction at this site. Growing evidence suggests the role for an additional calcium-sensitive step in hippocalcin, a molecule from a different family of calcium sensors that are expressed in hippocampal neurons (Kobayashi et al., 1992; O'Callaghan et al., 2003). Hippocalcin is normally freely diffusing in a cell until an increase in internal calcium concentration triggers a myristoyl switch at resident EF hands that allows it to translocate to the membrane (O'Callaghan and Burgoyne, 2003; Tzingounis et al., 2007; Andrade et al., 2012). Interestingly, hippocalcin can reversibly translocate to membrane regions in relation to spike-associated activity, and thus potentially respond to calcium increases to modify sAHP amplitude as required (Markova et al., 2008). While several lines of evidence point to a role for hippocalcin, gaining a strict sense of which data apply to CA1 pyramidal cells is difficult since many studies used a combination of dissociated cultured cells, CA3 pyramidal cells or neocortical pyramidal cells *in vitro*.

Potentially the closest comparison is work performed in CA3 pyramidal cells, even though one can expect a greater contribution by Kv7 channels to the sAHP. Thus, it was shown that the IsAHP is substantially reduced in CA3 pyramidal cells of hippocalcin KO mice, where it was also concluded Kv7.3 channels had a major role in generating the sAHP (Kim et al., 2012). The IsAHP was also reduced in hippocalcin KO mice tested in dissociated cells in culture or tissue slices (region unspecified) (Tzingounis et al., 2007). In dissociated rat hippocampal cultured cells the IsAHP was increased by transfecting hippocalcin but not when transfected with a mutant construct that could not be myristoylated (Tzingounis et al., 2007). Similarly, it was found that transfecting hippocalcin in organotypic cultures of neocortical neurons greatly enhanced the IsAHP, while the opposite occurred for transfection of hippocalcin shRNA (Villalobos and Andrade, 2010). The potential influence of more than one calcium sensor protein was indicated when transfection of neurocalcin-δ as an alternate member of this family also increased the IsAHP of neocortical neurons (Villalobos and Andrade, 2010). The site of action of these proteins, however, was not determined.

It is difficult to envision hippocalcin as a calcium sensor in a manner analogous to CaM since an elevation of calcium that triggers myristoylation effectively acts as a switch in function. Relevant here may be reports of hippocalcin acting as an intermediate to AMPA receptor internalization and synaptic plasticity (Palmer et al., 2005; Amici et al., 2009; Dovgan et al., 2010; Jo et al., 2010). Hippocalcin has also been shown to interact with PiP2 (O'Callaghan et al., 2005), leading to the possible transport of PiP2 to the membrane where it could augment Kv7 channel activation (Zhang et al., 2013; Kim et al., 2016, 2017). It is uncertain as to how these data pertain to CA1 pyramidal cells where Kv7 channels have not been recognized as significant contributors to the sAHP. Yet the role for hippocalcin or other members of this family (Haynes et al., 2006; Burgoyne and Haynes, 2012; Raghuram et al., 2012) with regard to the sAHP is a rich target for future work.

State of the Field

The intensive efforts of hundreds of studies trying to define the molecular identity of sAHP potassium channels have come across unique pharmacological traits that have slowed progress. The reasons for a marked difference between data reported in the King et al. (2015) and Wang et al. (2016) studies remains to be identified. However, in the authors' view the data for a role for IK channels are at least strong and have been repeated in more than one lab. The recent findings on the outcome of heteromeric combinations of IK and SK channel isoforms serve as another plausible contributing factor to the sAHP that awaits further analysis. The role for alternate calcium sensors opens up a wide range of possibilities for further analysis in the CA1 region.

CALCIUM SOURCES DRIVING THE CA1 PYRAMIDAL CELL SLOW AFTERHYPERPOLARIZATION

Equally important to identifying the molecular basis of an AHP are the properties of calcium sources that drive calcium-gated potassium channels. Given difficulties in identifying a direct blocker of "sAHP channels" attention shifted to factors that governed the activation of such a prolonged sAHP.

Slow Afterhyperpolarization Onset and Decay

A peculiar characteristic of the sAHP is a delayed onset to peak ∼500 ms after a stimulus, and a long rate of decay (τ ∼1.5 s) (Jahromi et al., 1999; Gerlach et al., 2004). It was interesting that these properties did not fit the reported fast activation rate of expressed SK channels (Kohler et al., 1996; Hirschberg et al., 1998; Xia et al., 1998). Early studies thus also focused on the calcium sources that drive the sAHP. An initial entry of calcium *via* voltage-gated calcium channels (VGCCs) was established early by block of the sAHP with external Cd^{2+} perfusion (Madison and Nicoll, 1984; Lancaster and Adams, 1986). Introduction through the electrode of EGTA, BAPTA, or other salts with calcium buffering effects reduced or sped the kinetics of the IsAHP (**Figure 1E**; Madison and Nicoll, 1984; Zhang et al., 1994, 1995; Velumian et al., 1997; Tzingounis et al., 2007). The response of the sAHP was not always as expected, however, where inclusion of low levels of calcium chelators in the electrode could induce a slow increase in amplitude or decay time of the IsAHP during equilibration of electrode contents (Zhang et al., 1995; Velumian and Carlen, 1999). In addition, photolytic activation of either DM-Nitrophen or Nitr-5 to increase internal levels of

calcium still evoked an apamin-insensitive outward current with a slow rate of activation to peak 200–300 ms later (Lancaster and Zucker, 1994; Sah and Clements, 1999). All together this led to the understanding of a pattern of relatively slow activation of calcium sources or calcium-dependent potassium channels that delayed the peak of the sAHP, with the duration of the sAHP presumably reflecting the time-course of diffusion/decay of the internal calcium increase (Knöpfel et al., 1990; Müller and Connor, 1991; Lancaster and Zucker, 1994; Sah and Clements, 1999; Gerlach et al., 2004).

Cav1.3 Calcium Channels

The search for VGCCs that could act as the calcium source established that the IsAHP in intact tissue was not affected by toxins or blockers of N-type (ω-Ctx-GVIA), P/Q-type (ω-Ctx-MVIIC, Aga IVA), T-type (SFTX-3.3, TTA-P2) or R-type (SNX-482) channels (Rascol et al., 1991; Tanabe et al., 1998; Borde et al., 2000; Lima and Marrion, 2007; Kaczorowski, 2011; Sahu et al., 2017). Rather, a role for L-type calcium channel isoforms was shown by a block of the sAHP/IsAHP by verapamil and the dihydropyridines nimodipine, nifedipine, and in particular, isradipine at levels as low as 1–2 μM (**Figure 6A**; Rascol et al., 1991; Moyer et al., 1992; Lima and Marrion, 2007). A greater specific influence by the Cav1.3 compared to Cav1.2 calcium channel isoform was found in a study of CA1 pyramidal cells of Cav1.x knockout (KO) mice. In these animals the area of the sAHP evoked following a 5 spike train was significantly reduced in Cav1.3 KO but not in Cav1.2 KO mice compared to *wt* mice (**Figure 6B**; Gamelli et al., 2011). Sahu et al. (2017) revisited this issue to test the role of Cav1 channels capable of activating IK channels in rat CA1 pyramidal cells. This was important in that IK channels were recognized as being highly sensitive to dihydropyridines in the concentration range traditionally used to block Cav1 channels. Thus, IK channels are blocked by dihydropyridines at reportedly low levels of nifedipine (IC_{50} 27 nM), nimodipine (IC_{50} 1 μM), nitrendipine (IC_{50} 27 nM) or verapamil (IC_{50} 28 μM) (Jensen et al., 1998; Wulff et al., 2007).

To selectively block Cav1.x but not IK channels Sahu et al. (2017) tested Cav1 calcium and IK channels expressed in tsA-201 cells. Isradipine proved to significantly reduce IK current at 1 μM or above, but had no effect at 500 nM (**Figure 6C**; Sahu et al., 2017). By comparison, 500 nM isradipine produced ∼50–60% block of Cav1 current in CA1 pyramidal cells and of Cav1.2 or Cav1.3 expressed in tsA-201 cells (**Figure 6C**). To test the role of Cav1 calcium channels in activating IK channels they recorded IsAHP in CA1 pyramidal cells with pharmacological isolation of IK channels, and perfused a suite of calcium channel toxins and blockers against low-voltage-activated (LVA) and all high voltage-activated (HVA) channels except Cav1 channels. The IsAHP was not significantly reduced upon perfusion of these calcium channel blockers, but was blocked by subsequent perfusion of 1 μM TRAM-34 (**Figure 6D**). Repeating this test in the presence of the same LVA/HVA calcium channel blockers followed by perfusion of 500 nM isradipine to selectively target Cav1 channels reduced the IsAHP by ∼40% (**Figure 6D**). These tests were important in establishing that a selective block of Cav1 calcium channels can reduce the IK-mediated IsAHP in pyramidal cells. Nonetheless, recent work on calcium currents involved in sAHP potentiation suggest that this distinction is not absolute (see below).

Delayed Facilitation of Cav1 Channels

The activation of VGCCs to promote the calcium influx that generates the sAHP was assumed to take place largely during a preceding spike train or command step. Since the sAHP was sensitive to L-type calcium channel blockers, Cloues et al. (1997) isolated single L-type channels in the on-cell patch mode to test their rate of activation/deactivation (**Figure 7**). They found that a long step command pulse from −60 to −20 mV evoked a bout of rapid channel openings over a period of ∼500 ms (**Figures 7A,B**). By comparison, a short 50 Hz train of spike commands activated L-type channels at the end of the stimulus with intense and persistent openings of up to ∼5–6 s (**Figure 7B**). Ensemble averages of channel openings after a short spike train revealed a delayed facilitation where calcium channel activity rose to an initial peak at ∼500 ms and decayed over a similar time as that of IsAHP. From this and related work it was concluded that the slow activation of sAHP potassium channels reflects at least the properties of the associated L-type calcium channels (Cloues et al., 1997; Marrion and Tavalin, 1998; Bowden et al., 2001). Sahu et al. (2017) later confirmed that the activation of IK channels strongly reflects the voltage-dependence of activation and conductance of either Cav1.2 or Cav1.3 when coexpressed in tsA-201 cells.

Cav1.x Isoform-Specific Actions

The mechanisms underlying delayed facilitation of Cav1 channel activity are not fully known. However, previous work has revealed Cav1.x isoform-specific properties that could contribute to delayed facilitation. Several studies have reported a transition in L-type channel activity following a depolarizing stimulus to one of long duration openings and high P(o) over time frames of 70–500 ms, although often in the presence of a DHP receptor agonist. Some of the first reports of different gating modes were in cardiac L-type channels (Pietrobon and Hess, 1990; Yue et al., 1990), hippocampal neurons (Fisher et al., 1990; Thibault et al., 1993), cerebellar granule cells (Slesinger and Lansman, 1991, 1996; Forti and Pietrobon, 1993; Koschak et al., 2007), and sensory and motor neurons (Ferroni et al., 1996; Hivert et al., 1999). These properties were reported as a voltage-dependent change in gating pattern (Pietrobon and Hess, 1990; Yue et al., 1990), voltage-dependent potentiation (Kavalali and Plummer, 1994, 1996), or anomalous gating/repolarization openings (Fisher et al., 1990; Slesinger and Lansman, 1991, 1996; Thibault et al., 1993; Koschak et al., 2007). Other work suggested that differential gating reflects two subclasses of channels referred to as Lp (potentiation) vs. Ls (standard), where a stimulus train selectively potentiated the activity of Lp channels beyond the stimulus for up to 200 ms (Kavalali and Plummer, 1994, 1996). Interestingly, the repolarization opening of L-type channels in cerebellar granule cells was traced to the Cav1.2 isoform (Koschak et al., 2007). Cav1.2 channels in ventricular myocytes also exhibit a PKA-dependent increase in membrane expression and formation of clusters through C-terminal linkage to enhance calcium current

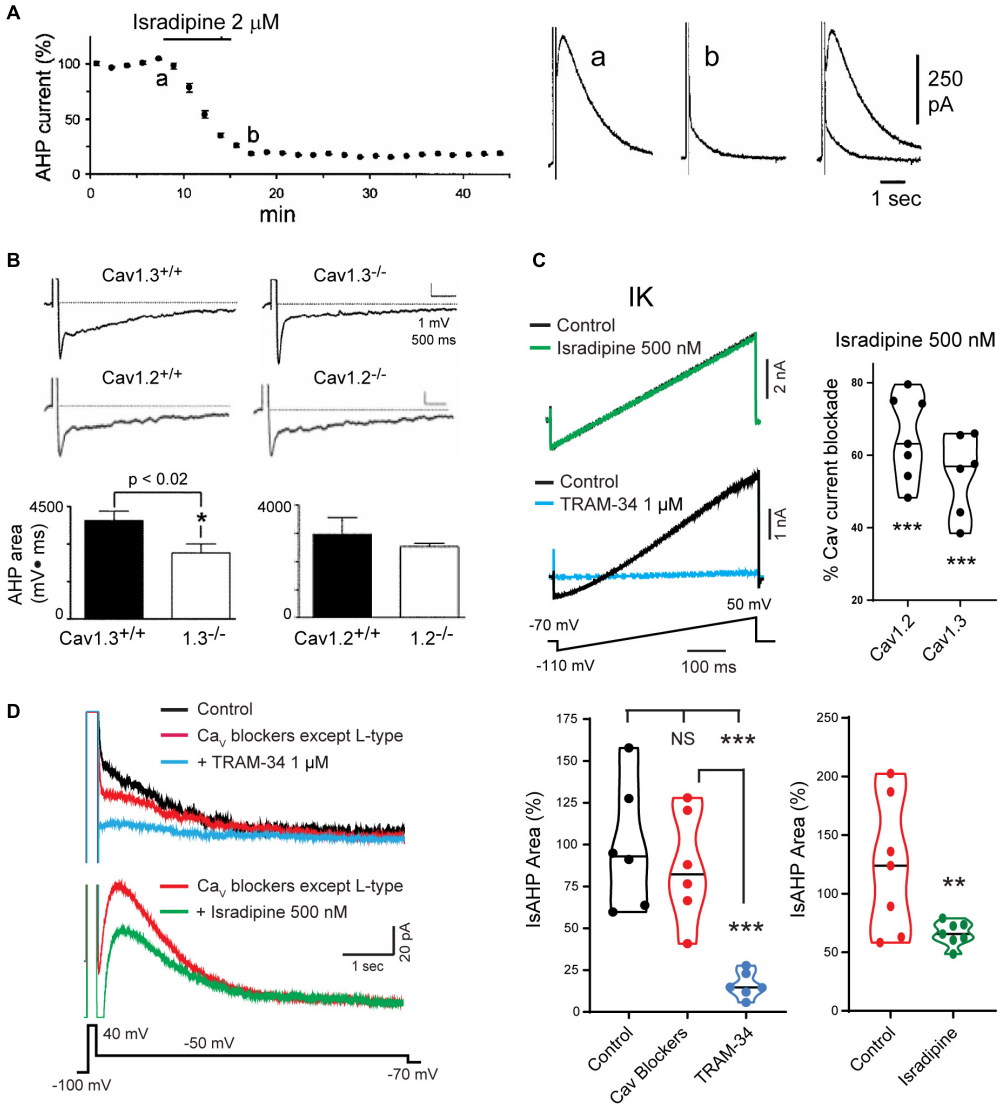

FIGURE 6 | Cav1 L-type calcium channels activate the sAHP. **(A)** The IsAHP is reduced by the L-type channel blocker isradipine. **(B)** The area of the sAHP evoked following a five spike train evoked by current injection is reduced in Cav1.3$^{-/-}$ but not Cav1.2$^{-/-}$ knockout mice. **(C)** Isradipine applied at 500 nM is below a level that affects TRAM-34-sensitive IK channels expressed in tsA-201 cells but provides a substantial block of Cav1.2 and Cav1.3 channel isoforms. **(D)** *Top traces:* The IsAHP in CA1 pyramidal cells with pharmacological isolation of IK channels is not significantly reduced by a suite of calcium channel blockers that excludes dihydropyridines (Cav2.1, ω-Ctx-MVIIC 200 nM; Cav2.2, ω-Ctx-GVIA 1 μM; Cav2.3, SNX-482 200 nM; Cav3.x, TTA-P2 1 μM). Verification of IK channel activation is obtained by a subsequent reduction of the IsAHP by 1 μM TRAM-34. *Bottom traces:* The IsAHP recorded in a separate pyramidal cell in the presence of all calcium channel blockers except L-type is reduced by perfusion of 500 nM isradipine to selectively block Cav3.x channels. Figures are modified from Tanabe et al. (1998) **(A)**, Gamelli et al. (2011) **(B)**, and Sahu et al. (2017) **(C,D)**. $^{**}p < 0.01$; $^{***}p < 0.001$, Student's paired *T*-test for mean ± SEM. The baseline temperature for recordings was: **(A)** 32°C (King et al., 2015), **(B)** 31°C (Gamelli et al., 2011), **(C)** 22°C (Sahu et al., 2017), and **(D)** 32–34°C (Sahu et al., 2017).

through cooperative gating interactions (Navedo et al., 2010; Dixon et al., 2012, 2015; Ito et al., 2019a). An analogous process of channel aggregation was reported for a Cav1.3S isoform that has a short C-terminus, but not for a Cav1.3L isoform with a longer C-terminus (Moreno et al., 2016). By forming clusters of 5–8 channels Cav1.3S channels exhibit cooperative gating to increase calcium influx. Yet the majority of studies on these increases in calcium channel activity following a stimulus report an increase in P(o) over a time frame of <500 ms, which is much shorter than that of delayed facilitation that can last ∼6 s (Cloues et al., 1997). Reports of a PKA- or isoproterenol-induced increase in Cav1.2 channel activity is also opposite to that reported of a block of delayed facilitation of Cav1 channel activity by beta adrenergic receptor activation (Cloues et al., 1997).

In CA1 pyramidal cells, a different process supporting CDF was found for the Cav1.3L isoform that contains a PDZ binding domain that interacts with the accessory protein densin. Densin is important as it acts as a bridge to Cav1.3L (but not Cav1.3S)

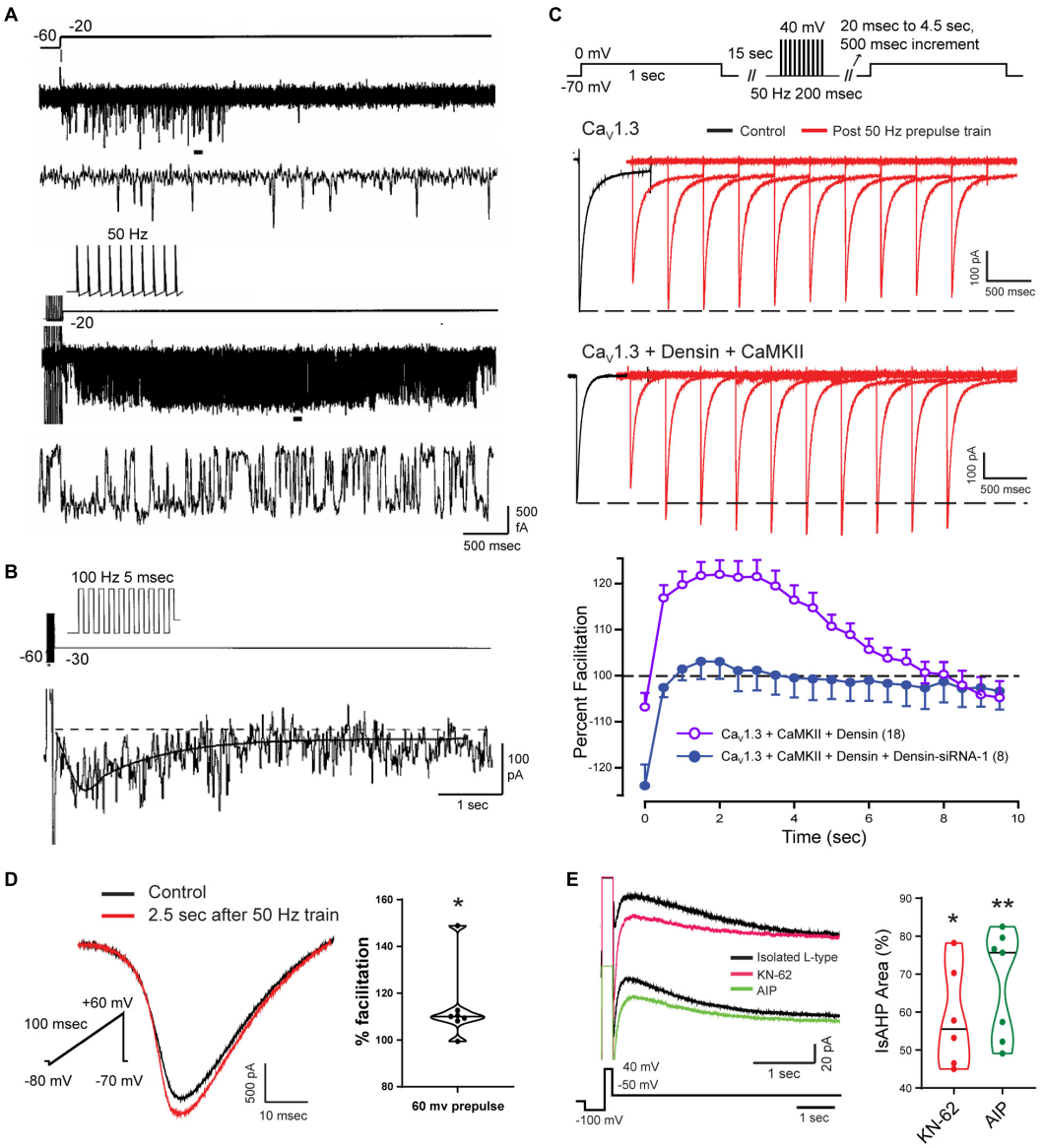

FIGURE 7 | Facilitation of Cav1 calcium current increases the magnitude of the sAHP. **(A,B)** On-cell patch recordings of L-type calcium channels at the soma of CA1 pyramidal cells acutely dissociated from P9-14 rat pups. Calcium channel activity undergoes a delayed facilitation induced by a short train of spike-like responses compared to a 40 mV step depolarization **(A)**. Expanded traces corresponding to horizontal bars show that delayed facilitation reflects an increase in the frequency and duration of openings. Ensemble average (27 sweeps) of L-type channel activity evoked by a 100 Hz train reveals a delayed peak (~600 ms) and prolonged duration of channel openings ($\tau \sim 1.6$ s) **(B)**. **(C)** Whole-cell recordings from tsA-201 cells subjected to step commands before and after presenting a 50 Hz train of spike-like steps. Cav1.3 channels exhibit a long duration calcium-dependent facilitation (L-CDF) that depends on coexpressing the linking protein densin and αCaMKII. Preincubation of tsA-201 cells with densin siRNA blocks L-CDF of Cav1.3 **(E)**. **(D,E)** Whole-cell recordings in rat CA1 pyramidal cells with L-type current isolated using blockers and toxins against other calcium channels (1 μM ω-conotoxin GVIA, 200 nM Agatoxin, 200 nM SNX-482, and 1 μM TTA-P2). L-type current is facilitated 2.5 s following a 50 Hz train of 200 ms steps to +60 mV **(D)**. The L-type IsAHP is reduced by CaMKII inhibitors KN-62 (10 μM) or AIP (20 μM) **(E)**. Average values in **(C)** are mean ± SEM, with violin plots for data sets in **(D,E)**. *$p < 0.05$, **$p < 0.01$; Student's paired *t*-test. Figures are modified from Cloues et al. (1997) **(A,B)** (Copyright [1997] Society for Neuroscience) and Sahu et al. (2017) **(C–E)**. The baseline temperature for recordings was: **(A,B)** 37°C (Cloues et al., 1997), **(C)** 22°C (Sahu et al., 2017), and **(D,E)** 32-34°C (Sahu et al., 2017).

to bind αCaMKII to promote phosphorylation. Jenkins et al. (2010) established in hippocampal neurons that the Cav1.3L-densin-CaMKII interaction increases net calcium current by reducing calcium-dependent inactivation (CDI) of the channel during repetitive stimulation. Sahu et al. (2017) used tsA-201 cells and CA1 pyramidal cells to test for any role of densin in modifying Cav1.3L function following a preceding train of spike-like commands at 50 Hz. They found that when Cav1.3L was coexpressed with densin and αCaMKII in tsA-201 cells it resulted in a long duration calcium-dependent facilitation (L-CDF) of

Cav1.3 channel current that could last for up to 8 s (**Figure 7C**). Moreover, the degree of facilitation increased over the first 500–1000 ms to peak 3–4 s post stimulus by at least 20% over the control test pulse (**Figure 7C**). As such, the L-CDF enabled by Cav1.3L, densin and αCaMKII effectively recapitulated the time course of an sAHP when coexpressed in tsA-201 cells. However, L-CDF was not evoked in the case of Cav1.3L expressed alone or when coexpressed with only densin or αCaMKII (**Figure 7C**). By comparison, L-CDF was not observed in tsA-201 cells expressing either Cav1.3S or Cav1.2 coexpressed in combination with densin and/or αCaMKII.

Complementary tests were conducted in CA1 pyramidal cells in the slice preparation using a 50 Hz pulse train followed by a brief test command. These experiments were also carried out in the presence of a suite of calcium channel toxins and blockers to isolate Cav1-mediated calcium current. Here an L-CDF process was detected that amplified Cav1 calcium current by up to 14% when tested 2.5 s after delivering a repetitive stimulus train to 60 mV (**Figure 7D**; Sahu et al., 2017). Moreover, the IsAHP evoked by a step command was reduced by application of either KN-62 or AIP as CaMKII inhibitors (**Figure 7E**). This work further supported an earlier report that a CaMKII knockin mouse with deficient autophosphorylation specifically reduced the synaptically evoked sAHP, leading to an increase in CA1 cell excitability (Sametsky et al., 2009).

Together these data suggest that a densin/CaMKII-mediated L-CDF of Cav1.3 channels represents one factor contributing to delayed facilitation that delays the peak and prolongs the duration of an evoked sAHP.

RYANODINE RECEPTORS

Functional Coupling of Cav1.3-RyRs

A delayed facilitation of L-type calcium channel activity is able to provide one solution to the long duration sAHP response. However, Cav1.1 and Cav1.2 calcium channels in skeletal and cardiac muscle are known to mediate excitation-contraction (E-C) coupling by linking to ryanodine receptors (RyRs) to enhance intracellular calcium concentration increases (Nakai et al., 1998; Avila et al., 2019). All three RyR isoforms are expressed in CA1 pyramidal cells, including reports of RyR1 and prominent expression of RyR2 and RyR3 (Furuichi et al., 1994; Giannini et al., 1995; Murayama and Ogawa, 1996; Mori et al., 2000; Kim et al., 2007). In other cells a RyR-mediated calcium increase was found to be central to generating the sAHP (Sah and McLachlan, 1991; Jobling et al., 1993; Cordoba-Rodriguez et al., 1999; Pineda et al., 1999; Vogalis et al., 2001). Early work reported variable results in assessing the role for RyRs in generating the sAHP in hippocampal neurons. A RyR-mediated contribution to the sAHP was described for CA3 pyramidal cells in organotypic cultures (Tanabe et al., 1998). In CA1 pyramidal cells some reported that blockers or agonists of RyRs had no effect on the sAHP (Zhang et al., 1995) or only slightly reduced the sAHP in cultured cells (Shah and Haylett, 2000). Yet overall, the data indicate that Cav1 channel isoforms in CA1 and CA3 pyramidal cells are functionally coupled to RyRs to invoke calcium-induced calcium release (CICR) in response to spike activity.

As evidence, calcium imaging in CA1 pyramidal cells established that brief application of caffeine as a RyR agonist evoked large calcium transients up to 600 nM above baseline (Garaschuk et al., 1997; Kim et al., 2007; Berrout and Isokawa, 2009). Caffeine-induced calcium elevations were blocked by higher concentrations of ryanodine or nimodipine, with sensitivity to cyclopiazonic acid (CPA) or thapsigargin to block endoplasmic Ca-ATPases (Garaschuk et al., 1997; Kim et al., 2007; Berrout and Isokawa, 2009). The ability to trigger RyR-mediated calcium increases physiologically came in findings that single or multiple spikes trigger transient elevations in calcium concentration that were sensitive to caffeine, ryanodine, thapsigargin, or CPA (Jacobs and Meyer, 1997; Sandler and Barbara, 1999). Spike-associated increases in internal calcium can also be very focal in that backpropagating spikes produce an L-type channel and RyR-mediated calcium increase localized to dendritic spines (Johenning et al., 2015).

Specific involvement of RyRs in generating the sAHP that follows a depolarizing stimulus has been demonstrated in multiple studies where application of higher levels of ryanodine reduced the amplitude and area of the sAHP/IsAHP along with a reduction in spike accommodation (**Figures 8A–C**; Torres et al., 1996; Borde et al., 2000; Kumar and Foster, 2004; Gant et al., 2006; van de Vrede et al., 2007; Sahu et al., 2019; Tedoldi et al., 2020). This was accompanied by data showing a significant block of the sAHP/IsAHP with effects on spike firing by dantrolene or ruthenium red as alternate blockers of CICR, and by thapsigargin or CPA (**Figure 8C**; Torres et al., 1996; Borde et al., 2000; Kumar and Foster, 2004; van de Vrede et al., 2007; Tedoldi et al., 2020). Evidence that RyR2-mediated calcium release can activate IK channels was reported by Sahu et al. (2017, 2019) when coexpressing Cav1.3, RyR2 and IK cDNA in tsA-201 cells produced an IsAHP-like response to a step command that is very similar to that of CA1 pyramidal cells (**Figure 8B**). Calculating the difference between test and control responses of IK recordings further revealed a rapid onset and long duration contribution of the ryanodine-sensitive component in both CA1 pyramidal and tsA-201 cells (**Figure 8B**; Sahu et al., 2019).

Together these studies established a functional interplay between L-type calcium channels and RyR-mediated CICR in CA1 pyramidal cells that can be triggered by spike-like depolarizations to provide the increase in internal calcium concentration required to activate an sAHP.

Cav1 Activation of Ryanodine Receptor Isoforms

Evidence has been gained that a block of the sAHP by modulators of CICR may involve specific RyR isoforms. van de Vrede et al. (2007) reported that RyR3 contributes to the sAHP in mouse CA1 pyramidal cells. Here a selective RyR3 antibody infused through the electrode blocked ∼70% of the IsAHP but found no effects when an anti-RyR2 antibody was infused. RyR3 was also implicated in a recent study by Tedoldi et al. (2020), although their results differed in finding that the

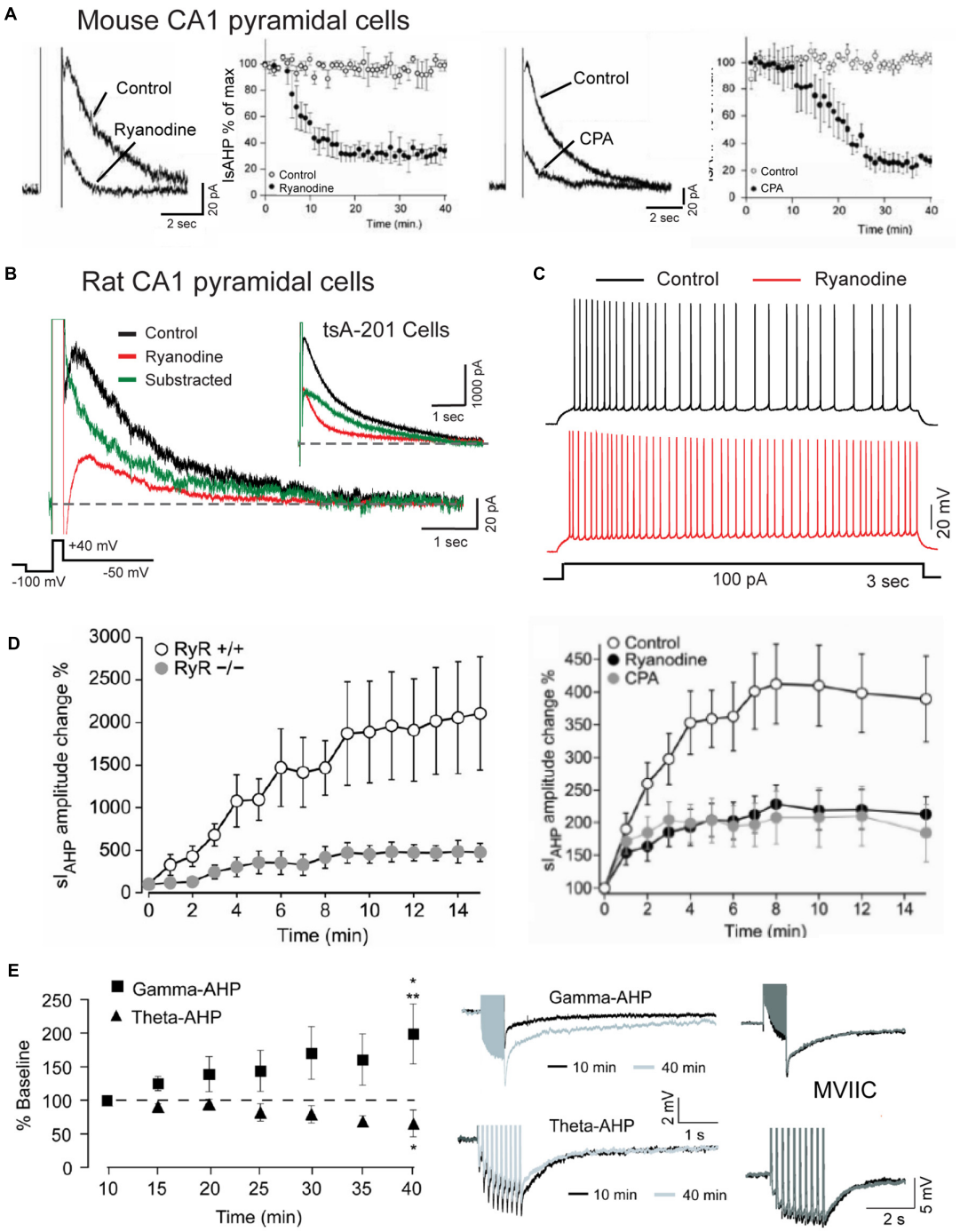

FIGURE 8 | The sAHP is augmented by RyR-mediated calcium release. **(A)** The IsAHP in mouse CA1 pyramidal cells is blocked by ryanodine or the SERCA pump inhibitor CPA. **(B)** Whole-cell recording of isolated IK as an IsAHP in a rat CA1 pyramidal cell before and after infusing 100 μM ryanodine through the electrode. Control and test recordings are superimposed with the difference response. *Inset* shows the same test conducted in tsA-201 cells where coexpressing Cav1.3, IK, and RyR2 produces the same rapid RyR-mediated contribution to an IsAHP-like outward current. **(C)** Spike accommodation in a CA1 pyramidal cell is blocked by 100 μM ryanodine infusion through the electrode. **(D)** A potentiation of the IsAHP in CA1 pyramidal cells with 0.5 Hz activation is blocked in RyR3$^{-/-}$ animals and reduced by pre-perfusion of 10 μM ryanodine or 50 μM CPA. **(E)** sAHP potentiation is selectively evoked by a gamma but not theta frequency pattern of postsynaptic spike firing. Gamma frequency sAHP potentiation is occluded by ω-conotoxin MVIIC (1 μM). Recordings in **(B,C)** were conducted in the presence of 100 nM apamin, 10 μM XE-991 and 2 mM CsCl to block SK, Kv7, and HCN channels, respectively. Average values in **(A,D,E)** are mean ± SEM. Figures are modified from van de Vrede et al. (2007) **(A)**, Sahu et al. (2017) **(B,C)**, Tedoldi et al. (2020) **(D)**, and Kaczorowski (2011) **(E)**. The baseline temperature for recordings was: **(A)** 20–22°C (van de Vrede et al., 2007), **(B)** 32–34°C (Sahu et al., 2017), **(D)** 22°C (Tedoldi et al., 2020), and **(E)** 33–34°C (Kaczorowski, 2011).

baseline sAHP in CA1 pyramidal cells in RyR3 KO mice was not significantly different from *wt* animals but still blocked by ryanodine, suggesting a contribution from other RyR isoforms. In the same year Kim et al. (2007) reported that Cav1.3 and RyR2 exhibit direct interactions. Here they used yeast two hybrid and coimmunoprecipitation assays to establish an association at the molecular level between the N-terminus of Cav1.3 (aa 45–115) and N terminus of RyR2 (aa 3150–3680). Interestingly, no coimmunoprecipitation was found between Cav1.2 and RyR2 (Kim et al., 2007), again indicating a more significant role for Cav1.3 compared to Cav1.2 in generating the sAHP. The Cav1.3-RyR2 interaction even extended into increases of RyR2 mRNA by 2 h of depolarization induced by a high K medium, with the mRNA increase reduced by nifedipine or ryanodine, and increased by the L-type agonist BayK 8644.

Remarkably, all aspects of Cav1.3-RyR2 functional coupling in Kim et al. (2007) were reproduced in the absence of extracellular calcium. These results are important in drawing comparisons to the voltage-dependent process found in skeletal muscle through a direct mechanical (non-calcium-dependent) interplay between Cav1.1 and RyR1 (Nakai et al., 1998; Avila et al., 2019). To our knowledge this result stands alone as the only report of a calcium-independent interaction between Cav1.3 and RyR2 or voltage-dependent activation of RyR2 in pyramidal cells. However, the Kim et al. (2007) study was also unique in using a combined high K/BayK 8644 medium to stimulate Cav1.3 channels, which can trigger multiple signaling cascades (Rienecker et al., 2020). The direct association between Cav1.3 and RyR2 established biochemically by Kim et al. (2007) is thus consistent with a role in generating the sAHP, with the proximity of these proteins now verified through FRET imaging (see below) (Sahu et al., 2019). However, it is more difficult to relate the function of a voltage-dependent Cav1.3-RyR2 interaction to generating the sAHP using a High K/Bay K8644 medium, as the initial 3–5 s phase of the sAHP has been shown repeatedly to be calcium-dependent (**Figures 1C–G**).

Activity-Dependent Potentiation of the Slow Afterhyperpolarization

An intriguing aspect of the sAHP is an ability to increase or decrease over time in an activity-dependent manner. The first report of this used 2 s current pulses to elicit spike firing presented at long interpulse intervals (Borde et al., 1995). They and others noted a graded reduction in spike firing with pulse intervals as low as 1 per min due to an increase in spike adaptation that was accompanied by an increase in the post train sAHP for up to 170 s (Borde et al., 1995, 2000). The responsible intrinsic factor was calcium-dependent, insensitive to TEA or 4-AP, but blocked by L-type channel blockers and ryanodine, implicating internal calcium release from RyRs as one potential contributor to potentiation (Borde et al., 2000). Indeed, Tedoldi et al. (2020) identified a role for the RyR3 isoform in recordings from a RyR3 KO mouse that revealed a selective reduction in IsAHP potentiation even though the baseline sAHP was not detectably altered (**Figure 8D**). They also reported that sAHP potentiation with low frequency activation (0.5 Hz) was reduced by either CPA or ryanodine, indicating a complex interplay between calcium release from RyRs and calcium stores, as suggested in control recordings in which the range of variation in degree of potentiation was reflected in values of S.E.M. (**Figure 8D**).

Kaczorowski (2011) tested the effects of physiologically relevant patterns of spike discharge on the degree of sAHP potentiation. One was designed to mimic a gamma frequency rate of spike discharge using a train of 50 pulses at 50 Hz. The second mimicked a theta burst pattern consisting of 10 bursts of 5 spikes at 100 Hz (interburst frequency 5 Hz). After delivering these stimuli every minute in isolation (or even in alternation) the amplitude of the sAHP was selectively and markedly potentiated by the gamma but not theta frequency train (**Figure 8E**). Gamma frequency potentiation was further occluded by pre-treatment with nimodipine (10 μM), but curiously, also by ω-conotoxin MVIIC (1 μM) applied as a putative combined N/P-Q type calcium channel blocker (**Figure 8E**). In addition, gamma frequency sAHP potentiation was blocked by pre-exposure to the HCN channel blocker ZD 7288 (25 μM), a result that was opposite to findings in Borde et al. (1995). However it is worth noting that ZD 7288 has been shown to also block T type calcium current in CA1 pyramidal cells (IC_{50} ∼40 μM) (Sanchez-Alonso et al., 2008). Calcium influx through different channel families might then find a common output by activating RyR3 (Tedoldi et al., 2020), but tests with ryanodine were not included in Kaczorowski (2011).

Intrinsic factors that support potentiation of the sAHP can also affect synaptic plasticity. Thus, the depression of intrinsic excitability induced by current-evoked sAHP potentiation was transferred to a reduction in synaptic responsiveness and spike discharge for 4–5 min (Borde et al., 1999). Conversely, low frequency repetitive synaptic stimulation incorporated postsynaptic sAHP potentiation to increase spike adaptation in response to subsequent trains of synaptic input, and the ability for other synaptic inputs to exhibit LTP (Borde et al., 1999; Le Ray et al., 2004). An early report of an augmented form of NMDA-independent (but nimodipine-sensitive) LTP of synaptic transmission in CA1 pyramidal cells of RyR3 KO mice may then reflect a reduction in postsynaptic IsAHP potentiation, although this was not tested (Futatsugi et al., 1999).

Together these results reveal that sAHP potentiation can be evoked entirely by intrinsic postsynaptic mechanisms that can be selectively recruited according to specific patterns of physiologically relevant spike discharge. They also suggest that calcium channel subtypes beyond L-type calcium channels mediate a gamma frequency-selective sAHP potentiation that can contribute to synaptic plasticity.

A Cav1-RyR-IK TRIPARTITE COMPLEX DRIVES THE SLOW AFTERHYPERPOLARIZATION

The functional coupling that had become apparent between calcium sources and the sAHP led Sahu et al. (2019) to more closely examine the spatial relationship between Cav1.x, RyR2, and IK channels using STORM super-resolution microscopy

in dissociated hippocampal neurons (**Figure 9**). By adding TIRF illumination they could further restrict fluorescent images to within 150 nm of the coverslip surface to focus on near membrane-associated labels. Dual color dSTORM-TIRF imaging found that ∼80–85% of clusters of Cav1.3, RyR2, and IK immunolabels exhibited overlapping emissions (**Figures 9A–C**). Calculations of the minimal distance to nearest neighbor cluster centroids confirmed close positioning between each of Cav1.3-IK, RyR2-IK, and Cav1.3-RyR2. Expression of eGFP- or mKate-tagged constructs in tsA-201 cells further revealed FRET between each of these pairs of labeled proteins, indicating an association of < 10 nm distance from one another (Sahu et al., 2019). Together these data support the existence of a tripartite complex of Cav1.3-RyR2-IK channels that are optimally positioned to allow calcium-gated activation of potassium channels underlying the sAHP.

Some support was gained for Cav1.2 to also form a similar complex with RyR2 and IK channels. Here morphological cluster analysis reported an even higher density of Cav1.2 than Cav1.3 immunolabel clusters in hippocampal cell membranes (Sahu et al., 2019), as earlier reported for channel expression levels in hippocampal pyramidal neurons (Hell et al., 1993). Yet Cav1.3 and IK immunolabel clusters exhibited overlap almost twice as often as for Cav1.2 and IK clusters. Cav1.3 channels are thus implicated again as a primary membrane-associated calcium source, although a role for Cav1.2 within a Cav1.2-RyR2-IK complex can not be ruled out. The potential role for RyR3 receptors to participate in these complexes was not tested.

Junctophilin 3/4 Proteins Link the Cav1-RyR-IK Complex at Endoplasmic Reticulum-Plasma Membrane Junctions

A striking aspect of a complex of this nature is that it draws on a Cav1.x calcium source expressed at the plasma membrane (PM) and RyRs positioned in the endoplasmic reticulum (ER). It is known from muscle physiology that subunits of Cav1-RyR complexes are maintained in close apposition by Junctophilin 1/2 (JPH1/2) proteins (Piggott and Jin, 2021). In contrast, hippocampal neurons express JPH-3&4 isoforms as one of several classes of linking proteins to help establish ER-PM junctions at distances as close as 30 nm (Nishi et al., 2003; Rowland and Voeltz, 2012; Wu et al., 2017). Indeed, previous studies established that Cav1 and RyR proteins are members of ER-PM junctions, and the mAHP in hippocampal cells is reduced in JPH3/4 KO mice by affecting RyR-SK interactions (Moriguchi et al., 2006; Johnson B. et al., 2018; Vierra et al., 2019). Sahu et al. (2019) used super-resolution imaging to report a close association between JPH-3&4 proteins and subunits of the Cav1.3-RyR2-IK complex (**Figures 10A–C**). Furthermore, knocking down JPH3/4 expression in cultured cells dissociated the multiprotein complex, as shown by a dramatic reduction in the overlap in cluster immunolabels and a bimodal histogram distribution for nearest neighbor clusters (**Figure 10D**).

To test the dependence of the sAHP on JPH3/4 protein expression and function, Sahu et al. (2019) conducted whole-cell recordings of the IsAHP in cultured hippocampal neurons and found that pretreatment of cultures with JPH3/4 shRNA for 48–72 h blocked the response, but not in the case of scrambled shRNA (**Figure 10E**). Similar results were observed in CA1 pyramidal cells in the slice preparation when JPH-3&4 antibodies were infused through the electrode, serving to block the IsAHP (**Figure 10F**) and spike accommodation mediated by the sAHP in response to current injection (**Figure 10G**).

DISCUSSION

The long-sought explanation for the molecular basis for a calcium-dependent sAHP in CA1 pyramidal cells has come closer to being resolved through the combined efforts of hundreds of studies over the past 30 years. The key stumbling blocks in identifying the underlying factors were a perplexing pharmacology and the delayed onset/long duration of the sAHP. Recent work now provides key answers to each of these issues in terms of the activity patterns and pharmacology of both potassium and calcium channels that contribute to the sAHP. In particular, the pharmacology of "sAHP channels" has many parallels to the properties of IK channels and a preferential heteromerization with SK1 channels (King et al., 2015; Turner et al., 2015, 2016; Higham et al., 2019). Secondly, the earlier finding of delayed facilitation of Cav1 calcium channels (Cloues et al., 1997; Bowden et al., 2001; Lima and Marrion, 2007) may reflect at least a densin-CaMKII-mediated interaction with Cav1.3 channels that underlies a novel form of L-CDF (Sahu et al., 2017). A tight functional coupling of IK channels to the activation and conductance of Cav1.3 channels (Sahu et al., 2017) will further ensure a coordinated interplay between these channels (Cloues et al., 1997; Lima and Marrion, 2007; Sahu et al., 2017). Finally, a long-recognized sequential activation of the sAHP by L-type calcium channels and RyR-mediated internal calcium release is explained by the existence of a triprotein complex between Cav1-RyR-IK channels (**Figure 11**). Proteins in this complex are juxtaposed with nanometer proximity through the actions of JPH3/4 proteins that link subunits at ER-PM junctions that will ensure efficient coupling of both calcium sources to the IK channel (Kim et al., 2007; Sahu et al., 2019; Tedoldi et al., 2020). The accumulated evidence suggests that specific attributes of each of the three members of the complex combine to create a slow onset and long duration calcium-dependent sAHP with unique pharmacological properties.

Pharmacology of Slow Afterhyperpolarization Potassium Channels

The sAHP in CA1 pyramidal cells defied an onslaught of pharmacological tests over the years to identify the underlying potassium channel isoform. However, advances in defining the pharmacology of IK channels (Wulff et al., 2000, 2007; Kaczmarek et al., 2017) and their expression in CA1 pyramidal cells (King et al., 2015; Turner et al., 2015, 2016) has supported

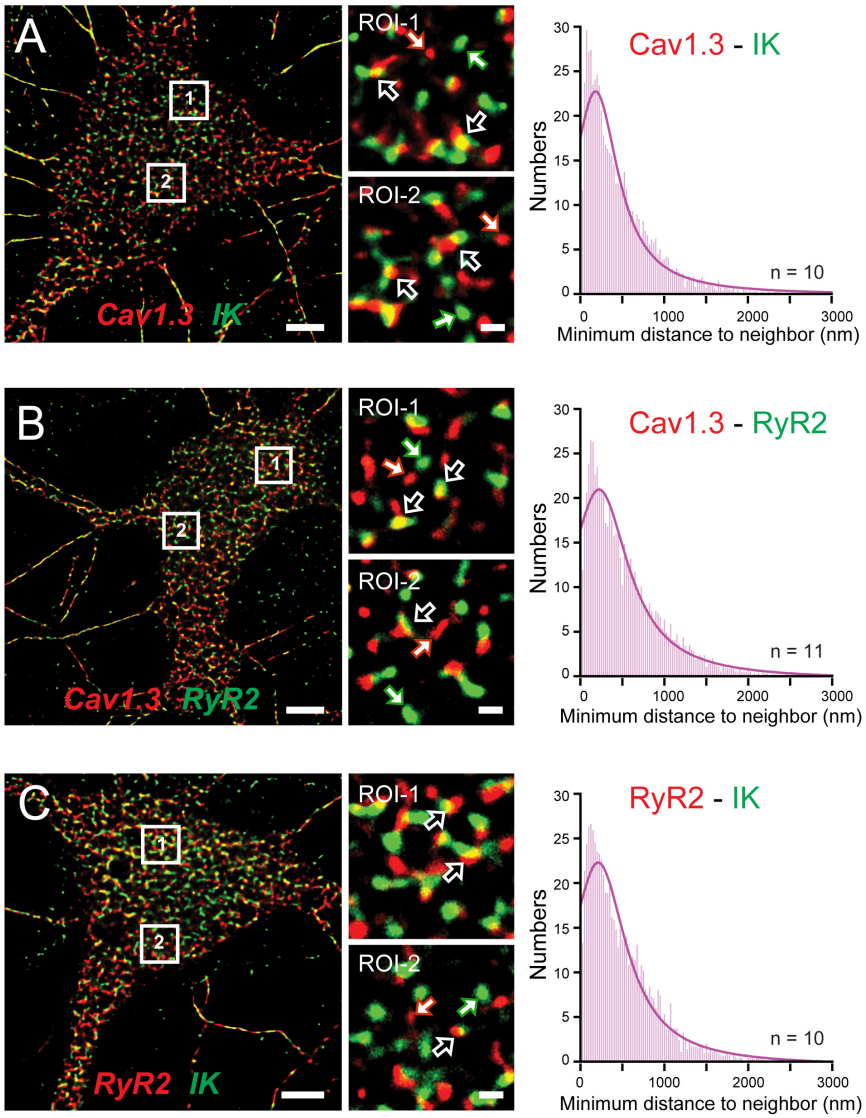

FIGURE 9 | A Cav1.3-RyR2-IK tripartate complex in hippocampal neurons. **(A–C)** dSTORM-TIRF images of dual immunofluorescent labeling in cultured hippocampal neurons for Cav1.3 and IK channels **(A)**, Cav1.3 and RyR2 **(B)**, and RyR2 and IK **(C)**. Representative ROIs identified by numbered boxes enlarged at center reveal clusters of fluorescent tags that present with overlap (*open arrows*) or as isolated signals (*solid white/colored arrows*). At right are histogram plots of the average minimal distance between nearest-neighbor cluster centroids for the indicated pair of labels (bin width, 25 nm). Scale bars: **(A–C)** 5 μm, (**A–C**, ROIs) 500 nm. Figures are modified from Sahu et al. (2019).

rapid progress over the past 6 years to provide a plausible molecular identity to at least one of the potassium channels that underlie the sAHP. The most recent work obtained in an expression system (Higham et al., 2019) is also pivotal in revealing a strong tendency for human IK and SK1 subunits to form heteromeric channels with characteristics that are remarkably similar to the hallmark identifiers of sAHP channels in CA1 pyramidal cells. Heteromeric IK-SK1 channels exhibit a single channel conductance of 30 pS, and importantly, an altered pharmacology that is insensitive to apamin and an IC_{50} for TRAM-34 of 557 nM – more than 10X higher than that of homomeric IK channels (IC_{50} 41 nM) (Higham et al., 2019). These data could then explain recent differences on the apparent efficacy of TRAM-34 as an IK channel blocker in CA1 pyramidal cells at 1 μM concentration (King et al., 2015; Turner et al., 2016; Wang et al., 2016). Notably, Tiwari et al. (2018, 2019) repeated the tests in CA1 pyramidal cells with bath-applied TRAM-34 at 5 μM, achieving a convincing block of the sAHP with no reported delay. Similar results were reported for Layer 5 neocortical pyramidal cells, where bath-applied TRAM-34 at 5 μM rapidly blocked an sAHP (Roshchin et al., 2020). The use of this higher level of TRAM-34 might then be required to act quickly on an IK-SK1 heteromeric channel population that exhibits a lower drug sensitivity than predicted from studies on

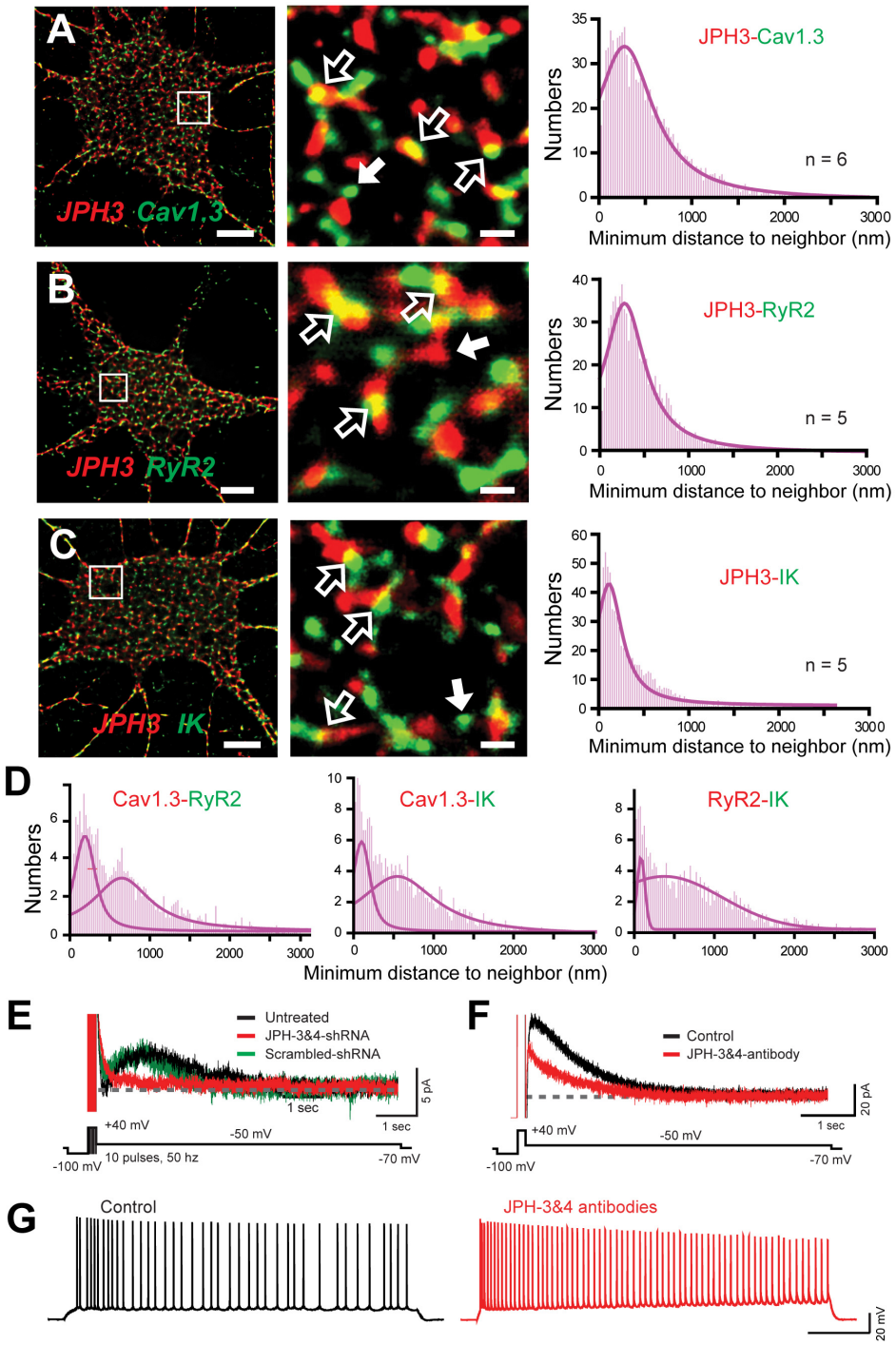

FIGURE 10 | The Cav1.3-RyR2-IK Complex colocalizes with the ER-PM linking protein JPH3. **(A–C)** dSTORM-TIRF images of dual immunofluorescence labeling in cultured hippocampal neurons for JPH3 and Cav1.3 **(A)**, JPH3 and RyR2 **(B)**, and JPH3 and IK **(C)**. ROIs indicated by boxes are enlarged at center, revealing clusters of JPH3 overlap with Cav1.3, RyR2, and IK (*open arrows*). Cases of individual clusters are shown by *solid white arrows*. At right are histogram plots of the average minimal distance between nearest-neighbor cluster centroids for the indicated pair of labels (bin width, 25 nm). **(D)** Minimal distance to neighbor plots from another experiment in which hippocampal cultures were pre-treated with JPH3&4 shRNA for 72 h. Dual dSTORM-TIRF images identified immunolabel clusters for each of Cav1.3, RyR2, and IK proteins. Histogram plots of minimal distance between the indicated protein pairs reveal a bimodal histogram reflecting less overlap of immunolabels, indicating dissociation of the complex without JPH3/4. **(E)** Whole-cell recordings from cultured hippocampal cells of the IK-mediated IsAHP evoked by a train of 10 pulses. Superimposed recordings are from separate cells either untreated or exposed for 72 h to intact or scrambled JPH3 and JPH4 shRNA. Knocking down JPH3/4 blocks the IsAHP. **(F)** Whole-cell recordings of the CA1 pyramidal cell of IK-mediated IsAHP in the slice preparation. The IsAHP is blocked

(Continued)

FIGURE 10 | within 10 min of internal infusion of anti-JPH3 and JPH4 antibodies (1:200) through the electrode. **(G)** Whole-cell recordings from CA1 pyramidal cells shows that spike accommodation is blocked within 10 min of infusing anti-JPH3/4 antibodies through the electrode. All recordings in **(A–D)** were conducted in the presence of 100 nM apamin and 10 μM XE-991 to isolate IK channels. Scale bars: **(A–C)** 5 μm, (**A–C**, ROIs) 500 nm. Figures are modified from Sahu et al. (2019), with recordings in **(E)** at 22°C (Sahu et al., 2019).

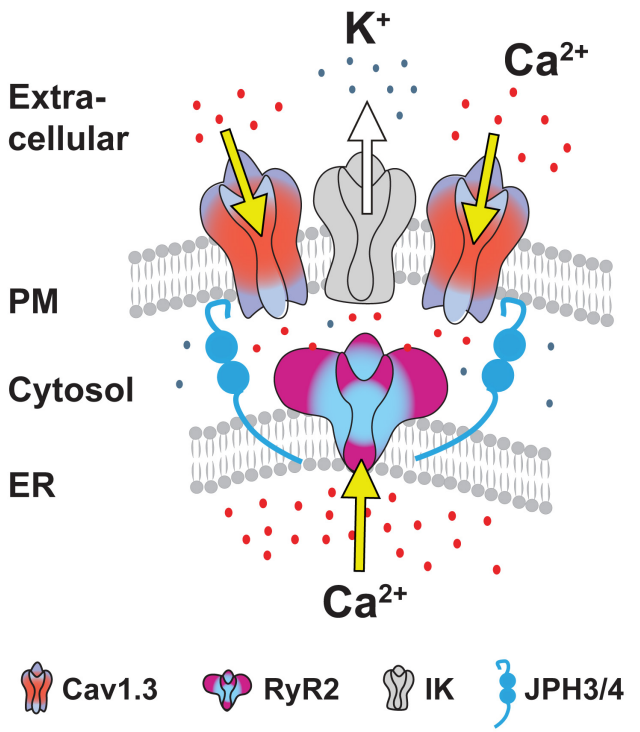

FIGURE 11 | A Cav1-RyR2-IK complex at ER-PM junctions coordinates two calcium sources to activate calcium-gated channels. A cartoon depiction of the tripartate protein complex at ER-PM junctions maintained by at least the linking proteins Junctophilin 3/4. All members of the Cav1-RyR-IK complex exhibit physical proximities at the nanodomain level to enable a tight functional coupling between membrane calcium influx through voltage-gated Cav1.3 calcium channels followed by calcium-induced calcium release by RyRs. A similar tight association with at least IK channels ensures a strong functional coupling between membrane channel voltage and internal calcium concentrations to activation of IK channels as a factor contributing to the sAHP in CA1 pyramidal cells. Figure is modified from Sahu et al. (2019).

homomeric IK channels in expression systems. The validity of this prediction or of any similar interactions with SK2 channels in CA1 pyramidal cells awaits further analysis.

Cav1 L-type Calcium Channels

Several lines of evidence have built over the years to implicate Cav1 channels, and in particular, the Cav1.3 isoform in generating the sAHP in CA1 pyramidal cells. A key element of calcium channel activity underlying the sAHP is the ability for a short train of high frequency input to invoke a delayed facilitation of Cav1 channel openings. One contributing mechanism to this process is the L-CDF of Cav1.3 (but not Cav1.2) channels that develops through an interaction between the Cav1.3L C-terminus and densin that acts as a bridge to CaMKII. An L-CDF of calcium current was detected in CA1 pyramidal cells, as was the influence of CaMKII on the sAHP and CA1 cell excitability (Sametsky et al., 2009; Sahu et al., 2017).

Despite a direct role for Cav1.3 in mediating L-CDF, there are other ways that Cav1.2 channels could contribute to this process. For instance, while the specific isoform that could account for Lp channels that exhibit late reopening in pyramidal cells was not identified, the Cav1 isoform that exhibits late reopening in dentate gyrus granule cells was traced to Cav1.2 (Koschak et al., 2007). Given that CaMKII has been shown to phosphorylate Cav1.2 channels to increase channel open probability (Yuan and Bers, 1994; Hudmon et al., 2005; Lee et al., 2006; Blaich et al., 2010) it is possible that the Cav1.3L-densin-CaMKII interplay could recruit Cav1.2 channel activity through phosphorylation. We note that PKA phosphorylation can also increase Cav1.2 channel activation (Kavalali et al., 1997; Hall et al., 2007), suggesting the potential for PKA to recruit Cav1.2 channel openings. This seems unlikely, however, given that delayed facilitation of Cav1 calcium channels was blocked by isoproterenol expected to increase PKA (Cloues et al., 1997). Finally, the possibility that an activity-dependent aggregation of Cav1.2 or Cav1.3S channels could contribute to delayed facilitation remains to be tested (Dixon et al., 2012, 2015; Moreno et al., 2016; Ito et al., 2019b). This action could be envisaged if JPH3/4 or other factors provide an activity-dependent change in ER-PM junctions or recruitment of subunits to the complex (de Souza et al., 2015; Johnson B.T. et al., 2018; Kirmiz et al., 2018; Tao-Cheng, 2018; Vierra et al., 2019). In this regard, Kv2 potassium channels have been found to be key factors in controlling Cav1 channel participation in ER-PM junctions that has not been tested yet in the context of the sAHP (Mandikian et al., 2014; Fox et al., 2015; Kirmiz et al., 2018; Johnson et al., 2019; Vierra et al., 2019).

It is also important to note that an equivalent role for Cav1 channels driving IK channels and the sAHP can not be assumed in other cells. For instance, neocortical pyramidal cells in Layers II/III that exhibit distinct firing patterns activate an sAHP with other calcium channel isoforms, and ryanodine blockers have differential effects on these cell types (Pineda et al., 1998, 1999). In cerebellar Purkinje cells IK channels colocalize with Cav3.2 T-type calcium channels to generate an sAHP ∼400 ms duration activated even by low amplitude subthreshold parallel fiber EPSPs (Engbers et al., 2012). The function of the IK channel-mediated sAHP is also different in Purkinje cells in establishing a frequency-dependent control of synaptic input (Engbers et al., 2012) and maintaining spike output at nodes of Ranvier (Gründemann and Clark, 2015). The roles for IK channels in controlling cell excitability in central neurons are thus only beginning to be identified.

RYANODINE RECEPTOR CONTRIBUTIONS TO A Cav1-RyR-IK COMPLEX

The extent of linkage between RyRs and Cav1 channels as part of a complex has steadily emerged, with an association detectable using yeast two hybrid analysis, coimmunoprecipitation, immunolabel localization, morphological cluster analysis, and FRET (Kim et al., 2007; Sahu et al., 2019; Vierra et al., 2019). The tight association of RyR2 with IK channels as part of a Cav1-RyR-IK complex that depends on JPH3/4 linking proteins further identifies their location at ER-PM junctions. The recent report of the RyR3 isoform contributing to sAHP potentiation reveals the functional specificity of different RyR isoforms (Tedoldi et al., 2020).

It is interesting to note that the Cav1-RyR association within a complex here has many similarities to the complex formed in cardiac and skeletal muscle for excitation-contraction (E-C) coupling. In skeletal muscle this reflects an association between Cav1.1 and RyR1, while in cardiac tissue it is Cav1.2-RyR2 retained at the interface of membrane and sarcoplasmic reticulum by JPH1/2 (Dirksen, 2002; Piggott and Jin, 2021). This is important, as years of work on E-C coupling have identified multiple levels of interaction between these subunits. In particular, it emerged that Cav1.x and RyRs exhibit a bidirectional interplay, such that calcium- or voltage-dependent Cav1 activation can modify RyR-mediated calcium release in a tissue-specific manner (Avila and Dirksen, 2000; Avila et al., 2001, 2019; Dirksen, 2002; Heck et al., 2021). Indeed, calcium released by RyRs can augment Cav1 channel activity (Mitterdorfer et al., 1996; Nakai et al., 1996; Grabner et al., 1999; Bers and Morotti, 2014), increase Cav1 channel expression levels (Avila et al., 2001), or reduce Cav1 channel conductance by promoting CDI (Bers and Morotti, 2014). Some of these effects in skeletal muscle have been tracked to specific residues on RyR1 that invoke voltage-dependent interactions and enhance Cav1.1 function (Nakai et al., 1998).

In the hippocampus this complex is replaced by at least a Cav1.3-RyR2-JPH3/4 combination (**Figure 11**). The full extent of interactions between these protein partners or their modulation to regulate the sAHP remains to be determined. Relevant to this may be reports of an interplay between mGluR activation and RyR-mediated augmentation of Cav1 current in cerebellar granule cells (Chavis et al., 1996). Interestingly, these effects appear to be distinct from those which might arise from calcium release by IP3Rs (McPherson et al., 1991; Johenning et al., 2015; Chen-Engerer et al., 2019), but the interactions between synaptic inputs, RyRs, and IP3Rs is complex and cell specific (Fagni et al., 2000; Miyazaki and Ross, 2013). Finally, the targets for phosphorylation by CaMKII associated with Cav1.3-densin have not been determined. This should be central to the entire process of delayed facilitation and L-CDF since CaMKII has been shown capable of phosphorylating RyR2 to increase open probability and calcium release (Meissner, 2004; Ai et al., 2005; Currie, 2009). The potential to directly phosphorylate Cav1.x channels, IK channels, or JPH3/4 in this process are all possible outcomes of CaMKII actions that could identify final effectors of delayed facilitation important to generating the sAHP.

FACTORS CONTRIBUTING TO SLOW AFTERHYPERPOLARIZATION PROPERTIES

Studies on the sAHP were met with continual frustration given a lack of pharmacological tools that investigators rely on to define the underlying basis of a response. As laid out above, identifying an expression of the IK channel isoform and potential heteromerization with other members of the KCNN family will help resolve this (Turner et al., 2015; Higham et al., 2019). Another significant development was identifying the role of the Na-K pump that overlaps and greatly extends the earlier calcium-dependent component (Gulledge et al., 2013; Tiwari et al., 2018). The magnitude of the Na-K pump contribution is also highly dependent on the history of spike firing, complicating efforts to define sAHP duration using only potassium channel blockers (Tiwari et al., 2018). Apart from an unrecognized contribution by the Na-K pump, a review of the conditions and techniques used to study the sAHP reveal several practices that have differed between labs over time. While many of these have been mentioned within this review, a brief summary for future investigators to consider is provided here.

An obvious difference that will have effects on the sAHP is the recording temperature that is typically set at either room temperature (22°C) or ∼32–34°C. It is known that the kinetics of ion channels can be markedly affected by temperature, as can the activity of kinases that can be required for their function. Temperature is also particularly relevant to the Na-K pump in which an increase of only 2°C from 34 to 36°C was reported to substantially increase the contribution of this component compared to the earlier calcium-dependent component (Gulledge et al., 2013). On the one hand this effect could be used to advantage if restricting recordings to ∼32°C help isolate the calcium-dependent component for study. But any studies conducted at temperatures approaching 36°C can be expected to incorporate both processes involved in generating the sAHP and need to consider how this might affect the apparent kinetics of the sAHP.

Early studies that first defined the sAHP used microelectrodes, followed later by almost exclusive use of patch clamp recordings. For most of a decade little attention was paid to the ionic constituents of the internal medium for whole-cell patch recordings before adjusting to ensure a reasonable equilibrium potential for key ions. Whole-cell recordings are also now recognized to wash out some component(s) required for the Na-K pump, leading to a return to the use of microelectrodes to see the full impact of this factor on the sAHP and the effects of

temperature and preceding spike patterns (Gulledge et al., 2013; Tiwari et al., 2018).

Another factor that came to be recognized is that of age, where several contributing factors such as Cav1 channel and RyR expression change rapidly during development or are elevated in aged animals (Campbell et al., 1996; Thibault and Landfield, 1996; Gant et al., 2006; Kim et al., 2007; Raza et al., 2007). Reports on the properties of the IsAHP conducted during the early years of whole-cell patch recordings must then consider the accepted practice at the time of preparing slices from animals ∼P14. A similar consideration clearly applies to recordings conducted in dissociated hippocampal cultures from cells at undetermined levels of very early development. Cell-to-cell variation in spike output properties has been reported depending on their position over the dorso-ventral axis of the hippocampus and thus different projection targets (Dougherty et al., 2012; Hönigsperger et al., 2015). Some of these differences could reflect the properties (amplitude, duration) of the sAHP found within these subpopulations. The pattern of spike output can in turn regulate the sAHP over a longer time frame, such as through potentiation of the sAHP by differential recruitment of RyRs (Borde et al., 1999; Tedoldi et al., 2020). It is worth noting that the rate of repeated current pulse injections capable of inducing LTP of the sAHP is surprisingly low (1/min) (Borde et al., 1995), which is well within the standard background repetition rate for many labs.

From the time of even some of the earliest studies, the sAHP was found to be remarkably sensitive to block by neurotransmitters that activate second messenger pathways, such that noradrenergic and muscarinic cholinergic agonists became standard pharmacological tools to study the sAHP (Madison and Nicoll, 1982, 1984; Lancaster and Nicoll, 1987). These studies were not summarized in the current review given the complexities of second messenger regulation, but an effort to summarize these findings is needed in the field. This is emphasized even more given that recent studies reveal that the Na-K pump component of the sAHP is also highly sensitive to several kinases and phosphatases (Mohan et al., 2019, 2021; Tiwari et al., 2019). This includes noradrenergic and cholinergic agonists, indicating the need to reevaluate the early results obtained using some of the only tools that were available to study the AHP and always interpreted in the context of calcium-dependent sAHP channels.

The hyperpolarizing influence of the Na-K pump at least partially overlaps the potassium-mediated component, so it is difficult to comment on factors that define the kinetics of the sAHP. However, the ability for both a Cav1-RyR-IK complex and a Na-K pump to generate an sAHP in the same cell provides an excellent example of the concept of degeneracy, defined as the ability for structurally different elements to provide an analogous function (Rathour and Narayanan, 2019; Goaillard and Marder, 2021). This process has become increasingly recognized as a means for a system to have alternate routes by which one can generate a functional result that could be subject to different modulatory influence (Tiwari et al., 2019). The debate that has existed on the role for SK or Kv7 channels in generating the mAHP in CA1 pyramidal cells likely reflects another example of degeneracy that emerges under different conditions to generate this response (Gu et al., 2005; Chen et al., 2014; Mateos-Aparicio et al., 2014). Finally, almost no consideration has been made of the potential for alternative calcium sources like IP3 or ligand-gated receptors, or STIM-ORAI interactions to potentially contribute to generating the sAHP that needs further consideration.

CONCLUSION

Defining the molecular basis for the sAHP in CA1 pyramidal cells has been a gradual process that unfolded over the course of 30 years since its initial description, with thousands of studies defining its properties and functions at the cellular, circuit, and behavioral levels. Recent work on both calcium- and calcium-independent components that drive the sAHP have resolved numerous complexities in its pharmacology and raised ever more questions on the number of factors that could modulate its activation. Yet differences in reported results remain, indicating that the final answer to the molecular identity of sAHP channels is still an active topic for research. Given these advances it is safe to assume that many more studies will be enabled to fully understand how a response as important as the sAHP contributes to regulating cell excitability.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Structural and Functional Coupling of Calcium-Activated BK Channels and Calcium-Permeable Channels Within Nanodomain Signaling Complexes

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Biochemical and functional studies of ion channels have shown that many of these integral membrane proteins form macromolecular signaling complexes by physically associating with many other proteins. These macromolecular signaling complexes ensure specificity and proper rates of signal transduction. The large-conductance, Ca^{2+}-activated K^+ (BK) channel is dually activated by membrane depolarization and increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$). The activation of BK channels results in a large K^+ efflux and, consequently, rapid membrane repolarization and closing of the voltage-dependent Ca^{2+}-permeable channels to limit further increases in $[Ca^{2+}]_i$. Therefore, BK channel-mediated K^+ signaling is a negative feedback regulator of both membrane potential and $[Ca^{2+}]_i$ and plays important roles in many physiological processes and diseases. However, the BK channel formed by the pore-forming and voltage- and Ca^{2+}-sensing α subunit alone requires high $[Ca^{2+}]_i$ levels for channel activation under physiological voltage conditions. Thus, most native BK channels are believed to co-localize with Ca^{2+}-permeable channels within nanodomains (a few tens of nanometers in distance) to detect high levels of $[Ca^{2+}]_i$ around the open pores of Ca^{2+}-permeable channels. Over the last two decades, advancement in research on the BK channel's coupling with Ca^{2+}-permeable channels including recent reports involving NMDA receptors demonstrate exemplary models of nanodomain structural and functional coupling among ion channels for efficient signal transduction and negative feedback regulation. We hereby review our current understanding regarding the structural and functional coupling of BK channels with different Ca^{2+}-permeable channels.

Keywords: BK channels, calcium-activated channels, calcium channels, NMDA receptors, nanodomain, coupling, calcium signaling

INTRODUCTION

Ca^{2+} and K^+ Signaling *via* Ion Channels

Cells need to sense and respond to changes in the extracellular environment and communicate with adjoining and distant cells. Cells use different signaling molecules to carry out these tasks. Ca^{2+} and K^+ cations are two signaling molecules that are abundant and used for cell signaling in all living systems. Ca^{2+} is a prominent second messenger molecule that is involved in nearly all biochemical, cellular, and physiological processes. It is essential for proper cardiac function, the structural integrity of bone, and muscular contraction, and it acts as a substrate for enzymatic signal in biochemical pathways. Cells invest vast amounts of energy into the regulation of intracellular concentrations of free Ca^{2+}. The speed and effectiveness of Ca^{2+} signaling builds upon the greater than 10,000-fold gradient between the Ca^{2+} concentrations inside (∼100 nM) and outside (∼2 mM) the cells. Rapid global (10-fold) and local (100-fold) increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentrations can be achieved by Ca^{2+} influx from extracellular mediums or Ca^{2+} release from intracellular Ca^{2+} stores *via* the activation of Ca^{2+}-permeable channels on the plasma membrane and intracellular organelle membranes, respectively.

Ca^{2+}-permeable channels passively diffuse Ca^{2+} ions down their electrochemical gradient. There is a wide variety of Ca^{2+}-permeable channels that differ in Ca^{2+} selectivity and activation mechanisms. Ca^{2+}-selective channels include voltage-gated Ca^{2+} (Ca_V) channels, including $Ca_V1.1$-4, $Ca_V2.1$-3, and $Ca_V3.1$-3; ligand-gated Ca^{2+} channels, including the store-operated Ca^{2+} channels (SOCCs or ORAI1-3) on the plasma membrane; and inositol 1,4,5-trisphosphate (IP3) and ryanodine receptors (IP3Rs and RyRs) on the endoplasmic reticulum (ER) membrane. Non-selective cation channels include mechanosensitive piezo channels, transient receptor potential (TRP) channels, cyclic nucleotide–gated ion channels (CNGs), acid-sensing ion channels (ASICs), and ionotropic receptors, including *N*-methyl-D-aspartate (NMDA) receptors, serotonin 5-hydroxytryptamine (HT3) receptors, and adenosine triphosphate (ATP)–activated P2X receptors.

K^+ is the most abundant cation in the intracellular fluid. The concentration of K^+ ions is usually about 25-fold higher on the cytoplasmic than on the extracellular side of the plasma membrane. K^+ channels selectively pass K^+ ions across membrane according to electrochemical driving forces. Channel-mediating K^+ signaling dynamically controls K^+ distribution across the cell membrane and is critical for normal cell function. The K^+ channel current dominates the ionic flow in a cell's resting state and thus is critical for setting a cell's resting membrane potential. K^+ channels are also involved in fluid secretion and cell volume regulation. In excitable cells, the K^+ channel's activities affect the action potential firing threshold, which is determined by the balance between inward Na^+ and outward K^+ currents and also underlies the repolarization, hyperpolarization, and after-hyperpolarization phases of the action potential. K^+ channels play a crucial role in all aspects of life by regulating the excitability of neurons and the heart, contracting muscles, secreting hormones, water homeostasis, and activating immune cells.

K^+ channels are the most diverse and abundantly expressed ion channels in living organisms. These channels are expressed in most excitable and non-excitable cells and perform numerous important functions, as is evident from the large number of genes (∼80 in mammals) encoding for the K^+ channels' pore-forming subunits. K^+ channels can be classified into four main groups: $Ca^{2+}-$activated K^+ (K_{Ca}) channels, tandem pore domain K^+ (K_2P) channels, voltage-gated K^+ (Kv) channels, and inwardly rectifying K^+ (Kir) channels. Each type of K^+ channel possesses unique electrophysiological and pharmacological properties. K_{Ca} channels activate in response to increases in $[Ca^{2+}]_i$ and thus cause changes in cell membrane potential toward the negative voltage direction *via* K^+ efflux. This common functionality enables K_{Ca} channels to play an important role in bridging cell excitability and the intracellular calcium concentration. K_{Ca} channels are a diversified group of channels with various biophysical and pharmacological properties. K_{Ca} channels are divided into 3 classes based on their single channel conductance: big conductance (BK, 200–300 pS), intermediate conductance (IK, 32–39 pS), and small conductance (SK, 4–14 pS).

BK Channels: General Properties and Function

The BK channel (also known as $K_{Ca}1.1$, MaxiK, and slo1) is a homotetrameric channel consisting of four identical subunits of the pore-forming, Ca^{2+}- and voltage-sensing α-subunit (BKα, encoded by a single gene *KCNMA1*) either alone or in association with regulatory β or γ subunits. The BKα channel (∼130 kDa) contains 7 transmembrane (TM) segments (S0–S6), a short extracellular N-terminus, and a large cytosolic C-terminus composed of two regulating conductance of K^+ (RCK) domains for Ca^{2+} sensing (Tao and MacKinnon, 2019) (**Figure 1**). The four auxiliary β subunits (β1–β4) and the four γ subunits (γ1–γ4) are double- and single-spanning membrane proteins, respectively, with tissue-specific expression patterns. For example, β1 is mainly in smooth muscles, γ1 is in secretory, non-excitable cells, and γ3 and β4 are in the brain (Solaro and Lingle, 1992; Behrens et al., 2000; Brenner et al., 2000; Cox and Aldrich, 2000; Yan and Aldrich, 2010, 2012; Zhang and Yan, 2014).

BK channels have an exceptionally large single-channel conductance, which is 10–20 times larger than that of most other K^+ channels. BK channel activation is regulated by membrane voltage and a wide range of $[Ca^{2+}]_i$ (from sub-micromole to hundreds of micromoles) *via* Ca^{2+} bindings at the RCK domains. When activated by Ca^{2+} channel–mediated $[Ca^{2+}]_i$ elevation, BK channels generate large K^+ efflux and, consequently, rapid membrane repolarization that can limit further Ca^{2+} flux through membrane repolarization–induced deactivation of Ca^{2+} channels. *Via* this negative feedback mechanism, BK channel–mediated K^+ signaling plays a powerful, integrative role in regulating cellular excitability and calcium signaling in electrically excitable cells (Ghatta et al., 2006; Salkoff et al., 2006).

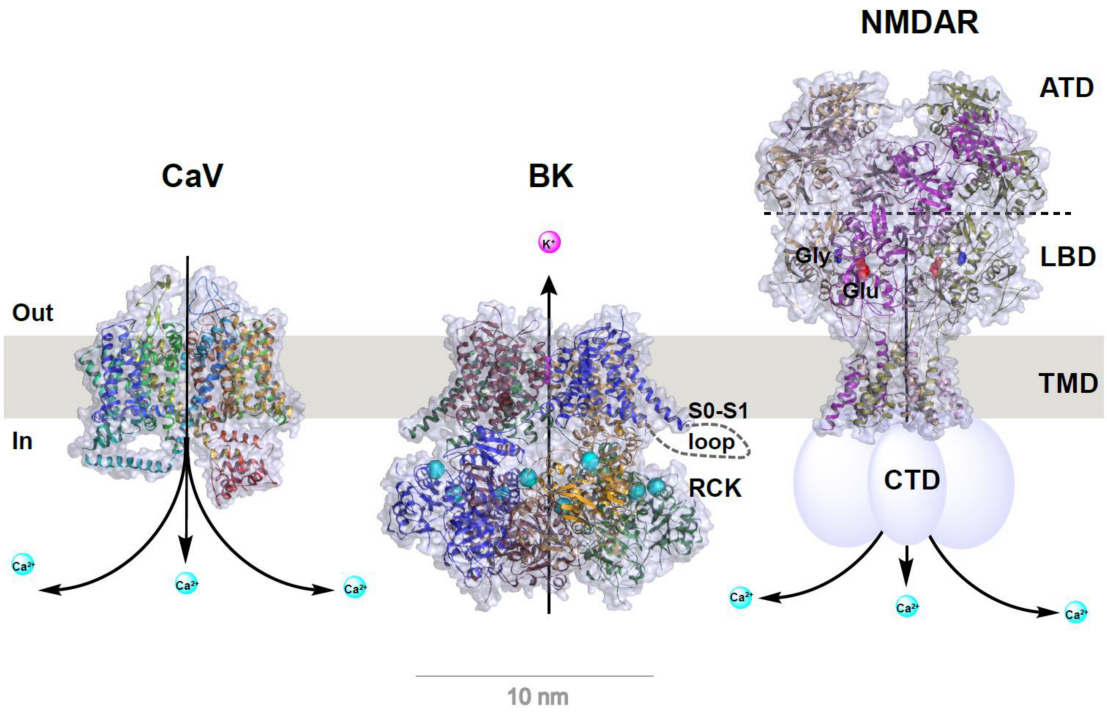

FIGURE 1 | Structural models of Ca_V and BK channels and NMDA receptor. Structures were redrawn from published structures of rabbit $Ca_V1.1$ (PDB ID: 5GJV) (Wu et al., 2016), human BK (PDB ID: 6V22) (Tao and MacKinnon, 2019), and rat GluN1a/GluN2B NMDA receptor (PDB ID: 4PE5) (Karakas and Furukawa, 2014). For clarity, auxiliary subunits of Ca_V and BK channels are omitted and the bound Ca^{2+} ions in BK channel's RCK domains are shown as enlarged cyan balls. Depiction in cartoon was added manually for the S0–S1 loop (only on one subunit) of BK channel and C-terminal domain (CTD) of NMDA receptor whose structures were unresolved. For NMDA receptor, the amino terminal domain (ATD), ligand binding domain (LBD), transmembrane domain (TMD), the bound glutamate (Glu) and glycine (Gly) are indicated on or near the corresponding structural parts.

The BK channels are critically involved in various cellular and physiological processes. In central neurons, BK channels mediate the repolarization and fast after hyperpolarization (fAHP) of action potentials (Shao et al., 1999; Womack and Khodakhah, 2002), shape dendritic Ca^{2+} spikes (Golding et al., 1999), and regulate neurotransmitter release at presynaptic terminals (Hu et al., 2001; Raffaelli et al., 2004; Xu and Slaughter, 2005; Samengo et al., 2014). In addition, they are involved in motor coordination (Sausbier et al., 2004), learning and memory (Matthews and Disterhoft, 2009; Ye et al., 2010; Typlt et al., 2013; Springer et al., 2014), the brain's intrinsic rhythmicity of the circadian clock (Meredith et al., 2006; Pitts et al., 2006; Montgomery et al., 2013; Farajnia et al., 2015) and respiration (Onimaru et al., 2003; Zhao et al., 2006; Zavala-Tecuapetla et al., 2008), frequency tuning of cochlear hair cells (Fettiplace and Fuchs, 1999), pain modulation (Chen et al., 2009; Cao et al., 2012; Zhang et al., 2012; Waxman and Zamponi, 2014), and neuroprotection under pathological conditions (Runden-Pran et al., 2002; Shen et al., 2007; Zhang et al., 2009; Mancini et al., 2014). Defects in or dysregulation of human neuronal BK channels can cause epilepsy and paroxysmal dyskinesia (Brenner et al., 2005; Du et al., 2005) and are implicated in intellectual disability (Higgins et al., 2008; Deng et al., 2013), autism (Laumonnier et al., 2006), and schizophrenia (Zhang et al., 2006).

The Necessity and Properties of Nanodomain Coupling of BK Channels With Ca^{2+}-Permeable Channels

In spite of multimode activation by cell membrane depolarization, a rise in $[Ca^{2+}]_i$, or both synergistically, BK channels are generally considered high-threshold channels. For the BK channels formed by the α subunit alone, a very high voltage of more than 100 mV, which is out of the physiological range, is needed for measurable BK channel activation under the cell's resting Ca^{2+} conditions ($\leq\sim$0.1 μM) (**Figure 2**). Under resting membrane voltage conditions (e.g., −60 to −80 mV), an extremely high ($\geq$100 μM) concentration of $[Ca^{2+}]_i$ is needed to produce channel activation (**Figure 2**). Under membrane depolarization conditions in excitable neuronal (Vm $\leq$ +40 mV) or smooth muscle cells (Vm $\leq$ +20 mV) during action potential, the required $[Ca^{2+}]_i$ concentration for significant BK channel activation is also approximately a few μM, which is generally higher than the cellular global $[Ca^{2+}]_i$ concentration in resting ($\leq\sim$0.1 μM) and excited ($\leq\sim$1 μM) states (**Figure 2**). In contrast, the other two types of K_{Ca} channels, IK and SK, have a much higher affinity for Ca^{2+} ($EC_{50} \approx$ 0.3–0.5 μM) owing to their constitutively bound Ca^{2+}-binding messenger protein, calmodulin (CaM). Therefore, BK channels had been considered to function mainly as a brake operating only under extreme

conditions, e.g., a pathological $[Ca^{2+}]_i$ overload repolarizing the membrane to limit further Ca^{2+} influx. Research progress in the last 2 decades has revealed that cells employ at least two effective biochemical strategies to allow BK channels to be activated under normal cellular or physiological conditions.

The first strategy is to modulate the voltage dependence of the channel activation *via* auxiliary proteins. The γ1 (LRRC26) subunit in particular gives BK channels an unusual capability to be constitutively active at physiological voltages and $[Ca^{2+}]_i$ levels in non-excitable cells by causing a large negative shift (∼ −140 mV) in the voltage dependence of the channel activation (Yan and Aldrich, 2010, 2012). The γ1 subunit is highly expressed in secretory epithelia cells in different organs and plays an important role in the resting K^+ efflux and fluid secretion in these cells (Yang et al., 2017; Gonzalez-Perez et al., 2021). The γ2 subunit also results in a great shift (∼ −100 mV) in BK channel voltage gating (Yan and Aldrich, 2012). The γ2 subunit is highly expressed in the testis and is a potent regulator of BK channel function in the cochlea's inner hair cells (Lingle, 2019). The β subunits overall do not strongly affect the thresholds of BK channel activation, despite their complex effects on BK channel voltage and Ca^{2+} gating and current kinetics. A detailed review of the BK channel β and γ subunits is beyond the scope of this work and can be found in previous articles (Zhang and Yan, 2014; Li and Yan, 2016; Latorre et al., 2017; Gonzalez-Perez and Lingle, 2019). The second strategy that cells use to activate BK channels is to position BK channels in proximity to some Ca^{2+}-permeable channels to gain immediate access to the local high concentrations of $[Ca^{2+}]_i$ during extracellular Ca^{2+} influx or Ca^{2+} release from intracellular Ca^{2+} store organelles.

Given the presence of endogenous Ca^{2+} buffers and the quick diffusion of Ca^{2+} ions, the distance to the Ca^{2+}-originating site determines the local $[Ca^{2+}]_i$ concentration. Thus, rapid and precise spatiotemporal control of the $[Ca^{2+}]_i$ concentration is found in local Ca^{2+}-signaling domains, in which Ca^{2+} channels and Ca^{2+}-effector sensors are within proximity, i.e., within the nanodomain or microdomain (Augustine et al., 2003; Eggermann et al., 2011) (**Figure 3**). In Ca^{2+} signaling, a nanodomain is a conceptual region of highly localized Ca^{2+} signals extending over a few tens of nm from the cytoplasmic mouths of the Ca^{2+} channels (Eggermann et al., 2011; Tay et al., 2012). Coupling between Ca^{2+} sensors and Ca^{2+} channels within nanodomains can achieve high efficacy, speed, and energy efficiency for Ca^{2+} signaling (Eggermann et al., 2011). Experimentally, the colocalization of Ca^{2+} channels and Ca^{2+}-effector sensors can be functionally probed with Ca^{2+} chelators, ethylene glycol tetraacetic acid (EGTA), and 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (**Figure 4**). Both EGTA and BAPTA compete with Ca^{2+}-sensing proteins for $[Ca^{2+}]_i$ and have similar steady-state binding affinities for Ca^{2+}. However, they differ greatly in their rates of Ca^{2+} binding (Naraghi and Neher, 1997). BAPTA has a binding rate constant 150 times higher than that of EGTA. At millimolar levels, BAPTA can efficiently prevent the spreading of free Ca^{2+} from the entry site. The slower chelator, EGTA, is relatively ineffective in sequestering Ca^{2+} within a short distance of the Ca^{2+} exit site but can intercept Ca^{2+} during Ca^{2+} diffusion over a longer distance. For convenience, the local Ca^{2+} signaling domain is classified as a Ca^{2+} nanodomain or microdomain depending on the sensitivity of the Ca^{2+}-sensing effector to EGTA and BAPTA. The Ca^{2+} nanodomain signaling process is effectively disrupted by millimolar levels (e.g., 2–10 mM) of BAPTA but not of EGTA, whereas in Ca^{2+} microdomains (e.g., from a few tens to a few hundred nm), EGTA also dissipates the signaling process effectively. Biochemically, the nanodomain or microdomain coupling of Ca^{2+}-sensing proteins to Ca^{2+} channels can be achieved by direct protein-protein physical interactions for nanodomain colocalization; indirect interactions mediated by other proteins, e.g., scaffold proteins (Sclip et al., 2018), for nano- or microdomain colocalization; and special membrane domains, e.g., lipid rafts (Ma et al., 2015), that concentrate and restrict their distribution for nano- or microdomain colocalization.

The distance to the Ca^{2+} source is a key factor determining BK channel activation and function. Given the presence of endogenous cytosolic Ca^{2+} chelating buffer that can be comparable to EGTA in capacity (Fakler and Adelman, 2008; Schwaller, 2010), the microdomain coupling is expected to be largely ineffective in BK channel activation. For example, in the presence of 2 mM EGTA, a BK channel must be within 50 nm of a Ca^{2+} source to sense an effective local $[Ca^{2+}]_i$ concentration of at least 2 μM (**Figure 4**) in order to achieve significant activation, e.g., an open probability (P_o) of 0.1 or greater, even at a maximally depolarized membrane voltage of +40 mV (**Figure 2**). Thus, under physiological conditions of limited membrane depolarization, native BK channels, particularly those in excitable cells that lack γ1 and γ2 expression, must colocalize with Ca^{2+} sources within nanodomains to be physiologically active and functional. Compared to other K_{Ca} channels, the requirement of nanodomain colocalization with Ca^{2+} source is specific to BK channels because the IK and SK channels' exquisitely high affinity for Ca^{2+} theoretically relieves them from the requirement of close nanodomain interaction with Ca^{2+} sources for functional coupling, even in the presence of EGTA (Fakler and Adelman, 2008).

From the above discussion, BK channels coupled with Ca^{2+} channels within the nanodomain are expected to demonstrate: (1) the Ca^{2+} channel–mediated BK channel currents that are largely resistant to Ca^{2+} chelating by millimolar levels of EGTA but could be fully or partially sensitive to millimolar levels of BAPTA; (2) physical formation of protein complex by the 2 channels that can be copurified or coimmunoprecipitated in the forms of channel-channel complexes or supercomplexes *via* direct or indirect physical interactions, or membrane domains with special lipid compositions and cytoskeletal support resistant to detergent disruption; and (3) physical colocalization *in situ* within a very short distance, which can be probed either directly with super resolution (under 100 nm) microscopy or more conveniently and indirectly *via* a nanometer distance-sensitive imaging analysis method such as förster resonance energy transfer (FRET) or proximity ligation assay (PLA) (see section "BK-NMDAR Colocalization and Functional Coupling Within the Nanodomain"). We review the evidence and findings

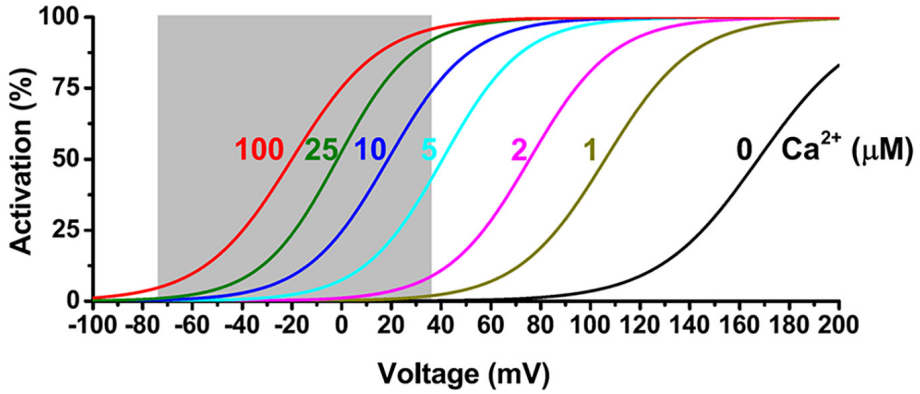

FIGURE 2 | Voltage and Ca^{2+} dependence of BKα channel activation. The shaded area indicates a physiological range of membrane voltages.

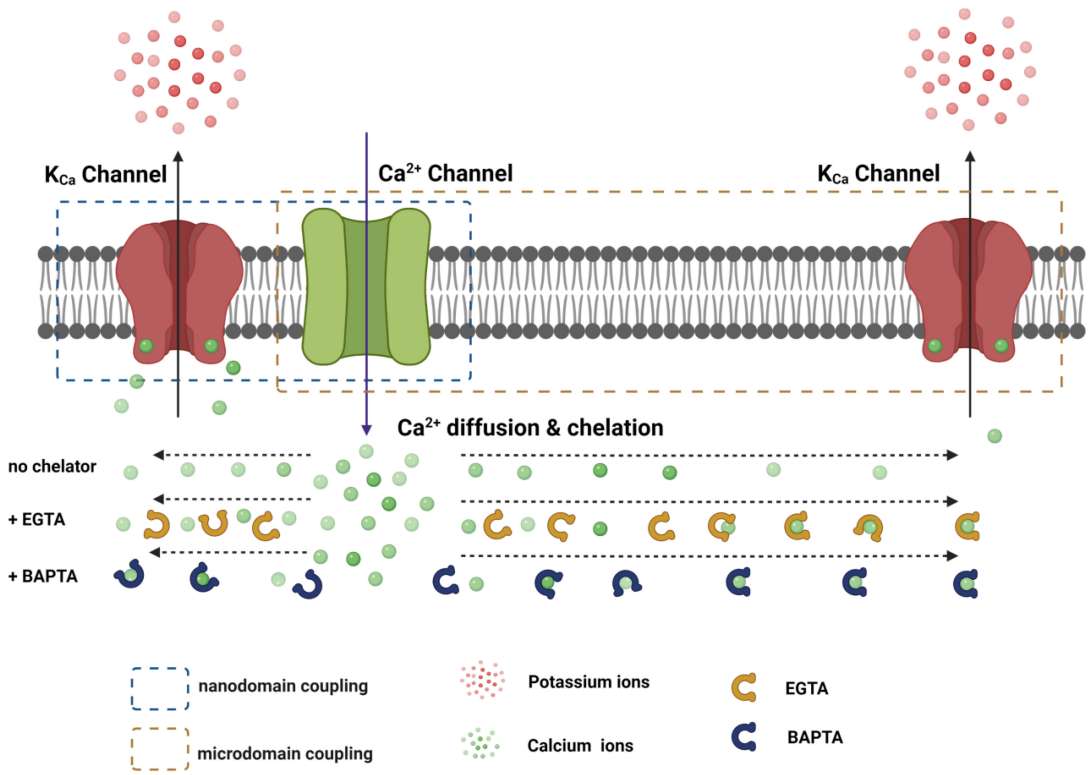

FIGURE 3 | Functional coupling of the K_{Ca} channel with the Ca^{2+} channel within nanodomain and microdomain. The distance-dependent effects of EGTA and BATPA on the availability of free Ca^{2+} ions during their diffusion from the Ca^{2+} channel pore are roughly depicted.

from these 3 directions in support of nanodomain coupling of BK channels with a specific Ca^{2+} channel.

BK-Ca_V COUPLING

Ca_V Channels

Of the different types of Ca^{2+} channels, voltage-gated calcium (Ca_V) channels play a vital role in providing intracellular Ca^{2+} ions to K_{Ca} channels. Ca_V channels are formed by central pore-forming α_1 subunits and regulatory auxiliary subunits and are responsible for membrane depolarization–induced Ca^{2+} entry into excitable cells (Zamponi et al., 2015; Nanou and Catterall, 2018). The auxiliary subunits, which contribute to calcium channel diversity, are encoded by 4 α2δ genes, 4 β-subunit genes, and 9 γ-subunit genes. The α_1 subunit forms the Ca^{2+} selective pore and consists of 24 TM α helices divided into 4 homologous domains, each containing 6 TM α helices (Wu et al., 2016) (**Figure 1**). Based on the electrophysiological and pharmacological properties of their ionic currents, Ca_V

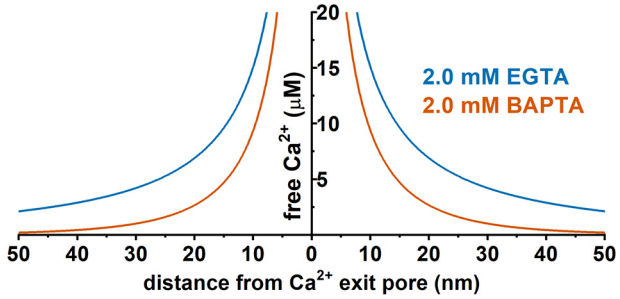

FIGURE 4 | Predicted distance dependence of the local free Ca^{2+} concentration originating from a hypothetic Ca^{2+} channel pore in the presence of 2 mM EGTA or 2 mM BAPTA. Prediction was performed using the CalC software (version 6.8.0) (Matveev et al., 2004) at 0.1 pA for the single-channel Ca^{2+} current. Chelator and Ca^{2+} parameters were taken from previous reports (Naraghi and Neher, 1997; Fakler and Adelman, 2008; Eggermann et al., 2011).

channels are classified into L-, N-, P/ Q-, R-, and T-types. Based on the amino acid sequence similarities of the pore-forming α_1 subunits, they are grouped into Ca_V1, Ca_V2, and Ca_V3 types. L-type ($Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$) channels are activated by high voltages and distinguished by long-lasting (L) activation, i.e., slow voltage-dependent inactivation and blocked by calcium antagonist drugs such as dihydropyridines and phenylalkylamines. L-type channels have diverse functions, including the initiation of contraction in muscle cells, hormone secretion in endocrine cells, and local calcium signaling for gene transcription. P/Q- ($Ca_V2.1$), N- ($Ca_V2.2$), and R- ($Ca_V2.3$) type channels are also activated by high voltages but have faster voltage-dependent inactivation. They are found primarily in neurons and are blocked by peptide toxins from spiders and snails. T-type ($Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$) channels are activated by low voltages (negative membrane potentials) and have fast deactivation upon repolarization and fast voltage-dependent inactivation. They are predominantly found in cardiac myocytes, sinoatrial nodes, and thalamic neurons.

BK-Ca_V Functional Coupling

Researchers studying BK channels from different types of neurons have found that channel activity depends on Ca^{2+}-influx through Ca_V channels and that blocking of the L-type (Storm, 1987; Prakriya and Lingle, 1999; Vivas et al., 2017), P/Q type (Edgerton and Reinhart, 2003; Womack et al., 2004), and N-type Ca_V channels (Marrion and Tavalin, 1998; Loane et al., 2007) with subunit-specific toxins suppresses neuronal BK channel currents. In addition, they discovered that BK-Ca_V coupling must occur within a nanodomain because the Ca_V-dependent BK channel activation was rapid (e.g., ∼1 ms) and not disrupted by the Ca^{2+} chelator EGTA when patch-clamp recordings were performed on channels heterologously expressed in *Xenopus* oocytes, Chinese hamster ovaries (CHO), and tsA-201 cells (Berkefeld et al., 2006; Berkefeld and Fakler, 2008; Vivas et al., 2017), native channels in chromaffin cells (Prakriya and Lingle, 2000; Berkefeld et al., 2006), or neurons (Gola and Crest, 1993; Robitaille et al., 1993; Edgerton and Reinhart, 2003; Sun et al., 2003; Muller et al., 2007; Vivas et al., 2017). During functional coupling, the apparent voltage dependence, current kinetics, and amplitude of BK channel activation are affected by the voltage dependence, conductance, and coupling strength of the Ca_V channels. Thus, different Ca_V channels confer different apparent gating properties for BK channel activation (Berkefeld et al., 2006; Vivas et al., 2017). The lower-voltage–activated $Ca_V1.3$ caused a much greater shift toward negative voltage in the activation of the BK channel than in that of the $Ca_V2.2$ channel (Vivas et al., 2017). Based on EGTA's and BAPTA's effects on the $Ca_V2.1$-induced BK channel's current amplitude and time course recorded in the heterologous expression system, the distance between these 2 channels was estimated to be approximately 10–15 nm (Berkefeld et al., 2006). Similarly, the diffusional distance for Ca^{2+} ions from the Ca_V channels to the BK channels in hippocampal granule cells was estimated to be 13 nm per linear approximation of buffer Ca^{2+} diffusion (Muller et al., 2007). The T-type $Ca_V3.2$ channel also provided Ca^{2+} for BK channel activation in a heterologous expression system of tsA-201 cells and in rat medial vestibular neurons (Rehak et al., 2013). However, the Ca_V3-evoked BK channel currents were sensitive to EGTA, suggesting weaker microdomain coupling than that observed for nanodomain coupling with $Ca_V1.2$, 1.3, 2.1, and 2.2 channels.

Molecular Organization of BK-Ca_V Nanodomain Coupling

Affinity purification of BK channels in rat brains with anti-BKα antibodies and subsequent mass spectrometry analyses identified the formation of macromolecular protein complexes between BK channels and $Ca_V1.2$ (L-type), $Ca_V2.1$ (P/Q-type), and $Ca_V2.2$ (N-type) channels (Berkefeld et al., 2006). Of the Ca_V channel proteins, $Ca_V2.1$ was the most abundantly copurified with BKα. The auxiliary subunits of BKβ2, BKβ4, Ca_Vβ1b, Ca_Vβ2, Ca_Vβ3, and Ca_Vβ4 were also identified in the complexes. By coimmunoprecipitation, BKα was found to form complexes with $Ca_V1.2$ and $Ca_V2.1$ as well in the heterologous expression system of *Xenopus* oocytes (Berkefeld et al., 2006). The coimmunoprecipitation of BK channels and $Ca_V1.3$ was also reported for native channels from rat brains (Grunnet and Kaufmann, 2004) and for heterologously expressed channels in tsA-201 cells (Vivas et al., 2017). These studies showed that BK and Ca_V channels formed complexes in both brain and heterologous expression systems, which rules out the requirement for neuron-specific protein to be present in order for the physical association to occur. Interestingly, the S0 TM segment of the BK channels was necessary for coimmunoprecipitation with $Ca_V3.2$ in spite of the likely microdomain coupling of these two channels (Rehak et al., 2013).

Given the predicted close distance (10–15 nm) in functional coupling (Berkefeld et al., 2006; Muller et al., 2007) and the large sizes of BK and Ca_V channels, which are ∼13 and ∼10 nm wide (parallel to the membrane), respectively (Wu et al., 2015; Tao and MacKinnon, 2019) (**Figure 1**), they could be positioned in close contact *via* direct physical interactions. However, no protein domains, regions, or residues are known to be involved

in BK-Ca_V interactions. Some reports support the possibility of indirect interactions between the BK and Ca_V channels. The colocalization of Ca_V and BK channels at the active zone of presynaptic nerve terminals was proposed to be mediated by the scaffold proteins, RIMs and RIMs binding proteins, which interact with Ca_V and BK channels, respectively (Sclip et al., 2018). Co-expression of the channels with a G protein-coupled receptor, β protein-co receptor, was reported to be needed for coimmunoprecipitation of BK $Ca_V1.2$ channels in HEK293 cells (Liu et al., 2004), which is in contrast with the reported BK-$Ca_V1.2$ complex formation without expression of any other exogenous protein in another heterologous system of *Xenopus* oocytes (Berkefeld et al., 2006). It is also unclear whether BK and Ca_V channels can directly interact with each other and form complexes in 1:1 stoichiometric relationships, as has been previously speculated (Berkefeld et al., 2006; Fakler and Adelman, 2008). Computational modeling of BK-Ca_V coupling at 1:1 (Cox, 2014) and other stoichiometric relationships (Montefusco et al., 2017) could reproduce the coupled electric activity observed in cells. A super-resolution microscopic study of BK and $Ca_V1.3$ in a heterologous system and in rat hippocampal and sympathetic neurons revealed the formation of homotypic multichannel clusters of both $Ca_V1.3$ and BK channels and a skewed, concentrated distribution of $Ca_V1.3$ clusters occupying areas adjacent to BK clusters (Vivas et al., 2017). The BK channel clusters had a median area of 1,600 nm^2 in tsA-201 cells, 2,000 nm^2 in hippocampal neurons, and 2,800 nm^2 in superior cervical ganglion neurons, containing roughly an average of 10–15 BK channels in a cluster. Most of the BK channel clusters were surrounded (within a radius of 200 nm) by a variable number (mean, ∼4) of $Ca_V1.3$ clusters (median area, 1,600 nm^2). Thus, surprisingly, the association between BK and $Ca_V1.3$ channels occurred mainly in multichannel clusters and was not fixed in stoichiometric or geometric relationships, which was contradictory to the concept of 1:1 stoichiometry in physical interactions. In hippocampal and superior cervical ganglion neurons, small portions (3 and 10%, respectively) of the BK channel clusters contained $Ca_V1.3$ channels, indicating the presence of some intimate BK-Ca_V coupling and interactions within a cluster. For BK-$Ca_V1.3$ coupling *via* the observed heterogeneous, multichannel clustering, the local Ca^{2+} source for BK channel activation varies greatly according to the number and locations of the surrounding activating Ca_V channels. Interestingly, clustering facilitates the cooperative opening of the $Ca_V1.3$ channels within a given cluster (Moreno et al., 2016), which could enhance BK-Ca_V functional coupling.

Localization and Roles of BK-Ca_V Coupling in the Nervous System

BK-Ca_V coupling has been recorded in a variety of different neurons and plays fundamental roles in the regulation of neural firing and transmission. The exceptionally large, single-channel K^+ currents and tight BK-Ca_V coupling allow BK channels to function as potent negative-feedback regulators of both membrane potentials and Ca^{2+}-influx. During the membrane depolarization–induced sequential activation of Ca_V and BK channels, BK channel-mediated large K^+ currents rapidly counterbalance membrane depolarization and Ca^{2+} influx by repolarizing the membrane potential, which also limits further Ca^{2+} influx *via* the repolarization-induced closure of Ca_V channels. For BK-Ca_V nanodomain coupling, the voltage threshold of BK channel activation tends to reflect the voltage threshold of the coupled Ca_V channels (Vivas et al., 2017). If BK channels are activated by Ca_V channels before the membrane potential reaches the action potential firing threshold, BK-Ca_V coupling can prevent neuronal firing, as that implicated for BK-$Ca_V1.3$ coupling in sympathetic neurons (Vivas et al., 2014, 2017). In most studies, the BK-Ca_V coupling was recorded on somatic membranes (**Figure 5**) and regulated neuronal firing by repolarizing action potentials and generating fAHP, such as those observed in Helix neurons (Gola and Crest, 1993), hippocampal neurons (Marrion and Tavalin, 1998), guinea pig sympathetic neurons (Davies et al., 1999), cerebellum Purkinje neurons (Edgerton and Reinhart, 2003), neocortical pyramidal neurons (Sun et al., 2003), hippocampal granule cells (Sun et al., 2003), and striatal cholinergic interneurons (Goldberg and Wilson, 2005). BK and Ca_V channels were also expressed on presynaptic terminals on which neurotransmitter release is tightly controlled by Ca^{2+} entry *via* Ca_V channels. The BK-Ca_V coupling at presynaptic terminals (**Figure 5**) negatively modulated the neurotransmitter release by negative feedback regulation of Ca_V-mediated Ca^{2+} influx. Such a role for BK-Ca_V coupling as a controller of neurotransmitter release at presynaptic terminals has been demonstrated in frog neuromuscular junctions (Robitaille et al., 1993), Xenopus nerve–muscle synapses (Yazejian et al., 1997), and mouse motor nerve terminals (Protti and Uchitel, 1997). For the $Ca_V2.3$ channel, it was previously reported to be absent in the isolated rat brain BK-Ca_V complexes and also failed in activating BK channels in the presence of EGTA in the heterologous expression system (Berkefeld et al., 2006). A later study showed co-immunoprecipitation of BK channels and $Ca_V2.3$ from mouse hippocampus and deletion of $Ca_V2.3$ in CA1 pyramidal cells reduced the BK channel function resulting in altered action potential waveforms which strengthens the synaptic transmission between CA1 and subiculum and increased the short-term plasticity (Gutzmann et al., 2019).

BK-NMDAR COUPLING

NMDARs

NMDARs are ligand- and voltage-gated, Ca^{2+}-permeable cation channels that function as coincidence detectors of elevated extracellular glutamate levels and membrane depolarization. Channel opening in NMDARs requires coincident glutamate binding–evoked channel activation and the membrane depolarization–induced release of pore blockades caused by extracellular Mg^{2+}. NMDAR activation results in prolonged Na^+ and Ca^{2+} influx that is critical to excitatory synaptic transmission and synaptic plasticity. Unlike the two other classes of ionotropic glutamate receptors, AMPA receptors (AMPARs) and kainate receptors (KARs), NMDARs are characterized

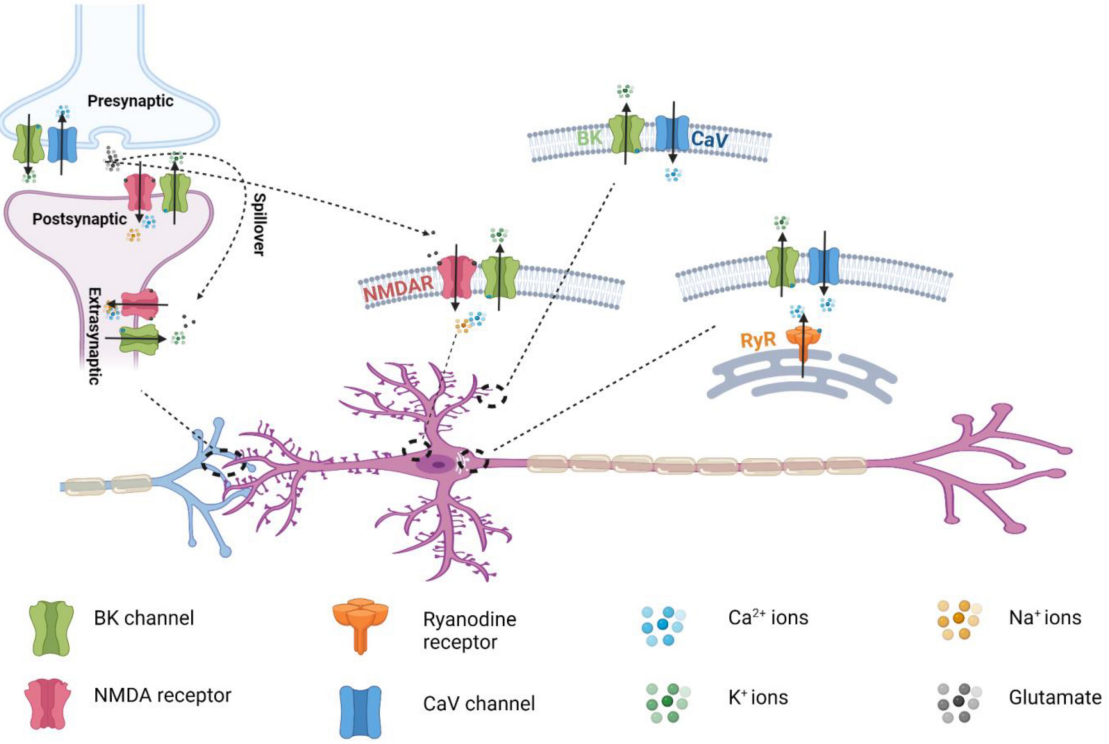

FIGURE 5 | Distribution of the nanodomain coupling of BK channels with different Ca^{2+} permeable channels in neurons.

by high Ca^{2+} permeability, slow deactivation, high affinity for glutamate, high unitary conductance (30–50 pS), and voltage-dependent blockading by extracellular Mg^{2+} (Zito and Scheuss, 2009; Paoletti, 2011; Paoletti et al., 2013). NMDARs are heterotetrameric channels composed of two obligatory GluN1 subunits and 2 modulatory GluN2 (A-D) or GluN3 (A–B) subunits (**Figure 1**). Each NMDAR subunit consists of 4 structural domains: 2 extracellular, tandem, globular, clamshell-like domains [the amino-terminal domain (ATD) for subunit assembly and the ligand-binding domain (LBD)] for the binding of glycine and glutamate; an ion channel pore TM domain made of 3 TM segments; and a highly variable, intracellular carboxyl-terminal domain (CTD) (Karakas and Furukawa, 2014) (**Figure 1**). NMDARs are involved in many aspects of synaptic transmission, dendritic integration, synaptic and neuronal maturation, and synaptic plasticity throughout the brain (Zito and Scheuss, 2009). They are also involved in various neurological and psychiatric diseases, including NMDAR hyperactivity–induced acute excitotoxicity (e.g., epilepsy and ischemic stroke) and chronic neurodegeneration (e.g., Alzheimer, Parkinson, and Huntington diseases and amyotrophic lateral sclerosis), NMDAR hypofunction–related neurodevelopmental disorders (e.g., schizophrenia), and many others (e.g., pain, depression, autism, white matter injury, and anti-NMDAR encephalitis) (Lakhan et al., 2013; Paoletti et al., 2013; Zhou and Sheng, 2013; Bourinet et al., 2014). In spite of their ubiquitous expression in the mammalian central and peripheral nervous systems, BK channels and NMDARs were traditionally considered to be localized in different compartments as presynaptic and postsynaptic channels, respectively. However, in an early 2001 study, BK channels were activated by NMDAR-mediated Ca^{2+} influx at extrasynaptic sites in rat olfactory bulb granule cells; this was a previously unknown neuronal activity-inhibitory role for NMDARs (Isaacson and Murphy, 2001).

Compared to the intensive research on BK-Ca_V coupling, the attention to BK-NMDAR coupling had been lacking. Questions regarding whether BK channels and NMDARs can directly associate to form protein complexes, whether such extrasynaptic BK-NMDAR coupling is also present in other neurons or brain regions, and whether this coupling occurs in other subcellular location were not addressed until more recently.

Biochemical Basis of BK-NMDAR Interactions

Affinity purification and mass spectrometry analyses of immunopurified BK channels and NMDA receptors from rat brains have found that they were mutually identified together (Zhang et al., 2018). Tandem immunopurification with anti-BKα and ant-GluN1 antibodies in the first and second rounds of purification, respectively, was able to purify the protein complexes containing both BKα and GluN1. The copurification of GluN2A and GluN2B with the BKα-GluN1 complex indicates the association of BK channels with functional NMDARs. In the heterologous expression system of HEK293 cells, it was found

that BKα specifically interacts with the NMDARs' obligatory GluN1 subunit, whereas there were no interactions observed between BKα and the NMDARs' GluN2A and GluN2B, the kainate receptors' GluK1 and GluK2, or the AMPA receptors' GluR1 and GluR2. With truncation constructs of BKα and GluN1 and chimeric constructs of GluN1/GluK2, the BKα-GluN1 interactions involved the former's S0–S1 long loop and the latter's TM regions, including the loops and cytosolic C-terminus. An engineered protein containing the GluN1's cytosolic regions (residues 563–587 for the M1–M2 loop region and residues 813–920 for the C-terminus), which was expressed in and purified from *Escherichia coli*, was able to interact directly with a synthesized peptide of the BKα S0–S1 loop region (residues 46–93) *in vitro*. Furthermore, the synthesized peptide of the S0–S1 loop region competitively disrupted the association between BKα and GluN1. These findings indicate that BKα and GluN1 directly interact with each other *via* their cytosolic regions, including the BKα S0–S1 loop region (**Figure 1**), which seems to be flexible in structure as its structure was undefined in the reported cryo-EM structures of the BK channels (Tao and MacKinnon, 2019).

BK-NMDAR Colocalization and Functional Coupling Within the Nanodomain

The colocalization of BK and NMDARs was probed with an *in situ* PLA (Zhang et al., 2018; Gomez et al., 2021). PLA displays point-like staining signals only when the 2 epitopes on the interacting proteins for primary antibodies are in proximity (<40 nm) (Soderberg et al., 2006), making PLA a suitable assay for the detection of protein colocalization within the nanodomain. With *in situ* PLA, BKα and GluN1 were found to be colocalized in both the heterologous expression system and, broadly, in different regions of mouse hippocampus (Zhang et al., 2018). Consistent with the sole requirement of GluN1 for protein complex formation with BK channels (Zhang et al., 2018), PLA with HEK293 cells co-expressing BK channels with GluN1/GluN2A or GluN1/GluN2B showed no preference in BK channels' proximity to these 2 types of NMDARs (Gomez et al., 2021). In the dentate gyrus, PLA signals of the BKα-GluN1 complexes were most abundant in the molecular layer region (Zhang et al., 2018), which is consistent with the predominant dendritic distribution of NMDARs. Glutamate-induced BK channel outward currents were observed when whole-cell voltage clamp recording was performed on mature dentate gyrus granule cells in mouse hippocampal slices upon application of glutamate toward soma (Zhang et al., 2018). The currents were blocked by the NMDAR antagonist (2R)-amino-5-phosphonovaleric acid (AP5) and pore-blocker MK-801, indicating involvement of the receptor's ion-conducting function. The glutamate-induced BK channel currents were insensitive to intracellularly applied EGTA but became significantly smaller in the presence of BAPTA, confirming nanodomain functional coupling of BK channels with NMDARs. Intracellular application of the synthesized S0–S1 loop peptide reduced the glutamate-induced BK channel outward currents, indicating that the physical interactions between these 2 channels play a role in their functional coupling. EGTA-insensitive coupling of BK channels with NMDARs was also recently observed in a subset of barrel cortex layer 5 pyramidal neurons (BC-L5PNs) but when NMDA was applied toward basal dendrites instead of soma (Gomez et al., 2021). In a heterologous expression system of HEK293 cells, similar results of BK-NMDAR (GluN1/GluN2A) functional coupling were revealed by whole cell voltage-clamp recording (Zhang et al., 2018). In excised inside-out membrane patches of HEK293 cells, BK channel openings at the single channel level were observed upon NMDAR activation using flash photolysis of caged NMDA (Zhang et al., 2018). In HEK293 cells, activation of NMDARs formed by GluN1/GluN2A or GluN1/GluN2B both resulted in large shifts in the apparent voltage dependence of BK channel activation (Gomez et al., 2021).

Localization and Roles of BK-NMDAR Coupling in the Nervous System

Both BK channels and NMDARs are widely expressed in different regions of the central and peripheral nervous system. Using a pull-down assay, Zhang et al. (2018) showed that BK-NMDAR complexes are ubiquitously present in the brain, as BKα and GluN1 mutually pulled down each other in all examined brain regions, including the hippocampus, cerebellum, cortex, thalamus, striatum, and olfactory bulb. The subcellular distribution of NMDARs is affected by their subunit composition. In the adult central nervous system, GluN2B is mainly expressed at extrasynaptic sites, whereas GluN2A-containting receptors are enriched at postsynaptic sites at synapses (Paoletti et al., 2013). Given that both GluN2A and GluN2B are present in neuronal BK-NMDAR complexes, these complexes are expected to exist both extrasynaptically and postsynaptically. NMDAR-induced BK channel activation has been observed at the extrasynaptic sites of olfactory bulb granule cells (Isaacson and Murphy, 2001) and dentate gyrus granule cells (Zhang et al., 2018). At extrasynaptic sites, functional BK-NMDAR coupling can be induced by accumulated glutamate spillover during repetitive synaptic activities (**Figure 4**), such as those observed in olfactory bulb granule cells (Isaacson and Murphy, 2001). At postsynaptic sites, NMDAR-coupled BK channels can be immediately activated by presynaptically released glutamate during synaptic transmission (**Figure 5**). BK channels are generally considered to be axonal channels (Misonou et al., 2006), but a significant portion has also been observed at postsynaptic terminals in the stratum radiatum and oriens of the rat hippocampus (Sailer et al., 2006). However, the mechanism of channel activation and the function of BK channels at postsynaptic sites were largely unknown until recently. Interestingly, with BKα knockout mice and pharmacological tools, BK channels were found to negatively regulate the excitatory postsynaptic potentials (EPSPs) of dentate gyrus granule cells evoked by single-pulse stimulation of presynaptic fibers from the in the middle of the molecular layer (Zhang et al., 2018). Such a regulatory effect of BK channels on synaptic transmission at perforant path–dentate granule cell synapses was found to be insensitive to EGTA and strictly dependent on NMDAR activity. Given that the BK-NMDAR coupling was induced by a single pulse stimulation, the coupling

must occur within the immediate reach of newly released glutamate in the synaptic cleft, i.e., at the pre- or postsynaptic terminal. A presynaptic effect of the BK channels was largely ruled out because of the lack of effect of paxilline and BKα knockout on presynaptic transmitter release. The postsynaptic dendritic location of the BK-NMDAR coupling was confirmed by the blockade effects of postsynaptically loaded paxilline and MK-801. When loaded intracellularly, the synthetic S0–S1 peptide also abolished the BK channels' effect on synaptic transmission, suggesting that physical interactions are necessary for functional coupling at postsynaptic terminals (Zhang et al., 2018). The postsynaptic BK-NMDAR interactions and coupling (**Figure 5**) provide a novel mechanism for the negative feedback regulation of synaptic transmission *via* NMDAR-mediated activation of postsynaptic BK channels by the presynaptically released neurotransmitter glutamate in the synaptic cleft. Dentate gyrus granule cells are the first checkpoints for cortical information entering the hippocampus, where learning and memory take place. The findings on NMDAR-mediated BK channel activation and the modulatory effect of postsynaptic BK-NMDAR coupling on synaptic transmission provide a new molecular basis for understanding the role of BK channels in hippocampal learning and memory (Matthews and Disterhoft, 2009; Ye et al., 2010; Typlt et al., 2013; Springer et al., 2014).

Postsynaptic BK-NMDAR coupling was also recently reported to occur in the basal dendrites of approximately 40% of BC-L5PNs (Gomez et al., 2021). The application of NMDA toward basal dendrites led to large NMDAR inward and BK channel outward currents recorded in soma, supporting dendritic locations of BK-NMDAR coupling (Gomez et al., 2021). Such large inward currents of NMDARs and outward currents of BK channels were typically observed when BK-NMDAR coupling occurred locally at the soma (Isaacson and Murphy, 2001; Zhang et al., 2018). Similar to that observed in dentate gyrus granule cells (Zhang et al., 2018), the NMDAR-mediated BK channel activity in the basal dendrites reduced the postsynaptic membrane potentials (Gomez et al., 2021). Interestingly, as compared to the BC-L5PNs lacking BK-NMDAR coupling, the basal dendrites displaying NMDAR-mediated BK activation had no Ca^{2+} spike and had shortened durations and reduced amplitudes of after hyperpolarization in current injection–elicited action potentials. Furthermore, these BC-L5PNs also had reductions in spike timing–dependent LTP (t-LTP). t-LTP levels were restored by blockading the BK channel with paxilline, and reductions in these levels were prevented when the frequency and number of pre- and postsynaptic stimulation pairings were increased (Gomez et al., 2021). It was proposed that the BK-NMDAR coupling increases threshold for induction of t-LTP by functioning as high-pass filters for incoming synaptic input (Gomez et al., 2021). These putative roles for postsynaptic BK-NMDAR coupling on the regulation of action potential and LTP are novel. However, it remains to be determined whether NMDARs and their nanodomain coupling to BK channels are the major contributors to the BK channel activities underlying the observed differences between these 2 types of BC-L5PNs. Application of the synthetic S0–S1 peptide to decouple BK-NMDAR interactions (Zhang et al., 2018) could potentially answer this question.

The recent discovery of BK-NMDAR complex formation and functional coupling helps explain previous reports of NMDAR-mediated, apamin-insensitive K_{Ca} currents in cultured, postnatal rat hippocampal neurons (Zorumski et al., 1989); the need for both NMDAR and BK channel activities in the inhibition of opioid release in the spinal dorsal horn (Song and Marvizon, 2005); and BK channel–mediated negative feedback on NMDAR-mediated dendritic spine Ca^{2+} transients in the cartwheel cells of the dorsal cochlear nucleus (He et al., 2014).

Comparison of BK-Ca_V and BK-NMDAR Coupling

Compared with BK-Ca_V coupling, BK-NMDAR coupling has distinct kinetic and functional properties. It produces unique neurotransmitter release–dependent K^+ signaling. NMDARs provide more sustained Ca^{2+} sources for BK channel activation than do Ca_V channels because of the slow rate of NMDAR deactivation (τ = ∼40 ms–2 s). Functional coupling of BK channels with NMDARs at extrasynaptic sites results in glutamate spillover–induced, extrasynaptic BK channel activation and neuronal activity inhibition. Postsynaptic NMDARs coincidently detect presynaptic glutamate release and postsynaptic membrane depolarization and can thus facilitate BK channel activation in a spike timing–dependent manner owing to the intrinsic properties of NMDAR-mediated Ca^{2+} influx. The resultant BK channel K^+ currents reduce the amplitude of EPSPs and promote Mg^{2+} blockade of NMDARs by repolarizing postsynaptic membranes. Given the involvement of BK channels in learning and memory (Matthews and Disterhoft, 2009; Ye et al., 2010; Typlt et al., 2013; Springer et al., 2014), BK channels may regulate long-term potentiation or depression *via* postsynaptic BK-NMDAR coupling. It is of note that the other types of K_{Ca} channels, e.g., SK channels, are also reported to be functionally coupled with Ca_V channels and NMDA receptors (NMDARs) (Marrion and Tavalin, 1998; Ngo-Anh et al., 2005; Wang et al., 2014). However, there is scarce evidence on the physical complex formation between Ca^{2+} channels and SK or IK channels, which is consistent with the lack of the requirement for strict, close colocalization for functional coupling. In spite of the physiological significance, the coupling of IK and SK channels with Ca^{2+}-permeable channels is beyond the focus of this review. Previous reviews of this topic can be found elsewhere (Fakler and Adelman, 2008; Gueguinou et al., 2014).

COUPLING OF BK CHANNELS WITH OTHER CHANNELS

Coupling of BK Channels With Intracellular Ca^{2+}-Release Channels

Ca^{2+}-release channels are intracellular Ca^{2+} channels responsible for the release of Ca^{2+} from endoplasmic and sarcoplasmic reticulum (ER and SR) which form the intracellular Ca^{2+} stores (Woll and Van Petegem, 2022). They are giant membrane proteins consisting of two evolutionarily related gene families, ryanodine receptors (RyRs) and

inositol-1,4,5-trisphosphate receptors (IP_3Rs). The three RyR isoforms (RyR1, RyR2, and RyR3) are each ∼ 5,000 amino acid residue polypeptides that assemble into ∼2.2 MDa homotetrameric channels. They are expressed in different tissues including brains. As they are mostly studied in the context of muscle contraction, RyR1 is known as the skeletal muscle isoform and RyR2 as the cardiac isoform. RyR3 is ubiquitously expressed. The three IP_3R isoforms (IP_3R1, IP_3R2, and IP_3R3) are each ∼2,700 amino acid residue polypeptides that assemble into ∼1.2 MDa homotetrameric or heterotetrameric channels. IP_3Rs have a broad tissue distribution with a high abundance in the cerebellum. Both RyRs and IP3Rs are sensitive to changes in cytosolic Ca^{2+} concentrations in that the channels are stimulated by rises in the cytosolic Ca^{2+} concentration but inhibited by high cytosolic Ca^{2+} concentration. RyRs are activated by Ca^{2+}-influx mediated by plasma membrane Ca_V channels *via* a mechanism of Ca^{2+}-induced Ca^{2+} release (CICR). IP3Rs are activated by the cytosolic IP3 molecule formed by protein lipase C (PLC) through hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into IP_3 and diacylglycerol (DAG). PLC is activated through a G-protein-coupled receptor (GPCR) or receptor tyrosine kinase (RTK) signaling pathway.

BK and Ca_V channels on somatic plasma membranes (PM) couple to RyRs on ER to form double (Ca_V-RyR and RyR-BK) PM-ER nanodomains in Ca^{2+} signaling in the cartwheel inhibitory interneurons of the dorsal cochlear nucleus (Irie and Trussell, 2017). The triad Ca_V/RyR/BK channel coupling (**Figure 5**) was reported to be EGTA-resistant and rapid (within the time of a single spike), and thus provided a mechanism for the rapid control of action potentials on a millisecond timescale (Irie and Trussell, 2017). Immunofluorescence analysis showed partial overlap of puncta of BK and RyR labeling in mouse cartwheel cells (Irie and Trussell, 2017). In mouse dentate gyrus granule neurons, knockout of the BK channel β4 subunit caused increased functional coupling between RyR and BK channels, resulted in an increase in the fAHP amplitude (Wang et al., 2016). A similar effect was observed by knockin of the seizure-prone gain-of-function (R2474S) RyR mutant channels. This study revealed different roles of the BK-Ca_V and BK-RyR coupling during action potential in that BK channel activation is dependent on L-type Ca_V channels in repolarization phase but RyRs during fAHP (Wang et al., 2016). In cerebral smooth muscle cells, another type of triad channel coupling, TRPV4/RyR/BK, has been reported for RyR-induced BK channel activation (Earley et al., 2005). Activation of TRPV4 by 11,12 EET in freshly isolated cerebral myocytes led to elevated Ca^{2+} spark from RyRs and transient BK activity which was unaffected by inhibition of Ca_V channels. The TRPV4-RyR-BK channel coupling accounts for smooth muscle hyperpolarization and arterial dilation *via* Ca^{2+}-induced Ca^{2+} release in response to TRPV4 activation by an endothelial-derived factor (Earley et al., 2005). However, whether the coupling also occurs within nanodomains is unclear, as no Ca^{2+} chelators were used to probe the strengths of the Ca^{2+} coupling.

Potential functional coupling of BK channels with IP_3Rs was reported in glioma cells *via* colocalization within lipid rafts (Weaver et al., 2007). With whole cell patch-clamp recording, it was observed that the voltage-induced BK channel activity was reduced upon lipid raft disruption with methyl-β-cyclodextrin. Pretreatment of glioma cells with thapsigargin to deplete the intracellular Ca^{2+} store or 2-aminoethoxydiphenyl borate to inhibit IP3Rs negated the effect of methyl-β-cyclodextrin. Stimulation of muscarinic acetylcholine receptors (mAChRs) with muscarine or acetylcholine (ACh), which was known to promote IP3 formation, elicited an increase in $[Ca^{2+}]_i$ that subsequently activated BK channels and caused cell hyperpolarization. Disruption of lipid rafts or inhibition of IP3Rs prevented the ACh-induced rise in $[Ca^{2+}]_i$ and the BK channel-induced hyperpolarization of the membrane. Both BK channels and IP_3Rs were found to associate or localize with lipid rafts as detected by immunoblot in the isolated lipid raft fractions or by immunofluorescence on cells. Given the presence of 10 mM EGTA in the pipette solution of the whole cell recording and the sensitivity to Ca^{2+} store depletion, it is likely that the BK-IP_3R coupling involves Ca^{2+} and the coupling exists within nanodomain. However, the lack of data for comparison with/without EGTA or BAPTA prevents drawing a solid conclusion. Another instance of potential BK-IP_3Rs interactions was observed in rat and mouse cerebral artery smooth muscle cells (Zhao et al., 2010). Activation of BK channels by IP_3 or its membrane-permeable analog occurred in both intact cells and excised membrane patches *via* cell-attached and inside-out patch-clamp recording configurations, respectively. Inhibition of IP_3Rs, knockout of IP_3R1, or application of an IP_3R1 antibody suppressed the IP_3-induced BK channel activation in inside-out recording of excised membrane patches. Immuno-FRET imaging analysis indicated BK-RyR1 colocalization. The BK channel α and β1 subunit were detected to be coimmunoprecipitated with RyR1. However, the IP_3-induced BK channel activation in inside-out recording was time-independent after membrane patch excision and was insensitive to both BAPTA and EGTA, indicating some SR Ca^{2+}-release independent mechanism. In another conflicting report, the IP3 was found to activate BK channel in pig coronary artery smooth muscle cells *via* an IP_3R-indepenent mechanism (Yang et al., 2013). Therefore, uncertainty exists whether there is Ca^{2+}-mediated BK-IP_3R coupling and caution is needed in interpretation of the data on IP_3-related BK channel activation.

Coupling of BK Channels With Transient Receptor Potential Channels

BK channels have also been reported to functionally couple with TRP channels. TRP channels are a superfamily of cation channels whose activation mechanisms are more diverse than those of any other group of ion channels. TRP channels play critical roles in sensory physiology, including vision, taste, olfaction, hearing, touch, and thermo- and osmosensation. The TRP superfamily is divided into 7 subfamilies: 5 group 1 TRPs (TRPC, TRPV, TRPM, TRPN, and TRPA) and 2 group 2 subfamilies (TRPP and TRPML). The transient receptor potential vanilloid receptor 1(TRPV1) channel is a non-selective cation channel activated by a variety of exogenous and endogenous physical and chemical stimuli, such as temperature and capsaicin. In the heterologous

expression system of HEK293 cells, BK channels were found to be activated by Ca^{2+} influx through TRPV1 channels in a largely EGTA-insensitive manner (Wu et al., 2013). The TRPV1-induced BK currents were also observed in dorsal root ganglion (DRG) cells (Wu et al., 2013). Coimmunoprecipitation showed formation of the BK-TRPV1 complex in both HEK293 and DRG cells. There have also been reports of the functional coupling of BK channels with TRPV4 in human bronchial epithelial cell lines (Fernandez-Fernandez et al., 2008) and with TRPC1 in vascular smooth muscle cells (Kwan et al., 2009). Activation of TRPV4 by activator, osmotic and mechanical stimulation led to BK channel activities, which was lost upon of TRPV4 knockdown (Fernandez-Fernandez et al., 2008). Coimmunoprecipitation and immunofluorescence showed complex formation between BK and TRPC1 channels in HEK293 cells and their colocalization in vascular smooth muscle cells (Kwan et al., 2009). The BK-TRPC1 coupling was found to play a role in agonist-induced membrane depolarization and vascular contraction in isolated rat mesenteric arteries (Kwan et al., 2009). Whether the BK-TRPV4 and BK-TRPC1 couplings occurred within nanodomain is unknown, as no data on EGTA-sensitivity were presented in both studies (Fernandez-Fernandez et al., 2008; Kwan et al., 2009) and no protein complex formation and colocalization was demonstrated in the BK-TRPV4 coupling case (Kwan et al., 2009). Coimmunoprecipitation of BK with TRPC3 and TRPC6 channels was observed in differentiated podocyte cell line and heterologous expression system of HEK293 cells (Kim et al., 2009). TRPC3 but not TRPC6 was found to increase the surface expression of BKα subunit splice variant ($Slo1_{VEDEC}$) (Kim et al., 2009). However, the possibility of Ca^{2+}-mediated functional coupling of the BK with TRPC3 or TRPC6 channels was not addressed in this report (Kim et al., 2009).

DISCUSSION

Reported studies have clearly demonstrated the nanodomain functional couplings of BK channels with Ca^{2+}-permeable channels, particularly the Ca_V and NMDAR channels, in a variety of different cells. These mechanisms of nanodomain couplings allow BK channels to play diverse cellular and physiological roles. Given the BK channel's widespread expression and critical physiological roles, directly targeting it can have unavoidable adverse side effects. Selective enhancement or disruption of the interactions between BK channels and Ca^{2+} permeable channels using small chemicals, peptidomimetic molecules, or genetic methods can be an effective way to modify BK channel activity or to indirectly intervene in Ca_V or NMDAR function by limiting or enhancing the negative feedback from BK channels. It will be important to know how they organize and interact on membrane within nanodomains for effective functional coupling. However, the biochemical bases of the interactions of BK channels with Ca^{2+} permeable channels, either direct or indirect, remain mostly unknown. The recent advances in the cryo-EM–based structural determination of ion channels have given us a better understanding of the structure-function relationship of ion channels. Future determination of the structures of whole coupling complexes should enable researchers to develop potent and specific inhibitors or activators of these complexes and thus design novel therapeutic interventions. The initial investigation into the biochemical basis of BK-NMDAR coupling found direct physical interactions between the BK and NMDAR intracellular regions and domains (Zhang et al., 2018). Encouragingly, the synthetic S0–S1 peptide of BK channels was shown to be effective in disrupting the BK-NMDAR interactions and couplings (Zhang et al., 2018).

BK-Ca_V nanodomain coupling intertwines the functions of these two types of channels in the regulation of neuronal excitability. Compared to the cellular and physiological roles of BK-Ca_V coupling, those of BK-NMDAR coupling in different cells are less studied and understood. Postsynaptic and extrasynaptic BK-NMDAR couplings (**Figure 5**) provide unique neurotransmitter-dependent Ca^{2+} sources for BK channel activation and function, which could be important for synaptic plasticity, as was noted in the recent study (Gomez et al., 2021). More studies in different brain regions or neurons under normal or pathological conditions could expand our understanding of the physiological and pathological roles of BK-NMDAR coupling. NMDARs play numerous physiological and pathological roles. The extent to which BK-NMDAR coupling contributes to NMDAR-mediated Ca^{2+} signaling remains to be determined. The development of pharmacological tools that specifically interrupt BK-NMDAR interactions, such as the synthetic S0–S1 peptide (Zhang et al., 2018), will be helpful in determining the physiological and pathological roles of BK-NMDAR coupling. For the structural and functional coupling of BK channels with other Ca^{2+} permeable channels within nanodomain, the reported studies remain sparse or very limited in evidence. More studies will be needed to establish the structural and functional coupling, determine the underlying biochemical mechanisms, and understand the physiological roles.

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All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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