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ATP-GATED P2X RECEPTORS IN HEALTH AND DISEASE

Topic Editors
Geoffrey Burnstock, Andrea Nistri,
Baljit S. Khakh and Rashid Giniatullin

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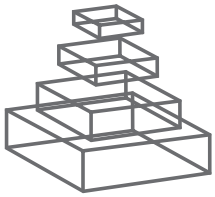

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ATP-GATED P2X RECEPTORS IN HEALTH AND DISEASE

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Extracellular ATP is currently recognized as one of the most widely distributed neurotransmitters and neuromodulators in the peripheral and central nervous system. ATP-gated P2X receptors are expressed by neurons, glial and many other non-neuronal cells and represent an attractive target for therapeutic interventions. Diverse molecular and cellular mechanisms have been identified for P2X receptor functioning, including the ability to enlarge the size of the ion pore associated with the release of several key immune molecules. A major recent breakthrough was the determination of the X-ray crystal structures of zebrafish P2X4 receptor in ATP-bound and ATP-free states. The P2X receptor research field is rapidly growing, as evidenced by the almost 2000 papers published in the last 5 years. However, despite the fundamental signalling function of extracellular ATP in the nervous system, the widespread roles of P2X receptors have not been widely elucidated and presented in textbooks. In this volume of papers we aim to gather a collection of high quality papers, detailing the latest insights from the most accomplished international P2X receptor researchers. Importantly, basic research into P2X receptors has a strong translational impact and our collection of articles could be a valuable guide for the development of new pharmacological and biotechnological tools addressing the function of P2X receptors. Within this collection we plan to cover receptor structure-function relationships, receptors trafficking, to highlight the special properties of P2X receptors and their pharmacological profiles, and to describe the translational aspects of cellular ATP signaling in pain and in other neurological and vascular diseases.

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EDITORIAL
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ATP-gated P2X receptors in health and disease

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This collection is devoted to the role of extracellular adenosine 5′-triphosphate (ATP) as a physiological transmitter in health and as an essential contributor to various pathologies. The present e-book contains 15 reviews (and 2 original papers) detailing the action of extracellular ATP on the peripheral and central nervous systems. Thus, this e-book provides a major repository of novel information on ATP-gated P2X receptors that are important transducers of the cellular effects of ATP.

ATP has long been known as the main source of energy in living cells. However, a further fundamental role of ATP was later identified, namely that extracellular ATP serves as a messenger for fast intercellular communications via binding to and activation of a set of membrane proteins termed P2X receptors. Unlike other classical neurotransmitters and neuromodulators, the extracellular actions of ATP are pleiotropic as they can affect almost all cell types (both neuronal and non-neuronal) in the body. Thus, ATP has rightly joined "the club" of traditional signaling molecules and is now considered to be a phylogenetically early and widely distributed endogenous agonist. The history and perspectives for this subject are presented in the opening paper "Introduction and perspective, historical note" written by Geoffrey Burnstock, the founding father of the field (Burnstock, 2013). The evolutionary thread of P2X receptors found in primitive organisms is discussed by Fountain (2013).

In the nervous system, ATP-gated P2X receptors are expressed in neurons, glia and vascular cells and are characterized by a variety of distinct properties. One intriguing issue is how these P2X receptors are activated by the natural agonist, ATP. Our understanding of ATP signaling was recently enhanced by the reports of the X-ray crystal structures of zebrafish P2X4 receptors in the closed and open states. Arising from these results, molecular dynamics studies of receptor structure are becoming an important tool for many studies of the molecular conformation of P2X receptors, as reviewed by Chataigneau et al. (2013). The mechanism of ion channel opening, which is indispensable for P2X function, as well as the properties of ion flow through it are presented by Samways et al. (2014). The next review explains, in detail, the process of assembling P2X subunits as either homo-trimers or hetero-trimers, with consequently major changes in their functional and pharmacological properties that depend on the final subunit composition (Saul et al., 2013). The trafficking and targeting of P2X receptors to the cell membrane, which are essential steps to express their function, are discussed by Robinson and Murrell-Lagnado (2013). Once inserted into the membrane, the activity of P2X receptors is modulated by phospholipids that play a substantial role in the receptor post-translational modifications (Bernier et al., 2013).

One unusual receptor property is the gradual formation of an increasingly larger membrane ion pore during the sustained activation of some ATP-gated P2X receptors (Rokic and Stojilkovic, 2013). This phenomenon is typically observed in the P2X7 subtype, but it can also be manifested in P2X2, P2X2/X5, and P2X4 receptors. In addition to the development of certain biophysical characteristics, this property is associated with important functions, including the release of powerful immunologically-relevant molecules.

In the current e-book collection, several reviews present a detailed analysis of the mechanisms and pathophysiological role of certain receptor subtypes. Thus, one review deals with the strong desensitization properties of P2X3 receptors (Giniatullin and Nistri, 2013). Desensitization (temporary inactivation of the channels by persistent agonist application) likely plays a filtering effect in the detection of physiological nociceptive signals induced by ATP. Abnormal pain signaling via P2X3 receptors is thought to contribute to neuronal sensitization and chronic pain, whose molecular mechanisms are amply discussed in the review by Fabbretti (2013). The therapeutic applications of P2X3 receptor antagonists to pathological states are subsequently discussed by Ford and Undem (2013).

The present e-book also contains reviews on fundamental aspects of P2X receptor translational application that includes new pharmacological approaches to target acute and chronic pain, and neurological and vascular diseases. Thus, the review on the role of ATP receptors in status epilepticus and excitability of mammalian brain neurons provides novel information on the control of cortical network activity (Henshall et al., 2013). A new function of ATP signaling has emerged in taste buds where, by acting on desensitizing P2X3 subunits co-assembled with non-desensitizing P2X2 subunits on receptor cells, ATP serves as a messenger between taste buds and afferent nerves (Kinnamon and Finger, 2013).

A detailed analysis of P2X4 receptors, which together with P2X7 receptors, are expressed by glial cells, indicates their contribution to neuroinflammation and chronic pain (Tsuda et al., 2013). The companion original article presents new data on the structural and functional properties of the rat P2X4 receptor extracellular vestibule during the gating process (Rokic et al., 2014). Furthermore, an important new role for P2X4 receptors as modulators of lung surfactant secretion has been recently identified (Miklavc et al., 2013). The dynamic micro-organization of P2X7 receptors revealed by PALM based single-particle tracking is elucidated in the original contribution by Shrivastava et al. (2013).

The field of P2X receptor studies is rapidly expanding as novel drugs based on their ability to selectively antagonize P2X receptor subtypes are developed. Furthermore, mechanisms of phenomena like large pore opening or desensitization, albeit incompletely understood, may represent future targets for designing specific new drugs. The extensive survey of P2X receptors found in the present e-book allows fast perusing of specific mechanisms related to distinct receptor subtypes, and represents a solid reference for future studies. We believe that this e-book is an excellent introduction for any scientist interested in the basic and clinical role of ATP receptors, and is also a very useful source of concentrated updated information for professionals.

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Post-translational regulation of P2X receptor channels: modulation by phospholipids

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P2X receptor channels mediate fast excitatory signaling by ATP and play major roles in sensory transduction, neuro-immune communication and inflammatory response. P2X receptors constitute a gene family of calcium-permeable ATP-gated cation channels therefore the regulation of P2X signaling is critical for both membrane potential and intracellular calcium homeostasis. Phosphoinositides (PIP_n) are anionic signaling phospholipids that act as functional regulators of many types of ion channels. Direct PIP_n binding was demonstrated for several ligand- or voltage-gated ion channels, however no generic motif emerged to accurately predict lipid-protein binding sites. This review presents what is currently known about the modulation of the different P2X subtypes by phospholipids and about critical determinants underlying their sensitivity to PIP_n levels in the plasma membrane. All functional mammalian P2X subtypes tested, with the notable exception of P2X5, have been shown to be positively modulated by PIP_n, i.e., homomeric P2X1, P2X2, P2X3, P2X4, and P2X7, as well as heteromeric P2X1/5 and P2X2/3 receptors. Based on various results reported on the aforementioned subtypes including mutagenesis of the prototypical PIP_n-sensitive P2X4 and PIP_n-insensitive P2X5 receptor subtypes, an increasing amount of functional, biochemical and structural evidence converges on the modulatory role of a short polybasic domain located in the proximal C-terminus of P2X subunits. This linear motif, semi-conserved in the P2X family, seems necessary and sufficient for encoding direct modulation of ATP-gated channels by PIP_n. Furthermore, the physiological impact of the regulation of ionotropic purinergic responses by phospholipids on pain pathways was recently revealed in the context of native crosstalks between phospholipase C (PLC)-linked metabotropic receptors and P2X receptor channels in dorsal root ganglion sensory neurons and microglia.

Keywords: P2X receptors, ligand-gated channel, purine nucleotides, PIP2, phospholipids, pain, calcium, phospholipases

P2X receptor channels are involved in a wide variety of physiological processes ranging from sensory transduction to neuro-immune interactions to synaptic modulation. Upon binding to their agonist ATP, conformational changes induce the opening of a non-selective cation channel, impacting cellular physiology through membrane depolarization and calcium influx (North, 2002). This process is tightly controlled by various allosteric regulatory mechanisms, some acting on extracellular or transmembrane regions of the channel subunits, as is the case for metals, pH, divalent cations or alcohols (Coddou et al., 2011). On the other hand, several mechanisms of posttranslational regulation are known to affect the efficacy of P2X activation by interacting with the intracellular N- or C-terminal tails of the subunits. Among them, plasma membrane-bound lipids such as phosphoinositides (PIP_n) were shown to have an important functional impact on P2X receptors, emerging as cofactors necessary for full channel activity. Here, we will review the recent evidence describing PIP_n-dependent functional modulation for various members of the P2X receptor channel family, the molecular determinants of the protein-lipid interaction as well as the impact of this novel regulatory mechanism at the cellular level.

FUNCTIONAL INTERACTIONS BETWEEN PIP_n AND ION CHANNELS

PIP_n are composed of two fatty acid chains esterified to a glycerol backbone, attached to a *myo*-inositol ring forming a polar head group. Poly-PIP_n are synthesized through phosphorylations by selective PI kinases and are found in low abundance in cellular membranes, with phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$; PIP_2) being the most common, but only representing 1% of total cellular acidic lipids (Toker, 1998; Prestwich, 2004). PIP_n are classically viewed as critical players in ubiquitous intracellular signaling pathways. Notably, activation of phospholipase C (PLC) catalyses the hydrolysis of PIP_2, giving rise to inositol trisphosphate (IP_3) and diacylglycerol (DAG), triggering multiple signaling cascades (Berridge, 1993; Monserrate and York, 2010). The different species of PIP_n can also serve

as membrane-bound anchors to various proteins, acting as a localization tag to specific organelles (Doughman et al., 2003; Heo et al., 2006). A third major signaling role of membrane PIP_n involves their direct functional regulation of integral membrane proteins (Suh and Hille, 2005; Gamper and Shapiro, 2007; Logothetis et al., 2010). Several families of channels and transporters have been demonstrated to be sensitive to PIP_n levels, among them are transient receptor potential (TRP) channels (Rohacs, 2007), inward-rectifier potassium channels (Kir) (Huang et al., 1998; Logothetis et al., 2007), KCNQ voltage-gated potassium channels (Hernandez et al., 2008b), cyclic nucleotide-gated (CNG) channels (Womack et al., 2000), epithelial sodium channels (ENaC; Kunzelmann et al., 2005), calcium release-activated calcium (CRAC) channels (Korzeniowski et al., 2009) and P2X receptor channels. Whereas direct protein-lipid binding was demonstrated for some of these families including P2X receptors, no consensus amino acid sequence has been defined to predict PIP_n binding to channels and transporters. However, several lines of evidence indicate that positive residues located on intracellular portions of the protein electrostatically interact with the negative head group of PIP_n to mediate the functional interaction (Rosenhouse-Dantsker and Logothetis, 2007; Whorton and Mackinnon, 2011).

FUNCTIONAL REGULATION OF P2X RECEPTORS BY PIP_n

P2X1

The initial observation of PIP_n-dependence for the P2X1 receptor subtype came from inside-out macropatch recordings in *Xenopus* oocytes expressing the receptor. Under this experimental condition, sequestration of $PI(4,5)P_2$ by application of polylysine led to a current rundown, which could further be rescued by the addition of a soluble $PI(4,5)P_2$ analog to the intracellular side of the membrane (Zhao et al., 2007). The regulatory role of PIP_n on P2X1 channel function was later confirmed when it was shown that blocking PI4 kinase (PI4K) activity negatively modulated P2X1 current amplitude and recovery from desensitization in whole-cell recordings performed on *Xenopus* oocytes expressing P2X1 (Bernier et al., 2008b). Current activation and desensitization rates were also slowed by $PI(4,5)P_2$ depletion, suggesting a modulatory effect of the lipids on channel gating kinetics. However, PI3 kinase (PI3K) blockade did not affect P2X1 responses, indicating a prevalent role of $PI(4,5)P_2$, as confirmed by the results showing a complete recovery of current kinetics and amplitude upon intracellular application of a soluble $PI(4,5)P_2$ analog in the recorded oocyte following pharmacological depletion. A direct interaction between the proximal C-terminal region of P2X1 and various PIP_n was shown by the binding of fusion proteins containing the region of interest to phospholipid species including $PI(4,5)P_2$ coated on nitrocellulose membranes (PIP strips). Mutating intracellular C-terminal basic residues into neutral glutamine (K359Q, K364Q) decreased the interaction affinity in this *in vitro* assay; P2X1 receptor containing these point mutations also exhibited decreased currents (Bernier et al., 2008b, 2012b). Interestingly, it was later shown that neutralizing lysine 364, as well as the positive arginine on position 360 also slowed the receptor recovery time after desensitization (Allsopp et al., 2013) in a mechanism that might implicate a decrease in PIP_n binding affinity.

P2X1/5

The sensitivity of the P2X1/5 heteromeric subtype to phospholipids was studied in recombinant form via tranfection in HEK293 cells as well as in native expression in murine astrocytes acutely isolated from brain slices. In both preparations, the current carried through the P2X1/5 channel was decreased following PIP_n depletion. Under whole-cell patch-clamp configuration, the decreased channel responses could be rescued by addition of a $PI(4,5)P_2$ analog inside the recording patch pipette. The direct positive regulation by the phospholipid, combined with the noticeable insensitivity of P2X5 homomers to PIP_n levels, is indicative of a critical dominant role of the P2X1 subunit in the regulation process (Ase et al., 2010). The extent to which calcium-permeable and $PI(4,5)P_2$-sensitive P2X1/5 ATP-gated channels contribute to glial function and synaptic transmission still remains to be explored.

P2X2

The first report of PIP_n-dependence of a P2X receptor channel came from Fujiwara and Kubo, who demonstrated that P2X2 channel gating was affected by pharmacological depletion of PIP_n with the PI3K blockers wortmannin and LY294002 (Fujiwara and Kubo, 2006). They observed that the relative absence of PIP_n accelerated current desensitization, an effect mimicked by mutating two positively charged lysine residues of the proximal C-terminal region (K365 and K369) into neutral glutamines, indicating that an interaction between these two residues and PIP_n promotes the stability of the open conformation of the channel. Some P2X subtypes including P2X2 display unique activity- and time-dependent changes in channel permeability. By measuring the permeability shift in N-methyl-D-glucamine ($NMDG^+$)-containing solutions, it was shown that this apparent pore dilation is also regulated by PIP_n. The direct nature of the interaction between the C-terminus of P2X2 and PIP_n was demonstrated in two ways. By generating fusion proteins containing a region of interest from the C-terminal tail and applying them to PIP strips, it was confirmed that a direct binding can occur with several PIP_n, including 3′ phosphorylated species dependent on PI3K activity. Using similar fusion proteins coupled to EGFP, the authors also reported association of the P2X2 proximal C-terminal tail to membrane PIP_n in COS-7 cells. Although these data lead to a primary role of PI(3)P and $PI(3,5)P_2$, inside-out macropatch recordings performed by the Logothetis group showed that the application of $PI(4,5)P_2$ can also directly rescue the rundown of P2X2 current induced by addition of polylysine, which binds and sequesters endogenous $PI(4,5)P_2$ (Zhao et al., 2007).

P2X3

As it was observed for all P2X homomers tested, the current rundown of the sensory P2X3 subtype expressed in *Xenopus* oocytes can be rescued by direct application of $PI(4,5)P_2$ to its intracellular domains (Zhao et al., 2007). Mo et al. (2009) then provided evidence of the $PI(4,5)P_2$-dependent regulation of the channel in native conditions, as ATP-evoked P2X3-mediated currents in

dorsal root ganglion neurons were significantly decreased after PI4K inhibition with the furanosteroid wortmannin at micromolar concentrations (Mo et al., 2009). However, the interaction between P2X3 and PIP_n might involve indirect modulation, as no direct binding was found between various P2X3 C-terminal regions and PIP_n on PIP Strips. Furthermore, when expressed in heterologous systems like HEK293 cells or *Xenopus* oocytes, only the rate of recovery from desensitization of the receptor was affected by $PI(4,5)P_2$ levels. The absence of direct PIP_n binding *in vitro* and the striking cell type-dependent difference in functional regulation strongly suggests that an unidentified associated protein expressed in DRG neurons provides a necessary link between P2X3 channels and phospholipids.

P2X2/3

The currents carried through the P2X2/3 heteromer were also shown to be modulated by pharmacological PIP_n depletion in *Xenopus* oocyte expression system and in native conditions in rat dorsal root ganglion neurons (Mo et al., 2009). Functionally, the P2X2/3 receptor channel retains the PIP_n sensitivity of the two subunits found in the heteromer. Blocking the formation of 3′ phosphorylated PIP_n with wortmannin reduced its current amplitude, as is the case for the P2X2 homomer. On the other hand, the blockade of the PI4K-dependent synthesis of 4′ phosphorylated isoforms also inhibited currents, similar to what is seen for the P2X3 homomer. Moreover, following PIP_n depletion, the P2X2/3 current amplitude can be partially rescued by addition of either $PI(3,4,5)P_3$ or $PI(4,5)P_2$.

P2X4

The PIP_n sensitivity of the P2X4 receptor channel has been extensively studied in both recombinant systems and native models. In inside-out macropatch, $PI(4,5)P_2$ increases currents carried through the P2X4 channel (Zhao et al., 2007), while whole-cell currents are inhibited by depletion of $PI(3,4,5)P_3$ or $PI(4,5)P_2$ (Bernier et al., 2008a). Subsequent intracellular injection of either of these two major signaling phospholipids leads to a recovery of the P2X4 current. Following PIP_n depletion, P2X4 also exhibits a slower recovery from desensitization as well as slower activation and desensitization rates, suggesting that PIP_n binding to the channel subunit triggers a conformational change that affects its gating, as was also observed with P2X1 and P2X2. P2X4-mediated ATP responses in BV2 murine microglial cells were recorded in patch clamp, and there again, PIP_n act as cofactors necessary for full current amplitude. Since the P2X4 channel has the highest calcium permeability in the P2X family (Egan and Khakh, 2004), P2X4-mediated calcium entry in microglia was also assayed and a similar inhibition following PIP_n depletion was observed. The capability of P2X4 to form a large conductance pore upon sustained activation was also found to be dependent on PIP_n when tested in a YO-PRO-1 uptake assay in primary mouse microglia and in an assay measuring time-dependent changes in $NMDG^+$ permeability of the channel in the *Xenopus* oocyte heterologous expression system (Bernier et al., 2012a). Binding between a proximal region of the P2X4 C-terminal tail and various PIP_n was demonstrated using PIP strips, and individual basic residues (K362, K363, K370, K371) were found to be critical for high-affinity lipid-protein (Bernier et al., 2008a, 2012b). In functional assays, mutating two of these residues to glutamine, therefore neutralizing their positive charge and decreasing the affinity of the P2X4-PIP_n binding, induced a current that showed a slower recovery from desensitization, a slower activation rate and a slower desensitization rate. To further confirm the ability of the P2X4 proximal C-terminal region (C360-V375) to bind to membrane PIP_n in cellular environments, a whole-cell patch-clamp experiment was performed in P2X4-expressing HEK293 cells, where fusion proteins containing the putative PIP_n-interacting peptide of P2X4 were introduced in the patch pipette. The addition of the P2X4 C360-V375 peptide led to a decrease in the activation and desensitization rates of the P2X4 channel, indicating a competition for PIP_n binding between the P2X4 receptor and its own PIP_n-binding domain. Conversely, introducing fusion proteins containing PIP_n binding loss-of-function mutations abrogated the effect. Overall, these results suggest that P2X4 binds directly to $PI(3,4,5)P_3$ and $PI(4,5)P_2$ via key lysine residues in the proximal C-terminal region, and that this interaction leads to conformational changes increasing the efficacy of channel activation.

P2X5

Functional data obtained in heterologous expression systems suggest that the P2X5 homomer is the only functional mammalian P2X subtype insensitive to PIP_n levels in the plasma membrane. A first report showed that when expressed in HEK293 cells, P2X5 was unaffected by pharmacological depletion of PIP_n (Ase et al., 2010). Expectedly, no direct binding between the P2X5 C-terminal tail and PIP_n could be observed using PIP strips. When various mutations were introduced on the P2X5 C-terminus to improve its binding affinity to PIP_n, the current carried by the P2X5 channel was greatly increased, and the mutated receptor acquired sensitivity to wortmannin-induced PIP_n depletion, implying that the relatively small currents obtained in WT P2X5 were due to a lack of PIP_n-dependent potentiation (Bernier et al., 2012b). Furthermore, the PIP_n-binding mutant P2X5 exhibited a faster recovery from desensitization, as well as faster activation and desensitization rates, whereas pharmacological PIP_n depletion led to a current phenotype similar to that observed with wild-type P2X5. The profound changes seen in the gating properties of the PIP_n-binding P2X5 mutant further confirms the important functional role phospholipids play on P2X function.

P2X7

The initial observation of PIP_n-dependence of the P2X7 receptor came from the Logothetis group, who demonstrated that pharmacological inhibition of $PI(4,5)P_2$ synthesis reduced the P2X7 current density in *Xenopus* oocytes and HEK293 cells (Zhao et al., 2007). PLC-dependent $PI(4,5)P_2$ hydrolysis induced by platelet-derived growth factor receptor (PDGFR) activation was also shown to partially inhibit P2X7 function and addition of $PI(4,5)P_2$ directly reversed the rundown of P2X7 current in inside-out macropatches. Furthermore, three positively-charged amino acid residues of the C-terminal domain were found to be critical in the $PI(4,5)P_2$-dependence of the P2X7 receptor. However, no direct binding was observed between the P2X7

C-terminal tail and PIP_n on PIP strip membranes, suggesting that the channel-lipid interactions might be indirect (Bernier et al., 2012b).

MOLECULAR DETERMINANTS OF THE PIP_n INTERACTION WITH P2X RECEPTORS

As all ATP-activated P2X subtypes except P2X5 were shown to be functionally regulated by PIP_n, it is critical to characterize the nature of the protein-lipid interaction. For all PIP_n-sensitive subtypes, several basic amino acid residues were shown through mutational assays to be necessary for the regulation. This is in accordance with most PIP_n-binding regions of other types of proteins; while no consensus sequence exists for PIP_n binding, the presence of positively-charged residues, often found in clusters, is necessary for an electrostatic interaction to take place with the negatively-charged head of the lipid (Rosenhouse-Dantsker and Logothetis, 2007; Whorton and Mackinnon, 2011). For P2X receptors, it is hypothesized that an intracellular domain containing a dual cluster of basic amino acids, mainly arginines and lysines located on the C-terminal tail 6 to 19 residues away from the second transmembrane domain, is required for PIP_n binding (Bernier et al., 2012b). Some subtypes, including P2X3, P2X7 and the PIP_n-insensitive P2X5 lack this characteristic domain and hence do not directly bind to PIP_n. Analysis of the basic and hydrophobic (BH) score of P2X C-terminal regions, using a quantification method that measures the lipid binding affinity of unstructured linear protein sequences (Brzeska et al., 2010), also predicts the presence of a lipid binding site on most P2X subunits (**Figure 1**). The high BH score region corresponds to the dual polybasic cluster motif experimentally confirmed (Bernier et al., 2012b). Furthermore, mutational analysis of P2X5 indicated that the regulatory dual cluster motif is not only necessary, but also sufficient for PIP_n-dependent regulation, as creation of the putative PIP_n binding site via neutral- or acidic-to-basic mutation induced a current phenotype functionally modulated by PIP_n. Similar regulatory PIP_n-binding motifs have been found on multiple PIP_n-dependent channels, including members of the TRPM family which bind to PIP_n via the TRP box, and M-type potassium channels (Kv7) interacting with PIP_n via a polybasic cluster (Rohacs et al., 2005; Nilius et al., 2008; Hernandez et al., 2008a; Hansen et al., 2011).

Whereas direct binding between phospholipids and the binding motif *in vitro* correctly predicts sensitivity to PIP_n and phenotype for most P2X subtypes, results obtained with P2X3 and P2X7 indicate that indirect PIP_n modulation can also influence channel properties. Several acidic residues are found within the PIP_n binding site of P2X3, disrupting the global positive charge of the clusters and reducing its binding affinity. On the other hand, the P2X7 sequence displays only one polybasic cluster. Accordingly, no direct binding to PIP_n was observed for the two subtypes (Mo et al., 2009; Bernier et al., 2012b). However, functional regulation of the P2X3 and P2X7 channels by PIP_n was observed in various models, leading to the hypothesis of an association with a cofactor acting as an indirect sensor of PIP_n levels in the plasma membrane (Zhao et al., 2007; Mo et al., 2009). Indirect interactions have been demonstrated for various PIP_n-sensitive channels: the potentiation of TRPV1 by PIP_n relies on *p*hosphoinositide *i*nteracting *r*egulator of *T*RP (PIRT) acting as a linker between both molecules, and N-methyl-D-aspartate (NMDA) glutamate receptors are regulated by PIP_n via α-actinin interacting with both the lipids and the NR1 and NR2b subunits to promote channel opening (Michailidis et al., 2007; Kim et al., 2008). Interestingly, the long P2X7 C-terminal domain directly associates with various proteins including α-actinin, possibly linking the channel subunit to PIP_n (Kim et al., 2001).

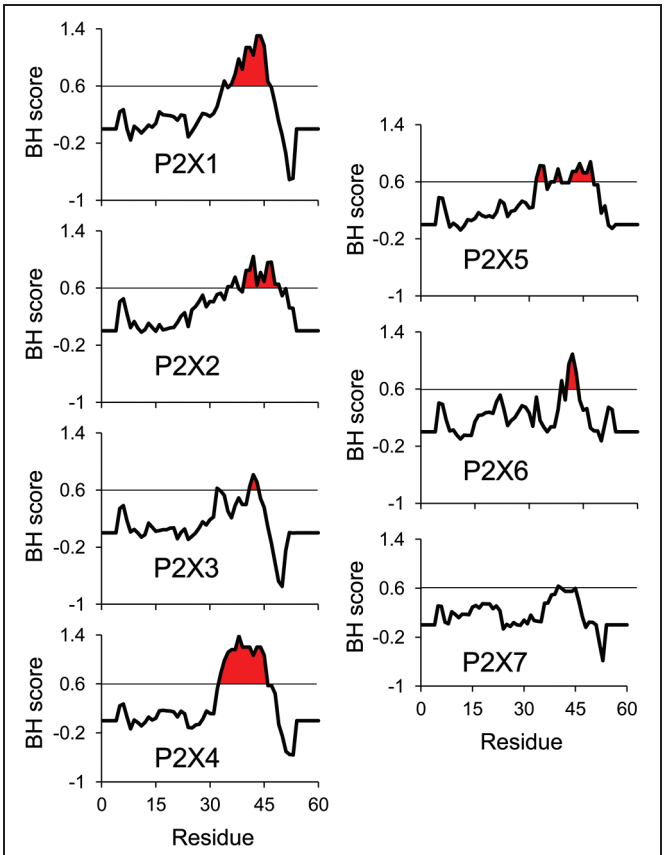

FIGURE 1 | BH scale analysis of P2X C-terminal regions. The analysis of all rat P2X C-terminal regions using the BH scoring method (Brzeska et al., 2010) predicts strong PIP_n affinity for the reported PIP_n binding region in the subunits experimentally shown to directly bind PIP_n. A BH score above 0.6 was demonstrated to accurately identify unstructured lipid-binding sites in proteins. The input sequence consisted of 60 amino acids of the C-terminal region, including the last 13 amino acids of the second transmembrane domain (starting at residue Gly324 in P2X1 numbering). The analysis was performed with a window size of eight amino acids.

The exact molecular mechanism by which protein-PIP_n binding induces changes in the functional phenotype of P2X channels remains elusive. However, it is likely that such interaction, whether it be direct or indirect, triggers a conformational change in TM2 linked to the proximal C-terminal domain. Recent evidence from crystallization of the PIP_n-binding and PIP_n-sensitive Kir2.2 channel in the presence of PIP_2 demonstrates that a similar channel-lipid interaction can lead to significant movements of the cytosolic domains, by as much as 6 Å (Haider et al., 2007; Hansen et al., 2011; Whorton and Mackinnon, 2011). The recent crystallographic data of P2X4 unfortunately do not contain the cytosolic C-terminal domain where the P2X PIP_n binding site is

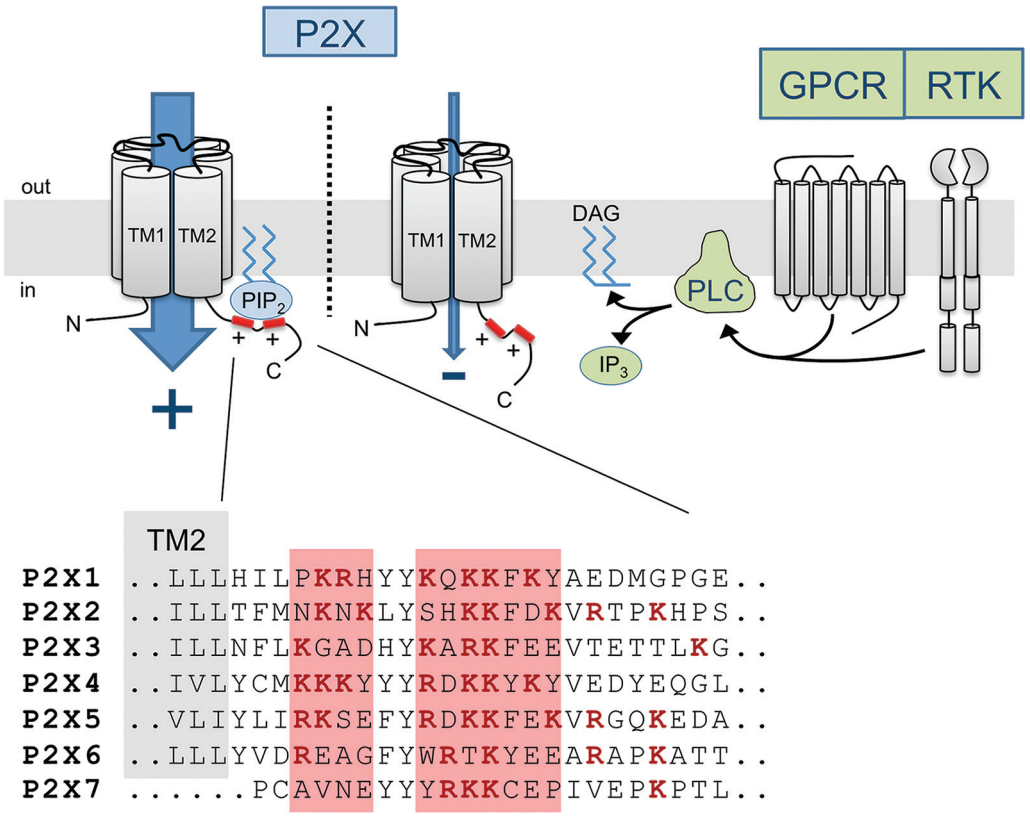

FIGURE 2 | Diagram of the PIP_n-dependent metabotropic regulation of P2X receptor channels. Membrane-bound PIP_n directly bind a dual poly-basic cluster motif found in the C-terminal region of certain P2X receptor subtypes, modulating the current carried through the channel. G protein-coupled receptor (GPCR) or receptor tyrosine kinase (RTK) activation induces PLC-mediated hydrolysis of $PI(4,5)P_2$, transiently reducing the levels of $PI(4,5)P_2$ and affecting P2X function. The amino acid sequence of the proximal C-terminal regions of P2X receptors shows the presence of two clusters of basic residues forming a regulatory PIP_n binding site in most subunits.

located (Kawate et al., 2009; Hattori and Gouaux, 2012). Interestingly, the regulatory PIP_n binding site, being in close proximity to the second transmembrane domain, is located only 15 to 20 amino acids away from the gate region of the pore controlling the ion conduction properties of the channel (**Figure 2**). Since conformational changes in the gate area upon ATP binding mediate the opening of the channel as well as its desensitization (Kracun et al., 2010; Li et al., 2010; Kawate et al., 2011; Du et al., 2012; Hattori and Gouaux, 2012), it is likely that forces generated by lipid binding affect the movements of the region and alter the gating kinetics of the channel, as PIP_n level-dependent changes in channel activation and desensitization rates have been observed in most P2X subtypes.

Furthermore, the ability of P2X2 and P2X4 receptor channels to dilate into a large conductance pore is also sensitive to PIP_n levels (Fujiwara and Kubo, 2006; Bernier et al., 2012a). This activity-dependent change in permeability is believed to be driven in part by major rearrangements in the interactions between the transmembrane domains (Eickhorst et al., 2002; Chaumont and Khakh, 2008; Shinozaki et al., 2009). For P2X2, 10 residues in the transmembrane domains were shown to be involved in the transition to the high permeability state, several of which are in close proximity to the PIP_n binding site (Fisher et al., 2004; Khakh and Egan, 2005). Also arguing for a role of PIP_n in regulating the large pore formation via conformational changes are reports demonstrating that the change in permeability requires rearrangements of the cytosolic domains where the channel-lipid interaction site is found. More specifically, a study using a chimeric P2X2 engineered with a PIP_2-binding pleckstrin homology (PH) domain fused to its C-terminal tail showed that PIP_2 can tether the domain to the membrane, thereby preventing the transition of P2X2 into a dilated state (Fisher et al., 2004).

While we can speculate that PIP_n binding induces a rearrangement of the C-terminal tail of P2X channels and in this way changes its functional properties, it remains unclear how specific the interaction is with regards to the various PIP_n species present in cellular environments. P2X1, P2X3 and P2X7 seem to be strongly potentiated by $PI(4,5)P_2$, P2X2 is mainly modulated by the 3′ phosphorylated PIP_n [PI3P, $PI(3,5)P_2$], while P2X4 depends on both $PIP(4,5)P_2$ and $PI(3,4,5)P_3$ as cofactors for full activation. Parallels will be drawn between P2X receptors and other families of PIP_n-dependent ion channels in terms of protein-lipid binding characteristics. Most phospholipid-dependent channels and transporters require $PI(4,5)P_2$ however some are modulated by PI3P and $PI(3,5)P_2$, like small-conductance Ca^{2+}-activated K^+ channels, or by $PI(3,4,5)P_3$, like

CNG channels and epithelial sodium channels (Zhainazarov et al., 2004; Pochynyuk et al., 2005; Srivastava et al., 2006). For P2X receptor channels, while subtype-specific variations in the primary sequence of the regulatory PIP_n site could provide some binding specificity to PIP_n species, it is likely that the regulation is mostly directed by the relative abundance of specific PIP_n in the membrane microenvironment surrounding the channel. Unlike PH domains which require a complex protein folding and basic residues scattered over a region of hundreds of amino acids, the shorter and more linear P2X PIP_n binding site only forms a cluster of positive charges electrostatically interacting with the negative head group of the lipid, providing a lower level of specificity.

A PHYSIOLOGICAL ROLE FOR PIP_n REGULATION OF P2X

Intracellular PIP_n levels can fluctuate very rapidly within the plasma membrane. Many surface receptors are coupled to the activation of PLC isoforms, transiently lowering the levels of $PI(4,5)P_2$ via hydrolysis, while enzymes involved in PIP_n synthesis, like PI3K or the phosphatase and tensin homolog (PTEN), are tightly regulated via multiple signaling cascades. Therefore, receptor channels requiring the presence of PIP_n as essential cofactor for complete function can be regulated by enzyme-driven depletion or addition of PIP_n species. Multiple examples of PIP_n-dependent ion channel regulation through metabotropic pathways exist, including M1 muscarinic receptor-mediated inhibition of KCNQ channels, Trk- and PLC-mediated inhibition of TRPM7 or G protein-coupled inwardly-rectifying potassium (GIRK) channels (Caulfield et al., 1994; Kobrinsky et al., 2000; Runnels et al., 2002; Cho et al., 2005; Brown et al., 2007; Falkenburger et al., 2010). The first report of P2X receptor channels being regulated through receptor-initiated depletion of $PI(4,5)P_2$ came from the Logothetis group, who showed that P2X7 currents are inhibited by co-activation of PDGFR in *Xenopus* oocytes (Zhao et al., 2007). The inhibition specifically depends on PIP_2 hydrolysis as it does not occur following activation of a PLCγ-deficient mutant PDGFR. Another multireceptor crosstalk involving PIP_n was later reported natively as cationic currents carried through the P2X3 receptors in isolated dorsal root ganglion neurons are reduced after activation of the UTP-sensitive P2Y2 G_q protein-coupled receptor (Mo et al., 2013). This interaction can be occluded by exogenous introduction of a PIP_2 analog as well as by pharmacologically uncoupling the P2Y2 receptor from PLC activation, indicating that P2X3 inhibition by P2Y2 directly relies on $PI(4,5)P_2$ hydrolysis. A third crosstalk was recently uncovered in microglia, where P2X4 receptor channels are functionally inhibited by co-activation of the G_q-coupled P2Y6 receptor also upregulated in neuropathic pain conditions (Bernier et al., 2013). UDP activation of P2Y6 leads to a PLC-dependent decrease in P2X4-mediated currents and calcium entry in both resting and LPS-activated microglia. The dilation of P2X4 into a large conductance pore was also inhibited by P2Y6 activation, all of these effects being highly similar to that of pharmacological depletion of PIP_n.

These recent results suggest that the requirement of PIP_n as a cofactor for P2X receptor is a critical regulatory mechanism involved in signaling crosstalks under physiological and pathological conditions. Many aspects of this post-translational modulatory mechanism remain to be investigated to understand its physiological significance. For example, it is still unclear if and how this modulatory pathway can be specific to one metabotropic receptor. Can P2X receptors be inhibited by any signaling event inducing PLC hydrolysis of $PI(4,5)P_2$? Interestingly, stimulating G_q-coupled P2Y1 ADP receptors or M3 muscarinic receptors failed to inhibit P2X4 responses in transfected HEK293 cells while stimulating P2Y6 receptors did, indicating some degree of specificity to the P2Y6-P2X4 crosstalk (Bernier et al., 2013). It is probable that subcellular localization plays a key role in controlling which receptors interact. $PI(4,5)P_2$ hydrolysis by PLC likely induces only a local depletion in $PI(4,5)P_2$ levels and could therefore preferentially affect adjacent P2X receptors. It would be interesting to investigate the role of lipid rafts in controlling the proximity of different receptors, considering that plasma membrane microenvironments have been reported to affect P2X physiology (Vacca et al., 2004; Vial and Evans, 2005; Allsopp et al., 2010). Other factors such as GPCR desensitization might also come into play given that PIP_n levels have a fast turnover rate. For example, P2Y6 displays a much slower desensitization pattern than other P2Y receptors and might induce a longer, more significant decrease in membrane $PI(4,5)P_2$ level (Robaye et al., 1997).

As an increasing amount of data highlights the role of PIP_n in P2X receptor activity and multireceptor crosstalks, such a post-translational regulatory mechanism might provide an innovative pharmacological target to treat chronic pain conditions or immune diseases where P2X receptors are known to be involved.

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Introduction and perspective, historical note

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P2 nucleotide receptors were proposed to consist of two subfamilies based on pharmacology in 1985, named P2X and P2Y receptors. Later, this was confirmed following cloning of the receptors for nucleotides and studies of transduction mechanisms in the early 1990s. P2X receptors are ion channels and seven subtypes are recognized that form trimeric homomultimers or heteromultimers. P2X receptors are involved in neuromuscular and synaptic neurotransmission and neuromodulation. They are also expressed on many types of non-neuronal cells to mediate smooth muscle contraction, secretion, and immune modulation. The emphasis in this review will be on the pathophysiology of P2X receptors and therapeutic potential of P2X receptor agonists and antagonists for neurodegenerative and inflammatory disorders, visceral and neuropathic pain, irritable bowel syndrome, diabetes, kidney failure, bladder incontinence and cancer, as well as disorders if the special senses, airways, skin, cardiovascular, and musculoskeletal systems.

Keywords: brain, skin, lung, gut, bladder, cancer, pain, inflammation

INTRODUCTION

Division of receptors for purines into P1 (adenosine) and P2 (ATP/ADP) families was proposed in 1978 (Burnstock, 1978). In 1985, P2 receptors were divided into two subtypes, P2X and P2Y receptors, on the basis of pharmacology (Burnstock and Kennedy, 1985). In the early 1990's, P2 receptors for purines and pyrimidines were cloned and characterized and second messenger mechanisms determined (see Abbracchio and Burnstock, 1994; Ralevic and Burnstock, 1998). $P2Y_1$ (Webb et al., 1993) and $P2Y_2$ (Lustig et al., 1993) G protein-coupled receptors were described initially and soon after P2X1 and P2X2 ion channel receptors were reported (Brake et al., 1994; Valera et al., 1994). Seven P2X receptor subunits have been identified. P2X receptors have been cloned from many eukaryotic species, including mammals, fish, parasitic trematode worms, amoeba, slime mould, and green algae (see Fountain and Burnstock, 2009; Burnstock and Verkhratsky, 2012a). The physiology and pathophysiology of P2X receptors in diseases of the special senses, urinary tract, gastrointestinal tract, pancreas, skin, and musculoskeletal system, as well as in cancer and inflammatory disorders will be discussed.

It was assumed for a long time that the main source of ATP acting on purinoceptors was damaged or dying cells. It is now clear, however, that ATP is released, without causing damage, from many cell types, including endothelial and urothelial cells, macrophages, astrocytes, odontoblasts and osteoblasts, in response to gentle mechanical disturbance, hypoxia, and some agents (Bodin and Burnstock, 2001; Lazarowski et al., 2011; Lazarowski, 2012). Release of ATP initiates purinergic mechanosensory transduction that is involved in bone remodeling (Orriss et al., 2010) and visceral pain via P2X3 receptors on nociceptive sensory nerves (Burnstock, 1999, 2007b). The mechanism of ATP transport from cells appears to be a combination of vesicular exocytosis and connexin and/or pannexin 1 hemichannels (see Lazarowski, 2012). Ectoenzymes are involved in the breakdown of released ATP into ADP, AMP, adenosine, inosine and hypoxanthine (see Zimmermann, 2006; Yegutkin, 2008). These enzymes include NTPDases, pyrophosphatase/phosphodiesterases, alkaline phosphatases, 5′- nucleotidase and monoamine oxidase.

P2X RECEPTOR SUBTYPES

Seven P2X subunits have been cloned and characterized. The P2X1 to P2X6 receptors are 379–472 amino acids long, while the P2X7 receptor is 595 amino acids long, due to the increased length of the COOH terminus. The molecular physiology of P2X receptors has been thoroughly reviewed (see North, 2002). Each subunit possesses two hydrophobic, transmembrane spanning regions that span the cell plasma membrane. A seminal study has been published describing the crystal structure of the P2X4 receptor (Gonzales et al., 2009; Kawate et al., 2009). When P2X7 receptors are occupied by ATP, cation channels are activated, but in addition with high concentrations of ATP large pores are formed which lead to uptake of Ca^{2+} leading to apoptotic cell death.

The seven P2X subtypes combine as trimers (Nicke et al., 1998), which form functional homo- and heteromultimers (see Burnstock, 2007a). P2X6 receptors do not form a homomultimer, while P2X7 receptors do not form a heteromultimer. P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, and P2X4/6 heteromultimers have been identified.

DISTRIBUTION OF P2X RECEPTORS

Detailed analyses of the distribution of P2X receptors on nerves and non-neuronal cells have been published (Burnstock and Knight, 2004; Burnstock, 2007b; see **Table 1**).

PHYSIOLOGY OF P2X RECEPTORS

ATP released as a cotransmitter with noradrenaline (NA) from sympathetic nerves was shown to act mainly via P2X1 receptors

Table 1 | Principal P2X receptors expressed by excitable tissues and non-neuronal cells (Compiled from Burnstock, 2007b).

NEURONAL	
Sympathetic neurons	P2X1-7
Parasympathetic neurons	P2X2, P2X3, P2X4, P2X5
Sensory neurons	P2X1-7, predominantly P2X3 and P2X2/3
Enteric neurons	P2X2, P2X3, P2X4, P2X7
Central nervous system	P2X2, P2X4 and P2X6 (perhaps heteromultimers) predominate, (P2X7?)
Retinal neurons	P2X2, P2X3, P2X4, P2X5, P2X7
MUSCLE CELLS	
Smooth muscle	P2X1-7, predominantly P2X1
Skeletal muscle	
-Developing	P2X2, P2X5, P2X6
-Adult	P2X1-7
Cardiac muscle	P2X1, P2X3, P2X4, P2X5, P2X6
NON-NEURONAL	
Osteoblasts	P2X1, P2X2, P2X5, P2X7
Osteoclasts	P2X1, P2X2, P2X4, P2X7
Cartilage	P2X2
Keratinocytes	P2X2, P2X3, P2X5, P2X7
Fibroblasts	P2X7
Adipocytes	P2X1
Epithelial cells (lung, kidney, trachea, uterus, cornea)	P2X4, P2X5, P2X6, P2X7
Astrocytes	P2X1-7
Oligodendrocytes	P2X1
Microglia	P2X4, P2X7
Müller cells	P2X3, P2X4, P2X5, P2X7
Enteric glial cells	P2X7
Sperm	P2X2, P2X7
Endothelial cells	P2X1, P2X2, P2X3, predominately P2X4
Erythrocytes	P2X2, P2X4, P2X7
Platelets	P2X1
Immune cells (thymocytes, macrophages, neutrophils, eosinophils, lymphocytes, mast cells, dendritic cells)	P2X4 and predominately P2X7, but some P2X1, P2X2, P2X5
Exocrine secretary cells	P2X1, P2X4, P2X7
Endocrine secretory cells (pituitary, pancreas, adrenal, thyroid, testis)	P2X1-7, predominately P2X2/6
Cholangiocytes	P2X2, P2X3, P2X4, P2X6
Interstitial cells of Cajal	P2X2, P2X5
Kupffer cells	P2X1, P2X4, P2X7
Special senses	
Inner ear	P2X1, P2X2, P2X3, P2X7
Eye	P2X2, P2X7
Tongue	P2X2, P2X3
Olfactory organ	P2X2, P2X4
Cochlea hair cells	P2X1, P2X2, P2X7

on both visceral and vascular smooth muscle to produce contractions (see Burnstock, 1990, 2009b) and ATP released together with acetylcholine (ACh) from parasympathetic nerves acts on P2X1 receptors in the urinary bladder (Burnstock et al., 1978; Burnstock, 2013). ACh acting via nicotinic receptors was established early as the neurotransmitter released from motor nerves supplying adult skeletal muscle, but later it was shown that during postnatal development of the neuromuscular junction, ATP is released as a cotransmitter together with ACh to act on P2X receptors (see Henning, 1997). An important advance was made when purinergic synaptic transmission between nerves was described in both the coeliac ganglion (Evans et al., 1992; Silinsky et al., 1992) and medial habenula in the brain (Edwards et al., 1992).

P2X receptors have also been shown to act presynaptically, for example P2X3 receptors on primary afferent sensory nerve endings in the dorsal spinal cord to enhance the release of glutamate (Gu and MacDermott, 1997) and on P2X receptors on sympathetic nerve varicosities in the vas deferens to enhance the release of NA (Queiroz et al., 2003).

P2X3 homomultimer and P2X2/3 heteromultimer receptors were identified on sensory neurons and nerve endings (Chen et al., 1995; Lewis et al., 1995) mediating both physiological reflex responses as well as nociception (see Burnstock and Verkhratsky, 2012b).

There is a wide distribution of P2X2, P2X3, P2X2/3, P2X4, and P2X7 receptors in the myenteric and submucous plexuses and on intrinsic and extrinsic sensory nerves of the enteric nervous system (see Burnstock, 2008b and **Figure 1**). These receptors are involved in reflex activities, including modulation of peristaltic reflexes (Bian et al., 2003; Wynn et al., 2003).

Expression of most P2X receptor subtypes have been localized in different regions of the central nervous system (CNS). Sensory nerves in the brain stem expressing P2X3 receptors and P2X2, P2X4, and P2X6 receptors, mostly in the form of heteromultimers, appear to be involved in both neurotransmission and neuromodulation (see Burnstock, 2007b; Burnstock et al., 2011b; Burnstock and Verkhratsky, 2012b; Lalo et al., 2012). The role of P2X7 receptors in the CNS is controversial. Behavioral studies have implicated roles for P2X receptors in memory and learning, sleep, locomotion and feeding (see Burnstock et al., 2011a).

In the heart, P2X1/3/4/5/6 receptor mRNA and protein are expressed in ventricles and P2X1-6 in atria (Hansen et al., 1999) leading to increase in contractility of cardiac myocytes (Shen et al., 2007). P2X receptor subtypes are widely expressed in different sites in the kidney (see Unwin et al., 2003). Preglomerular arterioles express P2X1 receptors, while glomerular mesangial cells express P2X4, P2X5, and P2X7 receptors, and podocytes express P2X1 and P2X7 receptors. Different regions of the kidney tubule are immunoreactive for P2X receptors: P2X1, P2X4, P2X5, and P2X6 on proximal tubules, P2X4 and P2X6 on distal tubules and P2X1, P2X4/6, and P2X5 on collecting ducts (Bailey and Shirley, 2009). In the collecting ducts P2X4 and P2X4/6 receptors are involved in control of sodium transport (Wildman et al., 2009).

Uptake of organic cations is mediated by P2X1 and P2X7 receptors in canine erythrocytes (Stevenson et al., 2009). The role

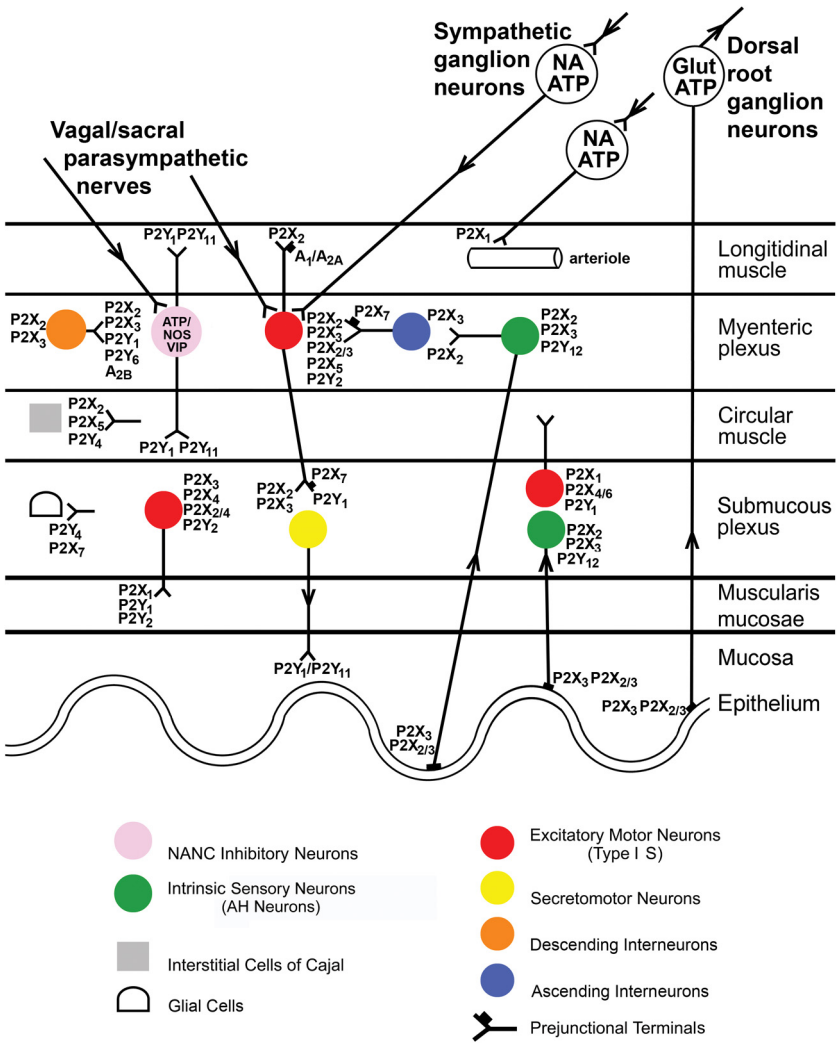

FIGURE 1 | Distribution of P2X receptor subtypes in the gut. Extrinsic vagal and sacral parasympathetic nerves connect with NANC inhibitory neurons in the myenteric plexus expressing P2X2 and P2X3 receptors, as well as with cholinergic motor neurons; these neurons are also activated by descending interneurons. Extrinsic sympathetic nerves modulate motility via excitatory motor neurons and constrict blood vessels in the gut via P2X1 receptors. Extrinsic sensory nerves arise from cell bodies in dorsal root ganglia and with subepithelial terminals expressing P2X3 and P2X2/3 receptors and mediate nociception. Intrinsic sensory neurons in both myenteric and submucosal plexuses express P2X2 and P2X3 receptors; they connect with motor pathways involved in peristalsis. Excitatory motor neurons express P2X2, P2X3, P2X2/3, and P2X5 receptors and connect with both interneurons and secretomotor neurons. Interneurons express P2X2 and P2X3 receptors. Enteric glial cells express P2X7 receptors, while interstitial cells of Cajal express P2X2 and P2X5 receptors. P2X7 receptors appear to act as prejunctional modulators of both motor and interneurons. [Modified from Burnstock (2008c), with permission from the BMJ Publishing Group Ltd].

of the P2X1 receptor expressed by platelets is unclear, although in P2X1 knockout mice there is a decreased level of thrombus formation and increased bleeding times (Nurden, 2007). P2X1 receptors have also been claimed to play a role in sensing bacteria (Kälvegren et al., 2010).

The P2X7 receptor is involved in immunomodulation responding to extracellular ATP at sites of inflammation and tissue damage (see Di Virgilio, 2013). P2X1 receptors promote neutrophil chemotaxis and play a significant role in host defense (Lecut et al., 2009). P2X7 receptors mediate cytokine release and chemokine expression via P2X1 and P2X3 receptors in mouse mast cells (Bulanova et al., 2009). P2X7 receptors in human dendritic cells mediate the release of tissue factor-bearing microparticles (Baroni et al., 2007).

Keratinocyte turnover in skin epidermis involves P2X receptors while $P2Y_1$ and $P2Y_2$ receptors in basal and parabasal layers mediate cell proliferation, P2X5 receptors in the granular layer mediate cell differentiation and P2X7 receptors at the stratum granulosum/stratum corneum border mediate apoptotic cell death (Greig et al., 2003c; Burnstock et al., 2012b). In the endocrine system, the posterior pituitary expresses protein for P2X2 and P2X6 receptors and P2X2, P2X3, P2X4, and P2X7 receptor channels are present on anterior pituitary cells and mediate hormone secretion (Stojilkovic et al., 2010). P2X7

receptors are expressed on osteoblasts, enhancing differentiation and bone formation and also on osteoclasts mediating apoptosis (see Orriss et al., 2010, 2012).

P2X receptors in the special senses mediate a variety of different functions (see Housley et al., 2009; Burnstock and Verkhratsky, 2012b). Nasal epithelium expresses P2X2, P2X5, and P2X7 receptors (Gayle and Burnstock, 2005), P2X1, P2X2, P2X3, and P2X2/3 are prominent receptors in the tongue (Bo et al., 1999) mediating both taste sensation and pain (Rong et al., 2000), and P2X receptors have multiple roles in the eye (see Pintor, 2006) and inner ear (see Housley and Gale, 2010).

PATHOPHYSIOLOGY OF P2X RECEPTORS

The involvement of P2X receptors is being investigated increasingly in relation to a wide variety of diseases (see Burnstock, 2006a,b, 2007b, 2008a).

DISEASES OF SPECIAL SENSES

P2X receptors are expressed by various structures in the eye and novel therapeutic strategies are being developed for glaucoma, dry eye, and retinal detachment (Pintor et al., 2003). P2X7 receptors are increased in retinal microvessels early in experimental diabetes. This suggests that purinergic vasotoxicity may play a role in microvascular cell death, characteristic of diabetic retinopathy (Sugiyama et al., 2004).

P2X receptors have been described in the vestibular system (Xiang et al., 1999), in particular on the endolymphatic surface of the cochlear endothelium, an area associated with sound transduction. It has been suggested that ATP may regulate fluid homeostasis, cochlear blood flow, hearing sensitivity and development, and therefore may be useful for the treatment of Ménière's disease, tinnitus, and sensorineural deafness (Housley, 2000). There is upregulation of P2X2 receptors in the cochlear occurs during sustained loud noise. P2X2 receptor expression is also increased in spiral ganglion neurons (Wang et al., 2003).

Purinergic receptors have been described in the nasal mucosa, including the expression of P2X3 receptors on olfactory neurons (Gayle and Burnstock, 2005). The induction of heat-shock proteins by noxious odor damage is prevented by the administration *in vivo* of P2 receptor antagonists (Hegg and Lucero, 2006).

DISEASES OF THE KIDNEY AND URINARY TRACT

Purinoceptors are expressed in different regions of the nephron, the glomerulus, and renal vascular system in the kidney and different subtypes are involved in the regulation of renin secretion, glomerular filtration and the transport of water, ions, nutrients and toxins (Unwin et al., 2003). Autocrine purinergic signaling enhances cyst expansion and accelerates progression of polycystic kidney disease (Schwiebert et al., 2002). P2X7 receptor expression is increased in cystic tissue from a rat model of autosomal dominant polycystic kidney disease (Turner et al., 2004). Increased glomerular expression of P2X7 receptors has been reported in rat models of glomerular injury due to diabetes and hypertension (Vonend et al., 2004). Human and experimental glomerulonephritis also showed increase in P2X7 receptor expression in the glomerulus (Turner et al., 2007).

P2X3 receptors are expressed by the suburothelial sensory nerves, and both the human and guinea-pig ureter urothelial cells release ATP in a pressure-dependent fashion when the ureter is distended (Knight et al., 2002; Calvert et al., 2008). P2X3 antagonists may be useful to alleviate renal colic (Rong and Burnstock, 2004).

Atropine will block at least 95% of parasympathetic nerve-mediated contraction in the healthy human bladder, showing neurotransmission that is predominantly cholinergic, although P2X1 receptors are present on the smooth muscle (Burnstock, 2001a). However, the purinergic component of parasympathetic cotransmission is increased in pathological conditions (see Burnstock, 2013). It is increased to 40% in interstitial cystitis, outflow obstruction, idiopathic detrusor instability and most types of neurogenic bladder. Release of ATP from distended bladder urothelial cells in patients with interstitial cystitis is significantly greater than from healthy cells (Tempest et al., 2004) and P2X1 receptor subtype expression is increased in obstructed bladder (Boselli et al., 2001).

Purinergic signaling also plays a role in afferent sensation from the bladder, involved in both the micturition reflex and pain. Release of ATP from urothelial cells occurs during distension (Vlaskovska et al., 2001) and it acts on P2X3 receptors on suburothelial sensory nerve endings (Cockayne et al., 2000). P2X3 receptors are therefore a potential target for pharmacological manipulation in the treatment of both pain and detrusor instability. In idiopathic detrusor instability, there is abnormal purinergic transmission in the bladder (O'Reilly et al., 2002). Voiding dysfunction involves P2X3 receptors in conscious chronic spinal cord injured rats, suggesting that P2X3 antagonists might also be useful for the treatment of neurogenic bladder (Lu et al., 2002). Drugs that alter ATP release or breakdown might also be considered as therapeutic targets (Chess-Williams, 2004). A recent review about purinergic signaling in the lower urinary tract is available (Burnstock, 2013).

CARDIOVASCULAR DISEASES

There is up-regulation of P2X1 receptor mRNA in the hearts of rats with congestive heart failure and an increase in expression of P2X1 receptors in the atria of patients suffering from dilated cardiomyopathy. P2X4 receptor mRNA was reported to be upregulated in ligation-induced heart failure and was claimed to have a beneficial life-prolonging role (Musa et al., 2009).

ATP, released as the purinergic component of sympathetic cotransmission, is increased in spontaneously hypertensive rats mediating vasoconstriction via P2X1 receptors (see Ralevic and Burnstock, 1998). There is upregulation of placental P2X4 receptors in mild preeclampsia (Roberts et al., 2007).

DISORDERS OF THE GUT

P2X receptors play major roles in diseases of the gut (see Burnstock, 2008a,b). P2X7 receptors, that mediate cytokine production, may play a role in the response of enteric glia to inflammation (Vanderwinden et al., 2003). Enhancement of P2X3 receptor-mediated purinergic signaling in an animal model of colitis has been described (Wynn et al., 2004). P2X3 receptor expression is also increased in the enteric plexuses in human irritable bowel syndrome (IBS), suggesting a role in dysmotility and pain initiation (Yiangou et al., 2001; Galligan, 2004; Shinoda

et al., 2009). Visceral hyperalgesia induced in a rat model of IBS was associated with potentiation of ATP-evoked responses and an enhanced expression of P2X3 receptors in sensory neurons in the colon (Xu et al., 2008). In aganglionic bowel from Hirschsprung's disease patients, P2X3 immunohistochemistry was demonstrated, suggesting that the sensory nerves may be involved (Facer et al., 2001).

Both intrinsic sensory neurons in the submucous plexus of the gut and extrinsic sensory nerves with cell bodies in the dorsal root ganglia (DRG), show positive immunoreactivity for P2X3 receptors (Xiang and Burnstock, 2004). It has been suggested that during moderate distension, low threshold intrinsic enteric sensory fibers are activated, via P2X3 receptors, by ATP released from mucosal epithelial cells resulting in reflexes concerned with propulsion of material down the gut (Burnstock, 2001b). Peristalsis is impaired in the small intestine of mice lacking the P2X3 receptor subunit, which supports this view (Bian et al., 2003). During substantial (colic) distension associated with nociception, higher threshold extrinsic sensory fibers may be activated by ATP released from the mucosal epithelial cells to pass messages through the DRG to pain centers in the CNS (Wynn et al., 2003, 2004). Sensitization of P2X3 receptors on vagal and spinal afferents in the stomach have been claimed to contribute to dyspeptic symptoms and to the development of visceral hyperalgesia (Dang et al., 2005). A recent review describing P2X receptors in the gut is available (Burnstock, 2012).

DISEASES OF THE REPRODUCTIVE SYSTEM

ATP induces a significant increase in sperm fertilizing potential and this has led to the use of ATP for treatment of spermatozoa during *in vitro* fertilization (Rossato et al., 1999). P2X1 receptor knockout mice appear normal, but fail to breed and this is associated with loss of the purinergic component of sympathetic cotransmission in the vas deferens (Dunn, 2000; Mulryan et al., 2000). P2X receptor subtypes are expressed at different stages during spermatogenesis in the adult rat testis, which may be novel targets for both fertility and contraception (Glass et al., 2001).

Low concentrations of ATP stimulate changes in transepithelial conductance in the human uterine cervix, the first phase mediated by $P2Y_2$ receptors and the second phase by P2X4 receptors (Gorodeski, 2002).

DIABETES

There is an enhancement of P2X7 receptor-induced pore formation and apoptosis in early diabetes in the retinal microvasculature (Sugiyama et al., 2004). P2X7 receptors are located on glucagon-containing α cells in pancreatic islets (Coutinho-Silva et al., 2001). In streptozotocin-diabetic rats P2X7 receptor-labeled α cells migrate centrally to take the place of the insulin-containing β cells, although the functional significance of this is unknown (Coutinho-Silva et al., 2003). Central neuropathic complications occur in diabetic neuropathy, including decreased cognitive performance and it has been shown that synaptic ATP signaling is depressed in streptozotocin-induced diabetic rats (Duarte et al., 2007). The density of P2X3/5/7 receptors was decreased in the hippocampal nerve terminals of diabetic rats. A recent review of the literature concerned with purinergic signaling in diabetes is available (Burnstock and Novak, 2013).

DISEASES OF THE AIRWAYS

Lung epithelial cells express P2X4 receptors that are involved in regulation of ciliary beat, manipulation of which may be of therapeutic benefit for cystic fibrosis (Zsembery et al., 2003). Vagal afferent purinergic signaling may be involved in the hyperactivity associated with asthma and chronic obstructive pulmonary disease (Adriaensen and Timmermans, 2004). Erythromycin, used for the treatment of upper and lower respiratory tract infections, blocks P2X receptor-mediated Ca^{2+} influx and may be involved in its anti-secretory effects in the treatment of chronic respiratory tract infections (Zhao et al., 2000).

A network of respiratory neurons in the ventrolateral medulla (VLM) is responsible for the generation of the respiratory rhythm and also functions as a chemoreceptive area mediating the ventilating response to hypercapnia. ATP acting via P2X2 receptors expressed on VLM neurons is involved in these functions (Gourine et al., 2003). P2 receptor synaptic signaling in respiratory motor control has been implicated by the multiple physiological effects of ATP in hypoglossal activity mediated by P2X2, P2X4, and P2X6 receptors in the nucleus ambiguous and the hypoglossal nucleus (Collo et al., 1996). ATP injected into the caudal nucleus of the solitary tract of awake rats produced respiratory responses (Antunes et al., 2005).

P2X7 receptors are expressed in alveolar macrophages, which play a pivotal role in the development of chronic lung inflammatory reactions, such as idiopathic pulmonary fibrosis, silicosis, asbestosis, hypersensitivity pneumonitis, sarcoidosis and mycobacterium tuberculosis (Lemaire and Leduc, 2004). Stimulation of P2X7 receptors results in activation of the proinflammatory interleukin (IL)-1 to IL-5 cytokine cascade and the formation of multinucleated giant cells, a hallmark of granulomatous reactions. A recent review describing purinergic signaling in the airways in health and disease has been published recently (Burnstock et al., 2012a).

DISEASES OF SKIN

An increase of P2X3 and P2X2/3 nociceptive receptors on sensory nerve endings in inflamed skin has been reported and antagonists are being explored as analgesics (Hamilton et al., 2001). A pathogenic role for keratinocyte-derived ATP in irritant dermatitis has been suggested (Mizumoto et al., 2003). There are changes in expression of purinergic receptors in the regenerating epidermis in wound healing (Greig et al., 2003a). Acceleration of skin barrier repair and prevention of epidermal hyperplasia induced by skin barrier disruption by P2X receptor antagonists has been reported (Denda et al., 2002). A review about purinergic signaling in skin in health and disease is available (Burnstock et al., 2012b).

IMMUNE SYSTEM AND INFLAMMATION

P2X7 receptors expressed by inflammatory and immune cells play a pivotal role in inflammation and immunomodulation (Di Virgilio, 2007, 2013). The treatment of neurogenic inflammation, rheumatoid arthritis, and periodontitis by purinergic compounds is being explored. P2X7 receptor-mediated apoptosis in macrophages results in killing of the mycobacteria

contained within them, unlike the macrophage apoptosis produced by other agents (Lammas et al., 1997). There is accumulation of macrophages expressing P2X4 receptors in rat CNS lesions during experimental autoimmune encephalomyelitis (Guo and Schluesener, 2005). It has been suggested that ATP may be mechanistically involved in human allergic/asthmatic reactions (Schulman et al., 1999). P2X7 receptors are expressed by alveolar macrophages, which, when activated, trigger pro-inflammatory activation of IL1-6 cytokines and granulomatous reactions (Lemaire and Leduc, 2004). A lower concentration of ATP activation of P2X7 receptors can result in cell proliferation (Di Virgilio et al., 2009). The functional expression of P2X7 receptors on B lymphocytes may be related to the severity of B-cell chronic lymphocytic leukaemia (Adinolfi et al., 2002).

ATP induces cell death in $CD4^+/CD8^+$ double-positive thymocytes during the acute phase of *Trypanosoma cruzi* infection in Chaga's disease and may play a role in the thymus atrophy that occurs in Chaga's disease (Mantuano-Barradas et al., 2003). *Schistosoma mansoni*, a parasitic blood fluke, also produces thymic atrophy, and the P2X receptor cloned from *S. mansoni* provided an example of a non-vertebrate ATP-gated ion channel and suggests a drug target for the treatment of schistosomiasis (Agboh et al., 2004).

CANCER

The use of adenine nucleotides as anticancer agents was first described by Rapaport (1983). ATP, injected intraperitoneally into tumor-bearing mice, resulted in anticancer activity against several fast-growing aggressive carcinomas (Agteresch et al., 2003). Evidence has been presented that extracellular ATP inhibits the growth of a variety of human tumors, including prostate, bladder, breast, colon, liver, ovarian, colorectal, oesophageal and melanoma cancer cells, partly via P2X7 receptors mediating apoptotic cancer cell death (Abraham et al., 2003; White and Burnstock, 2006). Studies have been carried out to determine the P2 receptor subtypes that contribute to ATP suppression of malignant melanomas (White et al., 2005a,b), basal and squamous cell tumors (Greig et al., 2003b) and prostate and bladder cancers (Calvert et al., 2004; Shabbir et al., 2008a,b). P2X5 receptors mediate cell differentiation, which in effect is antiproliferative and apoptotic cell death is mediated by P2X7 receptors. A review has been published recently entitled "Purinergic signaling and cancer" (Burnstock and Di Virgilio, 2013).

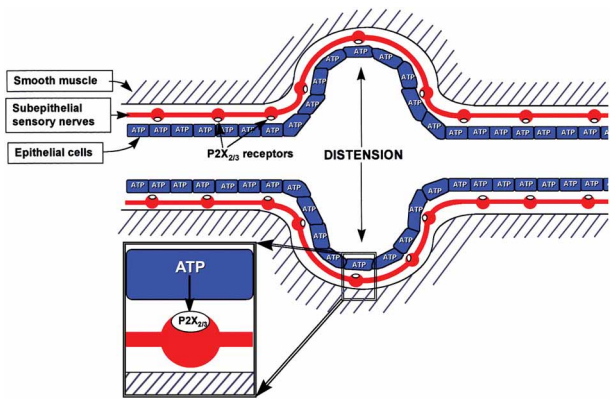

FIGURE 2 | Schematic representation of hypothesis for purinergic mechanosensory transduction in tubes (e.g., ureter, vagina, salivary and bile ducts, gut) and sacs (e.g., urinary and gall bladders, lung). It is proposed that distension leads to release of ATP from epithelium lining the tube or sac, which then acts on $P2X_3$ and/or $P2X_{2/3}$ receptors on subepithelial sensory nerves to convey sensory/nociceptive information to the CNS. [Reproduced from Burnstock (1999), with permission from Wiley].

MUSCULOSKELETAL DISEASES

Purinergic signaling is involved in bone development and remodeling (Hoebertz et al., 2003; Burnstock and Arnett, 2006; Orriss et al., 2010). Osteoclasts, osteocytes, osteoblasts and chondrocytes all express P2X receptors. Regulatory roles in bone formation and resorption by P2X7 receptors were revealed by studies of P2X7 receptor knockout mice. The purinoceptors on bone and cartilage represent potential targets for the development of novel therapeutics to inhibit bone resorption in musculoskeletal diseases, including rheumatoid arthritis, osteoporosis, tumor-induced osteolysis, and periodontitis (Komarova et al., 2001). The P2X7 receptor antagonist, oxidized ATP, reduced inflammatory pain in arthritic rats (Dell'Antonio et al., 2002).

Lymphoblastoid cells from Duchenne muscular dystrophy patients are sensitive to stimulation by extracellular ATP (Ferrari et al., 1994). Evidence has been presented for a role for P2X receptor-mediated signaling in muscle regeneration using the *mdx* mouse model of muscular dystrophy, which raised the possibility of new therapeutic strategies for the treatment of muscle disease (Ryten et al., 2004). A recent review about purinergic signaling in the musculoskeletal system is available (Burnstock et al., 2013).

DISORDERS OF THE CENTRAL NERVOUS SYSTEM

Recent reviews have focused on purinergic signaling in disorders of the CNS (Burnstock, 2008a; Burnstock et al., 2011a; Franke et al., 2012; Volonté and Burnstock, 2012).

Microglia and macrophages expressing P2X4 receptors accumulate following experimental traumatic brain injury and spinal cord injury. Activated microglia also show increase in P2X7 receptor expression, which initiate microglial proliferation and death. Lesions in the cerebellum result in upregulation of P2X1 and P2X2 receptors in precerebellar nuclei, and there is increased expression of several subtypes of P2X receptors after stab wound injury in the nucleus accumbens (Franke et al., 2006). P2X7 receptors are upregulated following ischaemia on neurons and glial cells in rat cerebral cortex, and become supersensitive in cerebrocortical cell cultures (Cavaliere et al., 2003). Ischaemic cell death was prevented by P2 receptor antagonists.

Involvement of P2X receptors in neurodegenerative diseases such as Parkinson's, Alzheimer's, Huntington's, amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) has been described (see Burnstock, 2008a). In the pathogenesis of Parkinson's disease, release of ATP from disrupted cells may cause cell death in neighboring cells expressing P2X7 receptors, leading to a necrotic volume increase. Upregulation of P2X7 receptors in human Alzheimer's diseased brains and in animal models has been reported (Parvathenani et al., 2003; McLarnon et al.,

2006) and stimulation of P2X7 receptors on human microglia and macrophages increased the degenerative lesions observed in Alzheimer's disease. In two different transgenic models of Huntington's disease, changes in P2X receptor-mediated neurotransmission in cortico-striatal projections were observed (Diez-Zaera et al., 2007). Both P2X4 and P2X7 receptors have been implicated in the transgenic superoxide dismutase 1 (SOD1) mouse model of ALS (Andries et al., 2007; Apolloni et al., 2013). In MS lesions in brain tissue, P2X7 receptors were detected on reactive astrocytes (Narcisse et al., 2005). Lesional accumulation of P2X receptors on macrophages in the CNS of the rat model of MS, experimental autoimmune encephalomyelitis, has been reported (Guo and Schluesener, 2005). P2X7 expression is elevated in astrocytes in MS patients (Narcisse et al., 2005).

P2X7 receptors on microglia, the immune cells in the CNS, are activated by purines to release inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-α (Di Virgilio, 2007). P2X7 receptors have been implicated in the formation of multinucleated giant macrophage-derived cells, a feature of chronic inflammatory reactions (Lemaire et al., 2006). Prion infection has been claimed to be associated with hypersensitivity of P2X7 receptors in microglia (Takenouchi et al., 2007). Microglial cell activation by pro-inflammatory bacterial lipopolysaccharide leads to a transient increase in ivermectin-sensitive P2X4 receptor currents (Raouf et al., 2007). Activation of astrocytes via P2X7 receptors increases chemokine monocyte chemoattractant protein-1 expression and it was suggested that this may be important for communication with haematopoietic inflammatory cells (Panenka et al., 2001).

Generalized motor seizures can be evoked by microinjection of ATP analogs into the prepiriform cortex (Knutsen and Murray, 1997). The prepiriform cortex expresses P2X2, P2X4, and P2X6 receptors and it was suggested that P2X receptor antagonists may have potential as neuroleptic agents. In chronic epileptic rats, the hippocampus showed abnormal responses to ATP, associated with increased expression of P2X7 receptors, which were upregulated in rats with chronic pilocarpine-induced epilepsy and may be involved in the pathophysiology of temporal lobe epilepsy. Enhanced immunoreactivity of the P2X7 receptor was observed in microglia from rat brain following kainate-provoked seizures (Rappold et al., 2006). A decrease of presynaptic P2X receptors in the hippocampus of rats that have suffered a convulsive period has been shown (Oses, 2006). Glutamate release from astrocytes induced by ATP has been described in epileptogenesis (Tian et al., 2005).

The P2X7 receptor gene has been implicated in both major depressive illness (Lucae et al., 2006) and bipolar affective disorders (Barden et al., 2006). In schizophrenia, the involvement of ATP receptors has been implicated in relation to reports that the antipsychotic drugs haloperidol, chlorpromazine and fluspirilene, inhibit ATP-evoked responses mediated by P2X receptors (Inoue et al., 1996). It has been suggested that ATP may have a facilitating role for dopaminergic transmission and that some antipsychotic drugs express their therapeutic effects by suppression of dopaminergic hyperactivity through inhibition of P2X receptor-mediated effects. Ethanol is probably the oldest and most widely used psychoactive drug. The cellular mechanisms underlying its actions are not well-understood, but some insights in relation to purinergic P2 receptor signaling have emerged in recent years (Davies et al., 2005). P2X receptor-mediated responses of DRG neurons are inhibited by ethanol by an allosteric mechanism. For P2X4 receptors, ethanol inhibition is altered by mutation of histidine 241. Ethanol differentially affects ATP-gated P2X3 and P2X4 receptor subtypes expressed by *Xenopus* oocytes.

Table 2 | Agonists and antagonists for the different P2X receptor subtypes.

Receptor Subtype	Agonists	Antagonists
P2X1	BzATP > ATP = 2-MeSATP = α,β-meATP = L-β,γ-meATP (rapid desensitization); PAPET-ATP	NF449 > IP_5I > TNP-ATP > RO 0437626 > NF279, NF023, RO1, MRS2159
P2X2	ATP ≥ ATPγS ≥ 2-MeSATP >> α,β-meATP (pH + zinc sensitive); β,γ-CF_2ATP	PSB-1011 > RB2, isoPPADS > PPADS > Suramin, NF770, NF778, Aminoglycoside
P2X3	2-MeSATP ≥ ATP ≥ Ap_4A ≥ α,β-meATP (rapid desensitization); PAPET-ATP; BzATP	TNP-ATP, isoPPADS > A317491 > NF110 > PPADS, Ip_5I, phenol red, RO4, RN-1838, Spinorphin, AF353
P2X4	ATP >> α,β-meATP >> CTP, 2-MeSATP Ivermectin potentiation	5-BDBD >> TNP-ATP, PPADS > BBG, Paroxetine, phenolphthalein, CO donor (CORM 2)
P2X5	ATP = 2-MeSATP = ATPγS >> α,β-meATP > AP_4A	BBG > PPADS, Suramin
P2X6	- (only functions as a heteromultimer)	–
P2X7	BzATP > ATP ≥ 2-MeSATP >> α,β-meATP	KN62, BBG, KN04, MRS2427, O-ATP, RN-6189, AZ10606120, A740003, A-438079, A-804598, GSK-1370319, Compound 31 (GSK), AZD-9056, CE-224,535

PAIN

There are reviews that have addressed this topic (see, for example, Burnstock, 2009c,d; Jarvis, 2010; Tsuda et al., 2010; Trang et al., 2012). Visceral pain is a common form of pain associated with pathological conditions such as renal colic, dyspepsia, inflammatory bowel disease, angina, dysmenorrhoea, and interstitial cystitis. P2X3 (homomultimer) and P2X2/3 (heteromultimer) receptors have been cloned and shown to be mainly located on small nociceptive sensory neurons in the DRG (Lewis et al., 1995).

It was proposed in 1999 that purinergic mechanosensory transduction occurred in visceral tubes and sacs, including ureter, bladder and gut, where ATP released from lining epithelial cells during distension acted on P2X3 and P2X2/3 receptors on subepithelial nociceptive sensory nerves to initiate impulses in sensory pathways to pain centers in the CNS (Burnstock, 1999) (**Figure 2**). P2X3 receptor knockout mice exhibited reduced inflammatory pain and marked urinary bladder hyporeflexia with reduced voiding frequency, suggesting that P2X3 receptors were involved in mechanosensory transduction underlying both inflammatory pain and physiological voiding reflexes (Cockayne et al., 2000). ATP was shown to be released from bladder urothelial cells during distension, and activity initiated in pelvic sensory nerves was mimicked by ATP and α,β-methylene ATP (α,β-meATP) and attenuated by P2X3 antagonists as well as in P2X3 knockout mice (Vlaskovska et al., 2001). Passage of a kidney stone through the ureter causes severe pain. P2X3 receptor immunostaining of sensory nerves in the suburothelial region was reported (Lee et al., 2000). Using a guinea-pig preparation, perfused *in vitro*, multifiber recordings of ureter afferent nerve activity were made (Rong and Burnstock, 2004). Distension of the guinea-pig ureter resulted in increased spike discharge in sensory nerves, which was mimicked by ATP and reduced by P2X3 receptor antagonists. Pressure-dependent release of ATP from urothelial cells to about 10 times the basal release levels resulted from distension of both the perfused guinea-pig and human ureters (Knight et al., 2002; Calvert et al., 2008).

Purinergic mechanosensory transduction in the gut initiated both physiological reflex modulation of peristalsis via intrinsic sensory fibers and nociception via extrinsic sensory fibers (Burnstock, 2001b). Distension of a pelvic sensory nerve-colorectal preparation led to pressure-dependent increase in release of ATP from mucosal epithelial cells and evoked pelvic nerve excitation. This excitation was mimicked by application of ATP and α,β-meATP and attenuated by selective P2X3 and P2X2/3 antagonists (Wynn et al., 2003).

P2X3 and P2X2/3 receptors located on primary afferent nerve terminals in inner lamina 2 of the spinal cord, also play a significant role in neuropathic and inflammatory pain (see Wirkner et al., 2007; Burnstock, 2009a). Dorsal horn neurons relaying nociceptive information further along the pain pathway express P2X2, P2X4, and P2X6 receptors (Bardoni et al., 1997). Microglial P2X4 and P2X7 receptors are also involved in neuropathic pain (Tsuda et al., 2003; Hughes et al., 2007), although the underlying mechanisms are still under investigation (Inoue, 2007; Trang and Salter, 2012). Neuropathic pain and allodynia are abolished in both P2X4 and P2X7 knockout mice, so there is much interest in finding selective antagonists that are suitable for therapeutic development (see McGaraughty et al., 2007).

ATP involvement in migraine was first suspected in relation to the vascular theory of this disorder with ATP released from endothelial cells in microvessels during reactive hyperaemia, which is associated with pain, following cerebral vascular vasospasm (that is not associated with pain; Burnstock, 1989). P2X3 receptor involvement in neuronal dysfunction in brain areas that mediate nociception in migraine, such as the trigeminal nucleus and thalamus, has also been proposed (Fabbretti et al., 2006), and may represent a novel target for antimigraine drugs (Fumagalli et al., 2006). Anti-nerve growth factor treatment suppressed responses evoked by P2X3 receptor activation in an *in vivo* model of mouse trigeminal pain (D'Arco et al., 2007).

P2X RECEPTOR AGONISTS AND ANTAGONISTS—THERAPEUTIC POTENTIAL

P2X receptors consist of a family of ligand-gated cation channels that are widely expressed in nerves and many non-neuronal cells. **Table 2** summarizes the selective agonists and antagonists currently available for the P2X receptor subtypes. With the recent discovery of their crystal structure (Kawate et al., 2009), medicinal chemists now have a detailed understanding of how the individual subunits that form the receptor interact with each other and are in a better position to prepare selective P2X receptor agonists and antagonists. P2X receptors change expression in pathological conditions, suggesting that they may be useful targets for treatment of diseases. The clinical manipulation of purinergic signaling is in its infancy. One of the main reasons why we do not yet have more purinergic therapeutic drugs is the scarcity of receptor-subtype-selective agonists and antagonists that can be used *in vivo*. Afferent Pharmaceuticals have recently developed some small molecules (AF-353 and derivatives) as P2X3 and P2X2/3 antagonists that are orally bioavailable and stable *in vivo* and which are currently in clinical trial (Gever et al., 2006, 2010). There has also been promising development of clinically relevant P2X7 antagonists recently, notably the Abbott compounds A438079 and A-317491 (McGaraughty et al., 2007). However, antagonists for some of the other P2X subtypes are still to be developed. Therapeutic strategies in the future are also likely to include agents that control the expression of P2 receptors, inhibitors of extracellular breakdown of ATP and enhancers and inhibitors of ATP transport.

TOPICS COVERED IN THIS SPECIAL ISSUE

Included in this Special Issue are papers by Elsa Fabbretti, Rashid Giniatullin and Anthony Ford about P2X3 receptors; Stanko Stojilkovic, Terrance Egan, Ruth Murell-Lagnado, Annette Nicke, Thomas Grutter and Philippe Seguela about the molecular physiology and targeting of P2X receptors; Sam Fountain about the evolution of P2X receptors; Manfred Frick and Kazu Inoue about P2X4 receptors involved in lung surfactant secretion and microglia-mediated neuropathic pain; David Henshall about P2X receptors as therapeutic targets for epilepsy; Gary Housley and Sue Kinnamon about P2X receptors in hearing and taste; and Antony Triller about P2X7 receptors.

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Exploring the ATP-binding site of P2X receptors

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P2X receptors are ATP-gated non-selective cation channels involved in many different physiological processes, such as synaptic transmission, inflammation, and neuropathic pain. They form homo- or heterotrimeric complexes and contain three ATP-binding sites in their extracellular domain. The recent determination of X-ray structures of a P2X receptor solved in two states, a resting closed state and an ATP-bound, open-channel state, has provided unprecedented information not only regarding the three-dimensional shape of the receptor, but also on putative conformational changes that couple ATP binding to channel opening. These data provide a structural template for interpreting the huge amount of functional, mutagenesis, and biochemical data collected during more than fifteen years. In particular, the interfacial location of the ATP binding site and ATP orientation have been successfully confirmed by these structural studies. It appears that ATP binds to inter-subunit cavities shaped like open jaws, whose tightening induces the opening of the ion channel. These structural data thus represent a firm basis for understanding the activation mechanism of P2X receptors.

Keywords: ATP, P2X receptors, binding site, gating, crystal structure, mutagenesis, engineered site-directed labeling

INTRODUCTION

ATP-gated P2X receptors are involved in a variety of physiological processes such as fast synaptic transmission, contraction of smooth muscle, regulation of neurotransmitter release, inflammation, and pain sensation (Surprenant and North, 2009; Burnstock, 2012; Khakh and North, 2012). They are also implicated in neurodegenerative and neuropsychiatric disorders (Burnstock, 2008, 2012; Lemoine et al., 2012) and are thus considered as important therapeutic targets.

P2X receptors belong to the super family of ligand-gated ion channels (LGICs), but they differ significantly from the other LGIC members -the Cys-loop and ionotropic glutamate receptors- by their molecular architecture and stoichiometry (Lemoine et al., 2012). They are trimeric channels permeable to cations with the exception of P2X5 receptor which is also permeable to chloride ions (Ruppelt et al., 2001; Bo et al., 2003; Kaczmarek-Hajek et al., 2012). In mammals, seven members (P2X1–7) have been cloned that arrange in homotrimeric or heterotrimeric P2X receptors and they are all characterized by an extracellular loop domain (ectodomain) and two transmembrane segments (TM1 and TM2) which are terminated by intracellular N- and C-termini (Coddou et al., 2011; Kaczmarek-Hajek et al., 2012; Lemoine et al., 2012).

Following ATP binding in the ectodomain which is about 280 amino acids long, a fast and large conformational change occurs throughout the receptor that results in pore opening (Evans, 2009; Jiang et al., 2013). Many different strategies such as site-directed mutagenesis, electrophysiological recordings, fluorescence-based approaches, and X-ray crystallography have contributed to the understanding of the mechanism by which ATP-binding is coupled to gating. An initial approach was to compare the binding site of P2X receptors with other ATP-binding proteins but it rapidly became evident that there is a lack of sequence homology between P2X receptors and these proteins; for instance, P2X receptors do not contain the Walker motif, which characterizes other ATP-binding proteins (Walker et al., 1982). However, the role of the conserved amino acids of the extracellular loop has been systematically investigated using site-directed mutagenesis. This method has led to the identification of short domains and specific residues such as lysine, arginine, and phenylalanine putatively involved in ATP recognition. But to clearly distinguish the participation of these residues in the recognition of ATP from the channel gating, which can also lead to loss of function, complementary methods have been developed. In this context, cysteine-reactive chemicals as well as photosensitive or cysteine-reactive ATP derivatives have been used to explore the putative residues involved in ATP recognition. Altogether, these investigations have yielded substantial information for the modeling of the interaction between ATP and its specific binding site.

The recent determination of X-ray structure of the zebrafish (zf) P2X4 receptor in a closed state and in an ATP-bound open-channel state confirmed many key experimental data, including the mechanism of ATP binding (**Figure 1**). It is now possible to understand at an unprecedented level of precision the mechanism by which ATP is selectively recognized. Indeed, as detailed later, the ATP-bound open-channel structure revealed that the ATP triphosphate tail is coordinated by the residues K70, K72, K316, N296, and R298 (zfP2X4 numbering), while K193 residue indirectly interacts with the α-phosphate group. The residues T189, L191, and I232 are responsible for the coordination of the adenine moiety of ATP whereas L217 is involved in the recognition of the ribose ring of ATP. Therefore, both

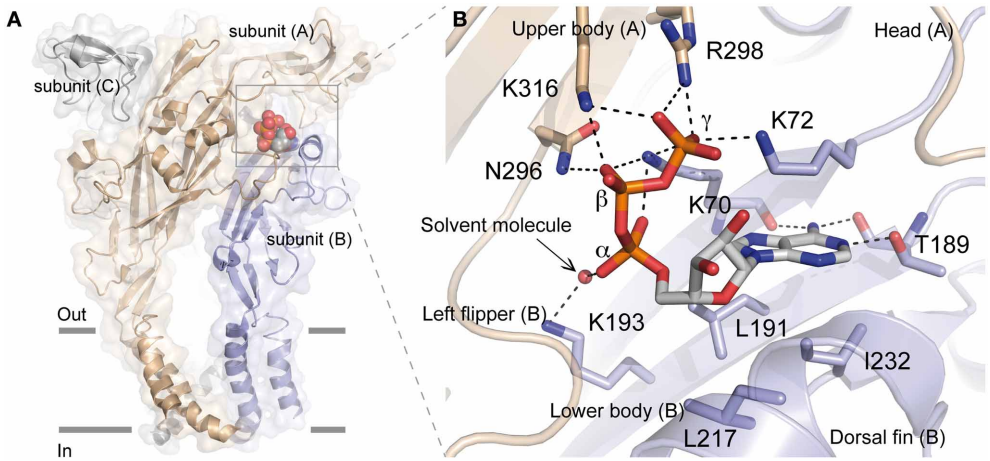

FIGURE 1 | Crystal structure of zfP2X4 receptor bound to ATP. (A) Lateral view of the trimeric structure. Each subunit, displayed in both surface and ribbon representation, is shown in a different color. Only one bound ATP molecule is shown. **(B)** Close-up view of the ATP-binding site. The oxygen atom from the solvent molecule (glycerol) is shown in sphere representation. Black dashed lines indicate hydrogen bonding. (Modified from Hattori and Gouaux, 2012).

positively charged and hydrophobic amino acid residues that belong to different structural domains of the receptor surround bound ATP molecule. Because the overall structure of each receptor subunit resembles a "leaping dolphin," these domains were named upper body from chain A, and lower body and dorsal fin from chain B (**Figure 1B**). Decoding the mechanism of this exquisite specificity will certainly be useful to designing new drugs for these receptors recognized as therapeutic targets.

In this review, we present different experimental approaches that have been used to explore the ATP-binding site in P2X receptors and discuss the results in the light of the recent ATP-bound crystal structure.

MUTAGENESIS-BASED ANALYSIS OF ATP BINDING SITE: A COMPARISON WITH THE CRYSTAL STRUCTURE OF THE ATP-BOUND zfP2X4 RECEPTOR

Considering the lack of sequence homology between P2X receptors and other ATP binding proteins, the analysis of the binding site has been performed mainly with the help of systematic site-directed mutagenesis combined with electrophysiological characterization to explore the role of conserved residues in the binding site of ATP in P2X receptors (Evans, 2009). In this long quest, site-directed mutagenesis of conserved ectodomain residues has been one of the most employed approaches. ATP-induced responses have been mostly assessed for P2X receptors composed of mutated subunits heterologously expressed in either HEK 293 cells with the patch-clamp technique or *Xenopus laevis* oocytes with the use of two-electrode voltage-clamp recordings (Fischer et al., 2007; Evans, 2009, 2010). A role of the amino acids in the binding of ATP has been evaluated by the alteration of ATP potency, a combined measure between affinity and gating (Roberts and Evans, 2004).

ROLE OF POSITIVELY AND NEGATIVELY CHARGED RESIDUES

It was initially suggested that highly conserved positively charged residues of the extracellular loop of P2X receptors could participate to the binding of negatively charged ATP through coordination of the phosphate groups as found for lysine residues in the Walker motif of other ATP-binding proteins (Ennion et al., 2000). Positively charged residues of human (h) P2X1 and rat (r) P2X2 receptors were mainly substituted for alanine to neutralize the positive charges whereas substitution with arginine allowed the conservation of the positive charge for comparison (see **Table 1**). Pioneering studies identified positively charged amino acids such as lysine residues in hP2X1 and rP2X2 receptors, corresponding to residues K70, K72, K193, and K316 in zfP2X4 receptor, as crucial residues for the binding of ATP (Ennion et al., 2000; Jiang et al., 2000). As initially shown by Digby et al. (2005), the KxKG sequence including the two amino acids K70 and K72 (zfP2X4 numbering), which is a highly conserved motif of P2X receptors, plays a major role in ATP recognition; these findings were confirmed in hP2X1, hP2X2, and hP2X3 receptors (Fischer et al., 2007; Roberts et al., 2008; Allsopp et al., 2011; Bodnar et al., 2011) as well as in rP2X1, rP2X2, rP2X3, heteromeric rP2X2/3, rP2X4, and rP2X7 receptors (Wilkinson et al., 2006; Yan et al., 2006; Zemkova et al., 2007; Roberts et al., 2008; Jiang et al., 2011). The participation of residues K193 and K316 (zfP2X4 numbering) to agonist recognition has also been described in hP2X1, hP2X2, hP2X3, and hP2X7 receptors (Worthington et al., 2002; Roberts and Evans, 2007; Roberts et al., 2008, 2009; Bodnar et al., 2011), as well as in rP2X2, rP2X2/3 and rP2X4 receptors (Yan et al., 2005; Wilkinson et al., 2006; Yan et al., 2006; Zemkova et al., 2007; Roberts et al., 2008; Jiang et al., 2011). It is noteworthy that lysine residues appear to also have a highly conserved role in non-mammalian P2X receptors since the two mutations K67A and K289A (at positions equivalent to K72 and K316 of zfP2X4, respectively) significantly decreased the ATP potency of the amoeba *Dictyostelium discoideum* P2X receptor (Fountain et al., 2007).

Thiol-reactive methanethiosulfonate reagents such as (2-aminoethyl)methanethiosulfonate hydrobromide (MTSEA, positively charged compound) and sodium (2-sulfonatoethyl)

Table 1 | Effects of mutations on ATP-induced activation of P2X receptors.

Receptor	Type of residues	Mutations	Corresponding residues in zfP2X4 receptor	Effect (fold decrease in ATP potency)	References
hP2X1	Positively charged residues	K190A	**K193**	5-fold	Ennion et al., 2000
		K70A and K70R	**K72**	5-fold and 18-fold, respectively	
		R292K and R292A	**R298**	90–120-fold	
		K309R and K309A	**K316**	25-fold and 1400-fold, respectively	
		K68A	**K70**	>1800-fold	
		K68R	**K70**	Non-functional	
	Polar residues	T186A	**T189**	6-fold	Roberts and Evans, 2006
		N290A	**N296**	60-fold	
	Aromatic residues	F185A	**F188**	10-fold	Roberts and Evans, 2004
		F291A	**F297**	160-fold	
	Glycine residues	G71A	**G73**	6-fold	Digby et al., 2005
		G96A	E98	Non-functional	
		G250A (plus G250P, G250C, G250D, G250F, G250I, G250K, and G250N but not G250S)	**G253**		
		G301A (but not G301P or G301C)	D307		
	Proline residues	P272A (but not P272F, P272G, or P272I)	**P275**	Non-functional	Roberts and Evans, 2005
	Cysteine residues	C217A	**C220**	8-fold	Ennion and Evans, 2002
		C227A	**C230**	45-fold	
	E181 to V200 segment	K190C	**K193**	5-fold	Roberts et al., 2009
		F188C	L191	7.5-fold	
		T186C	**T189**	8-fold	
	S286 to I329 segment	G288C, F297C, F311C	G294, Y303, Y318	5–10-fold	Roberts and Evans, 2007
		R292C	**R298**	17-fold	
		F291C	**F297**	50-fold	
		N290C	**N296**	71-fold	
		K309C	**K316**	195-fold	
	E52 to G96 segment	K70C	**K72**	10-fold	Allsopp et al., 2011
		F92C	I94	100-fold	
		K68C	**K70**	>3000-fold	
rP2X1	Residue in the first intercysteine region (segment A118 to I125)	E122C	Not aligned	10-fold	Lorinczi et al., 2012
	Positively charged residues	K68A	**K70**	Non-functional	Wilkinson et al., 2006

(Continued)

Table 1 | Continued

Receptor	Type of residues	Mutations	Corresponding residues in zfP2X4 receptor	Effect (fold decrease in ATP potency)	References
hP2X2		F183C, T184C, F289C	**F188, T189, F297**	4–10-fold	Roberts et al., 2008
		N288C, R290C, K307C	**N296, R298, K316**	Major decrease in ATP potency	
		K69C, K71C	**K70**, **K72**	Non-functional	
rP2X2	Positively and negatively charged residues; polar residues	D259A, K71A, Q108A, T184A, K188A, N288A, R290A, R304A	D265 **K72**, **Q114**, **T189**, **K193**, **N296**, **R298** **R312**	Major decrease in ATP potency	Jiang et al., 2000
		K69A, K308A	**K70**, **K316**	Non-functional	
	Glycine residues	G247A	**G253**	Non-functional	Nakazawa and Ohno, 1999
		G248V (but not G248A)	**G254**		
	Cysteine residues	C113A, C124A, C130A, C147A, C158A, C164A, C214A	**C119, C129, C135, C152, C162, C168, C220**	9–30-fold	Clyne et al., 2002
		C224A	**C230**	Non-functional	
	D57 to K71 segment	K71C	**K72**	1000-fold	Jiang et al., 2000
		K69C	**K70**	Non-functional	
		E84C, Q138C	A90, R143	Potentiation of ATP potency	Jiang et al., 2011
		E85C, F183C, F291C, A309C, Y310C, T184C, L306C	E85C, **F188, F299** G317, Y318 **T189, L314**	5–12-fold	
		G139C, Y287C	**G144, Y295**	Major decrease in ATP potency (>25-fold)	
		G141C, L186C	**G146**, L191		
		N288C, F289C, R290C	**N296, F297, R298**		
		K69C, K71C, K308C	**K70, K72, K316**	Non-functional	
hP2X3	Residues in nucleotide binding domains (NBD-1-4)	F174A, K284A K65A, G66A T172A, N279A, F280A	L191, K301 **K72**, **G73** **T189**, **N296**, **F297**	Reduction of α,β-methylene potency	Bodnar et al., 2011
		K63A, K176A, R281A, R295A, K299A K65A/G66A, F171A/T172A, N279A/F280A, F280A/R281A	**K70**, **K193**, **R298**, **R312**, **K316** **K72**/**G73**, **F188**/**T189**, **N296**/**F297**, **F297**/**R298**	Abolition of α,β-methylene-induced currents	
	Conserved positively charged residues	K65A	**K72**	12-fold (α,β-methylene potency)	Fischer et al., 2007
		R281A	**R298**	60-fold	
		K63A, K176A, R295A, K299A	**K70**, **K193**, **R312**, **K316**	Non-functional	
rP2X2/3	Positively charged residues	rP2X2 (K69A)	**K70**	Non-functional	Wilkinson et al., 2006
		rP2X2 (K308A)	**K316**		
		rP2X3 (K63A)	**K70**		
		rP2X3 (K299A)	**K316**		
		rP2X2 (K69A + K308A)	**K70, K316**	Functional receptor	
		rP2X2 (K69A) + rP2X3	**K70**	No modification of α,β-methylene potency	

(Continued)

Table 1 | Continued

Receptor	Type of residues	Mutations	Corresponding residues in zfP2X4 receptor	Effect (fold decrease in ATP potency)	References
		rP2X2 (K308A) + rP2X3	**K316**	Slight decrease of α,β-methylene potency	
		rP2X2 + rP2X3 (K63A)	**K70**	Major decrease of α,β-methylene potency	
		rP2X2 + rP2X3 (K299A)	**K316**		
		rP2X2 (K69A + K308A) + rP2X3	**K70**, **K316**		
rP2X4	Charged and aromatic residues	F294A	**F297**	8-fold	Zemkova et al., 2007
		F230A (but not F230W or F230Y), R278A (but not R278K), D280A (but not D280E)	F233 R281 D283	Non-functional	
		K67A (and K67R), F185A (but not F185W), K190A (but not K190R), R295A (and R295K), K313A (and K313R)	**K70** **F188** **K193** **R298** **K316**		
	K180 to K326 segment	R318A (but not R318K)	R321	20-fold	Yan et al., 2005
		K190A (but not K190R), F230A (but not F230W), R278A (but not R278K), D280A and D280Q (but not D280E)	**K193** F233 R281 D283	>1666-fold	
	K313 to I333	G316S	G319	9-fold	Yan et al., 2006
		Y315A, G316A (but not G316P), R318A	Y318, G319 R321	>16-fold	
		K313A	**K316**	10,000-fold	
		K313R	**K316**	Major decrease (value not indicated)	
		F185C	**F188**	20-fold	Roberts et al., 2008
		T186C	**T189**	50-fold	
		K67C, K69C, N293C R295C, K313C	**K70**, **K72**, **N296** **R298**, **K316**	Major decrease in ATP potency	
hP2X7		K193A, K311A	**K193**, **K316**	Non-functional	Worthington et al., 2002
DdP2X		K67A	**K72**	>10-fold	Fountain et al., 2007
		K289A	**K316**	Major decrease	
		D330A		Non-functional	

The results have been classified according to an increasing rank of order of inhibition for the mutations indicated in each study.

For the corresponding residues in the zfP2X4 crystal structure:

— conserved residues are in bold;

— underlined residues have been identified to participate to the binding of ATP;

— normal characters indicate residues that are not conserved among species.

The modification of α,β-methylene potency is also indicated. Decrease of agonist potency not exceeding 5-fold has not been taken into account.

The lack of function of G250A-containing hP2X1 receptors most likely results from a failure in normal processing of the receptor (Digby et al., 2005). K307C-containing hP2X2 receptors were expressed at lower levels in cells (Roberts et al., 2008). R295A-, and K299A-containing hP2X3 receptors were expressed at lower levels in cells (Bodnar et al., 2011). F230A-containing rP2X4 receptors were expressed at lower levels (Zemkova et al., 2007). A reduction of trafficking of N293C-containing rP2X4 receptors to the cell surface has been proposed (Roberts et al., 2008).

methanethiosulfonate (MTSES, negatively charged compound) have proved very useful in examining the role of charges in the ATP-binding site (Jiang et al., 2000; Roberts and Evans, 2007; Roberts et al., 2008, 2009). Indeed, this strategy is based on the fact that these compounds have the capacity to form disulfide bonds when amino acid residues are strategically substituted with cysteine; this procedure consequently modifies the recognition of ATP if the mutation is performed at crucial residues in the binding site. Interestingly, positively charged MTSEA introduces a positively charged side chain of similar length to that of lysine and thus helps to verify the critical role of positively charged residues in the recognition of the phosphate groups (Fountain and North, 2006). In this context, mutational studies and MTS reagents-based experiments have shown the importance of positively charged residues for ATP binding and action, in particular lysine residues of hP2X1, rP2X2, and rP2X4 corresponding to the residues K70, K72, K193, and K316 of zfP2X4 (Roberts and Evans, 2007; Roberts et al., 2008, 2009; Allsopp et al., 2011).

In addition to lysine, numerous studies have also highlighted the major role of arginine residues in hP2X1, hP2X3, rP2X2, and rP2X4 receptors (all corresponding to R298 in zfP2X4, **Table 1**) in agonist recognition by interaction with a phosphate group of ATP (Ennion et al., 2000; Jiang et al., 2000; Fischer et al., 2007; Zemkova et al., 2007). The contribution of this residue to the ligand binding site has also been confirmed using partial agonists such as 2′,3′-*O*-(4-benzoyl)-ATP (BzATP) and P(1),P(5)-di(adenosine 5′)-pentaphosphate (Ap(5)A) in hP2X1 (Roberts and Evans, 2004). It is notable that a recent study in rP2X2 receptor has also indicated that a salt bridge between the residues R290 (R298 in zfP2X4) and E167 stabilized the closed state of the receptor and that, after spatial rearrangement and release of this electrostatic coupling, a new ionic interaction took place between R290 and ATP, contributing to the coordination of ATP in its binding site (Hausmann et al., 2013).

All these results have now been confirmed by the recent resolution of the structure of the ATP-bound zfP2X4 receptor (**Figure 1**) (Hattori and Gouaux, 2012). This study revealed how the positively charged residues K70, K72, K316, and R298 are critically involved in direct coordination of the ATP triphosphate tail, while K193 residue indirectly interacts with the α-phosphate group through a glycerol solvent molecule (used for crystallization purpose). It has been proposed that water molecules substitute glycerol under physiological conditions (Hattori and Gouaux, 2012) (**Figure 1B**). The X-ray structure also shows how the three phosphate groups of ATP interact with the positively charged residues, providing a plausible explanation of why ADP has almost no effect on the activation of P2X receptors (Hattori and Gouaux, 2012). Furthermore, it is noteworthy that K70 residue, for which it has been shown that alanine mutation induced the largest inhibitory effect on ATP potency (see **Table 1**), occupies a very crucial position in ATP binding since it coordinates oxygen atoms of the α, β, and γ phosphate groups (**Figure 1B**). In addition, the X-ray structures of zfP2X4 definitively confirmed the intersubunit location of the ATP-binding site (Kawate et al., 2009; Hattori and Gouaux, 2012). The identification of the role of the conserved positively charged residues K68, K70, R292, and K309 (hP2X1 receptor) in ATP recognition initially led to the proposition that these residues, organized in two clusters, could form the ATP binding site by interacting either within a P2X receptor subunit or between adjacent subunits (Ennion et al., 2000). It was thereafter proposed that the heteromeric rP2X2/3 receptor was probably composed of one rP2X2 and two rP2X3 subunits and that the residues from two different subunits were able to interact in the ATP binding site (Wilkinson et al., 2006). Another decisive demonstration for the intersubunit position of the ATP binding site in P2X receptors came from the observation that the mutations K68C and F291C in rP2X1 (corresponding to K70 and F297 in zfP2X4 receptor, respectively) led to the formation of disulfide cross-link into trimers (Marquez-Klaka et al., 2007). In addition, it was shown that the disulfide bond formation between K68C and F291C was prevented in the presence of ATP (Marquez-Klaka et al., 2007). This study clearly demonstrated that residues from adjacent subunits contribute together to the formation of the ATP-binding site in P2X receptors.

It is assumed that ATP forms complexes with Mg^{2+} (Ashcroft and Gribble, 1998; Ennion et al., 2001; Li et al., 2013). For this reason, it has been postulated that the negatively charged amino acids of P2X receptors could contribute to the binding of ATP (Ennion et al., 2001). However, none of the mutations of the conserved negatively charged residues (aspartate and glutamate) had an effect on ATP potency, indicating that they are not involved in ATP recognition in hP2X1 (Ennion et al., 2001). In agreement, no negatively charged residues were found to interact directly with ATP in the crystal structure (**Figure 1B**). In addition, no Mg^{2+} ions were resolved near bound ATP, a result fully consistent with recent data showing that ATP^{4-} activates all subtypes of homomeric P2X receptors, whereas $MgATP^{2-}$ activates only P2X1 and P2X3, but not P2X2 and P2X4 receptors (Li et al., 2013). Thus, ATP^{4-} seems to be the primary ionic form that activates P2X receptors.

POLAR AMINO ACIDS

The possibility that conserved polar residues such as glutamine, asparagine, and threonine play a role in ATP recognition at the ATP binding site had also been investigated because they may form hydrogen bonds with ATP (Roberts and Evans, 2006). As shown in **Table 1**, only mutations of residues corresponding to T189 and N296 (zfP2X4 numbering) had a significant effect on ATP potency. The residue T189 is in part responsible for the coordination of the adenine moiety of ATP whereas N296 participates to the coordination of the β–phosphate groups (**Figure 1B**) (Hattori and Gouaux, 2012). It must be noticed that the mutations with alanine at the residues corresponding to T189 and N296 in zfP2X4 had already been shown to significantly reduce ATP potency in rP2X2 receptors (see **Table 1**) (Jiang et al., 2000).

AROMATIC AMINO ACIDS

The fact that aromatic residues have been identified to play key roles in the coordination of ATP in other ATP-binding proteins stimulated the investigation of alanine-based substitution of conserved extracellular aromatic amino acids in P2X receptors (Roberts and Evans, 2004). It was concluded that the residues corresponding to F188 and F297 (zfP2X4 numbering) are involved in the coordination of ATP (see **Table 1**) whereas the substitutions

of both tryptophan and tyrosine residues had no effect (Roberts and Evans, 2004). It is noteworthy that the residue F297 belongs to the highly conserved NFR sequence in which the two conserved amino acids (N296 and R298 in zfP2X4) participate to the coordination of the β- and γ-phosphate groups of ATP, respectively (Hattori and Gouaux, 2012). However, the crystal structure revealed that the side chain of F297 does not interact directly with ATP, but it may help to contribute to the general shape of the binding site. Altogether, these data confirm the importance of the NFR sequence in ATP binding (Jiang et al., 2000, 2011; Roberts and Evans, 2004, 2006, 2007; Fischer et al., 2007; Marquez-Klaka et al., 2007; Zemkova et al., 2007; Roberts et al., 2008; Bodnar et al., 2011). Furthermore, as determined in rP2X1 receptors, the residue F291 (F297 in zfP2X4) contributes to the formation of the ATP binding site between neighboring subunits in P2X receptors (Marquez-Klaka et al., 2007). The mutation of the surrounding amino acids (F289A and F293A) had, however, no effect suggesting that only the conserved NFR sequence has importance in this region (Roberts and Evans, 2004).

Another region between residues F185 and K190 in hP2X1 (region F188-K193 in zfP2X4) has also been indicated to contribute to the effect of ATP (Roberts and Evans, 2004). Indeed, at the neighboring position to residue T186 of hP2X1 (T189 in zfP2X4), F185 (F188 in zfP2X4) has been shown to participate to agonist-evoked conformational change of the receptor as determined with MTS experiments (Roberts et al., 2009) but this residue does not directly interact with ATP according to the crystal structure (Hattori and Gouaux, 2012).

The particular position of the two phenylalanine residues probably explains the important alteration of ATP potency after their substitution as it is estimated that adjacent residues are also involved in the control of ATP binding (Bodnar et al., 2011).

GLYCINE, PROLINE, AND CYSTEINE RESIDUES

The small and achiral amino acid glycine commonly confers flexibility to protein structures. In addition, in P2X receptors the possibility that the GGxxG motif (residues 250–254 in hP2X1 numbering) participates in ATP binding was investigated because it has similarities to the conserved GxGxxG motif found in around 95% of human protein kinases and nucleotide-binding proteins (Spitaler et al., 2000; Digby et al., 2005). Studies designed to investigate the putative role of these conserved glycine residues in the extracellular loop concluded that these amino acids do not participate to the ATP-binding site in hP2X1 (Ennion and Evans, 2002; Digby et al., 2005; Roberts and Evans, 2005). Interestingly, mutation of the glycine residue corresponding to G253 (zfP2X4 numbering) induces the formation of non-functional hP2X1 and rP2X2 receptors (Nakazawa and Ohno, 1999; Digby et al., 2005). These results can be explained by the fact that the mutation induces a defect in the level of expression of the receptor at the cell surface (Digby et al., 2005).

Proline residues have been proposed to play a major role in the secondary structure of proteins (Brandl and Deber, 1986; Yamaguchi et al., 1999; Sansom and Weinstein, 2000; Labro et al., 2003; Roberts and Evans, 2005). The alanine-based substitutions of proline residues of the extracellular loop of hP2X1 had only negligible effects on ATP potency indicating that these residues are not involved in the ATP-binding site (Roberts and Evans, 2005). Nevertheless, mutations at P272 in hP2X1 produced variable effects on ATP-potency depending on the nature of the residue used for the substitution suggesting that the effect of ATP is possibly sensitive to the variation of conformation in this region of the receptor (Roberts and Evans, 2005). Because this residue is localized at around 18 Å from the ATP-binding site, it can be postulated that it is more probably involved in the gating of the pore (**Figure 2**).

Ten conserved cysteine residues are present that can form disulfide bonds in the extracellular loop of P2X receptors; their alanine-based substitution had some effects on ATP potency and the mutations at positions homologous to the residue C230 (zfP2X4 numbering) considerably reduced ATP-induced responses (Clyne et al., 2002; Ennion and Evans, 2002). The crystal structure confirmed that the two residues C220 and C230 form a disulfide bond, which rigidifies the dorsal fin, a critical component of the ATP-binding site (**Figure 2**).

GROUPS OF AMINO ACIDS ORGANIZED IN NUCLEOTIDE BINDING DOMAINS

The alanine-based substitution of residues from four defined nucleotide binding domains of hP2X3 has indicated that the mutation of residues adjacent to identified amino acids, which are crucial for agonist response (i.e., lysine, asparagine, threonine), induced further alterations of agonist potency, in this case

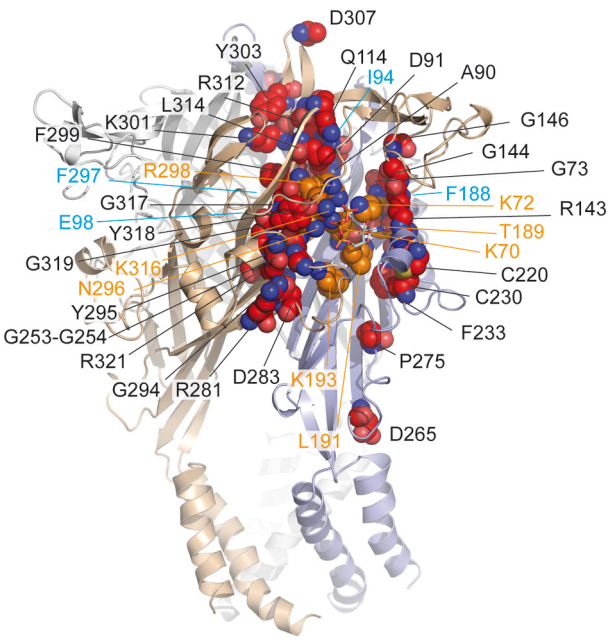

FIGURE 2 | Mapping in the zfP2X4 receptor of residues identified by site-directed mutagenesis. Corresponding residues (indicated in red spheres) previously identified by site-directed mutagenesis in different P2X receptor subtypes (see **Table 1**) are mapped on the crystal structure of the zfP2X4 receptor. The ATP molecule is shown in stick representation. Note that only few residues contact (in orange) the ATP molecule. Oxygen, nitrogen, and sulfur atoms are colored, respectively, in light red, blue, and yellow. Hidden residues are indicated in magenta.

α,β-methylene ATP (see **Table 1**) (Bodnar et al., 2011). Indeed, the double mutants K65A/G66A, F171A/T172A, N279A/F280A, and F280A/R281A (K72/G73, F188/T189, N296/F297, and F297/R298 in zfP2X4 receptor, respectively) were all insensitive to α,β-methylene ATP, in contrast to the single mutants which were responsive to the agonist (Bodnar et al., 2011). These results have led to the concept that groups of amino acids, rather than individual amino acids, are responsible for the recognition of ATP (Bodnar et al., 2011). The nucleotide binding domains overlap the essential residues for ATP binding, as determined from the X-ray structure of ATP-bound zfP2X4 receptor (**Figure 2**).

With the characterization of hundreds of alanine and cysteine mutants in the extracellular domain of P2X receptors, it was found that several mutations induced a decrease in ATP potency and were found close to the ATP molecule. However, among these mutations, only some (less than ten) correspond to crucial residues directly involved in ATP binding (**Figure 2**). In an MTS-based study, it was postulated that non-conserved residues play a regulatory role in the effect of ATP (Roberts et al., 2008). The participation of several non-conserved residues in ATP recognition was revealed by the crystal structure of the ATP-bound zfP2X4 receptor (Hattori and Gouaux, 2012). However, to our knowledge, the mutation-based analysis failed to determine two residues i.e., I232 and L217 (zfP2X4 numbering) which are involved, respectively, in the recognition of the adenine base and ribose ring of ATP (**Figure 1B**). In addition, the main limitation of site-directed mutagenesis was the difficulty to distinguish clearly the direct modification of ATP binding from alterations in channel gating, which can also lead to a loss of function (Colquhoun, 1998). Indeed, ATP potency is dependent upon both the affinity and gating. The mutations of residues which do not participate in ATP recognition in the binding site can, however, lead to changes in ATP potency because these residues are able to induce conformational alterations associated with gating of the pore in response to ATP binding (Evans, 2010). For this reason, complementary methods have been employed to distinguish clearly residues participating in the ATP-binding site from those involved in gating. In this context, allosteric reporter mutations in combination with single-channel recordings, cysteine-reactive chemicals as well as ATP-derivatives with photosensitive or cysteine-reactive moieties have been used to explore the putative residues involved in the coordination of both the negatively charged phosphate groups and the adenine ring of ATP. Altogether, these investigations provided new insights into the understanding of the mechanism of ATP-binding.

ADDITIONAL METHODS FOR THE INVESTIGATION OF THE ATP-BINDING SITE

ALLOSTERIC REPORTER MUTATIONS

The first evidence distinguishing ATP binding from gating in P2X receptors was provided by Cao et al. (2007), where the authors combined single-channel recordings and the use of a mutated receptor considered as an "allosteric reporter." They took advantage of the fact that the deep pore mutation T339S in the P2X2 receptor produced spontaneous openings of the channel in the absence of agonist. This mutation most likely modifies the allosteric equilibrium between the closed and open states, and thus reports gating properties. Introducing the K69A or K308A mutation into the T339S background, the authors showed that K308A mutation alters considerably the spontaneous channel openings and consequently the gating properties of the receptor, whereas the nearby K69A mutation leaves these spontaneous activities unaffected (Cao et al., 2007). Given the fact that both mutations do not respond to ATP, these elegant experiments identified K69 as a critical residue for direct recognition of ATP and K308 as important one for channel gating, in addition to its contribution to the binding site. As stated above, the crystal structure definitively confirmed the particular location of K69 (K70 in zfP2X4) in coordination of the phosphate tail, thus validating such a "genetic" approach, which can be applied to other ion channels.

STRATEGY OF SITE-DIRECTED AFFINITY LABELING

In the attempt to precisely localize the ATP-binding site, a "chemical" strategy has been developed which consists of site-directed affinity labeling to create covalent bonds between a synthesized ATP-derived thiol-reactive rP2X2 agonist, 8-thiocyano-ATP (NCS-ATP), and single cysteine mutants engineered in the putative binding cavities of the rP2X2 receptor (**Figure 3A**) (Foucaud et al., 2001; Jiang et al., 2011). The 26 residues to be substituted were chosen on the basis of rP2X2 homology model because they protrude in the binding cavity (Jiang et al., 2010). By combining whole-cell and single-channel recordings, it was shown that NCS-ATP labeled only two cysteine mutants, N140C and L186C, which are separated by about 18 Å in the closed state of the receptor. While irreversible binding at N140C decreased both ATP efficacy and open probability (NP_o) of ATP-activated rP2X2 receptors, labeling of L186C induced a potentiation of the ATP responses (Jiang et al., 2011). It was proposed that the potentiating effect would occur only at one or two of the three binding pockets per receptor, producing strong cooperativity for further ATP binding (Jiang et al., 2011). Considering all these results, models were constructed in which the residues previously identified by site-directed mutagenesis to be crucial for ATP recognition (see previous chapter) appeared in close proximity to docked NCS-ATP (Jiang et al., 2011). From this work, it was concluded that the inter-subunit cavities found in the closed X-ray structure of zfP2X4 (Kawate et al., 2009) correspond to the ATP-binding sites and that this strategy has helped define the involvement of two non-conserved residues, N140 and L186 in the coordination of the adenine ring of ATP (Jiang et al., 2011) (**Figure 3B**). The ATP-bound crystal structure of zfP2X4 confirms the close proximity (4.3 Å) of L191 (L186 in rP2X2) to the ATP site and shows that this residue is indeed involved in hydrophobic interactions with the adenine base of ATP (**Figure 3C**) (Hattori and Gouaux, 2012). However, it is surprising to note that the other NCS-ATP labeled residue, N140, (corresponding to D145 in zfP2X4) is located at ∼14 Å from position 8 of the adenine ring of ATP (**Figure 3C**). Interestingly, this residue is close to the region that accommodates NF770, a suramin derivative that is the most potent P2X2 receptor antagonist described so far (Wolf et al., 2011). Thus, an

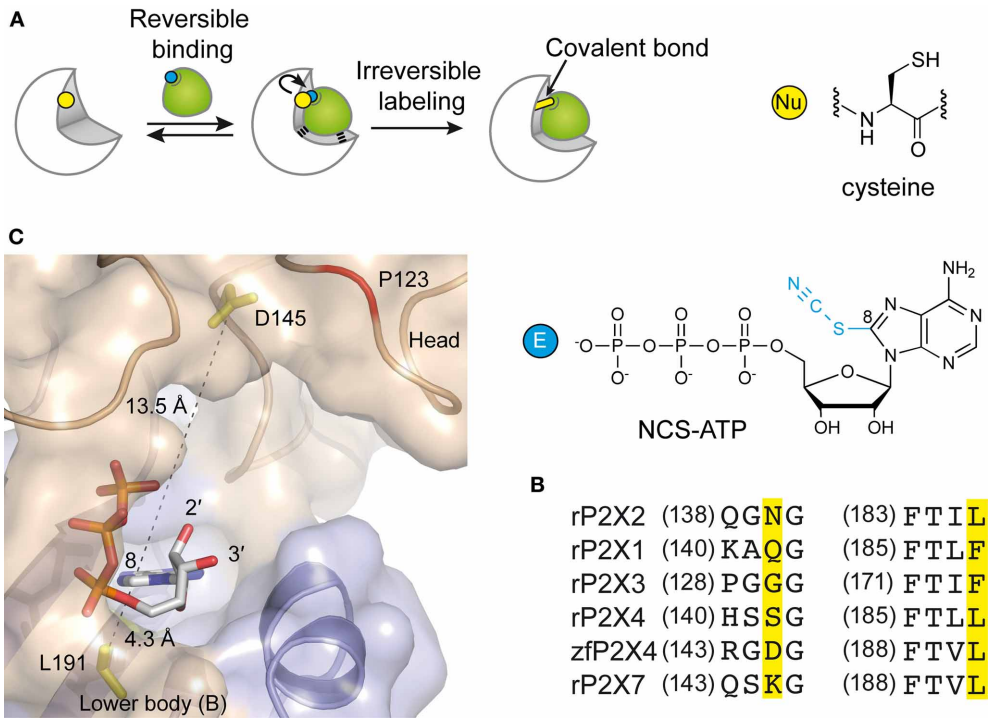

FIGURE 3 | Alternative methods to identify residues involved in the ATP-binding of P2X receptors. (A) Principle of engineered affinity labeling strategy. Chemical structures of the affinity label NCS-ATP and cysteine are shown. **(B)** Sequence alignment of identified segments containing NCS-ATP-labeled residues in the P2X2 receptor (N140 and L186). **(C)** Spatial location of the corresponding labeled residues in the X-ray structure of the ATP-bound zfP2X4 receptor.

attractive hypothesis that deserves further experiments is that the N140-containing region contributes, in part, to the competitive antagonist binding site.

SITE-DIRECTED FLUORESCENCE LABELING AND VOLTAGE-CLAMP FLUOROMETRY

Another approach is the use of fluorescent strategies. Tetramethyl-Rhodamine-Maleimide (TMRM), a sulfhydryl-reactive fluorescent dye on cysteine mutants, has the capacity to label cysteine-substituted residues that are accessible to the solvent (Lorinczi et al., 2012). The simultaneous measurement of ionic currents and fluorescence by voltage-clamp fluorometry, after site-directed fluorescence labeling, allowed to resolve ligand interactions and structural modifications which are associated with the conformational transitions of channels (Lorinczi et al., 2012). The effect of systematic substitution with cysteine residues in the cysteine-rich head domain of rP2X1 (A118 to I125) was examined because this region projects over the proposed ATP binding site (Kawate et al., 2009; Lorinczi et al., 2012). This work provided evidence that TMRM tethered to E122C reports 2′,3′-*O*-(4-benzoylbenzoyl)-ATP (Bz-ATP) binding, most probably by sensing Bz moiety of the ligand (Lorinczi et al., 2012). Because E122 is not aligned with a defined zfP2X4 residue (Hattori and Gouaux, 2012), it would correspond to a region close to P123 in zfP2X4. Of note, the ATP-bound crystal structure shows that the solvent-exposed oxygen atoms 2′ et 3′ of the ribose point toward P123, a fact that is fully consistent with the hypothesis that tethered TMRM interacts directly with bound Bz-ATP (**Figure 3C**).

PHOTOAFFINITY LABELING EXPERIMENTS

Photoaffinity labeling is a powerful technique that is used extensively in other ligand-gated ion channel studies to probe important binding sites (Lemoine et al., 2012). At P2X receptors, the UV light-reactive ATP analog, ^{32}P-2-azido ATP was employed to assess the effects of mutations of various residues on agonist binding (Roberts and Evans, 2007; Agboh et al., 2009; Roberts et al., 2009; Allsopp et al., 2011). The fact that photoincorporation of radiolabeled 2-azido ATP is reduced following mutations has contributed toward ascertaning the participation of residues K68, K70, K190, K309, and T186 to the coordination of ATP in the binding site of hP2X1 receptor (Roberts and Evans, 2007; Roberts et al., 2009; Allsopp et al., 2011). More recently, the partial agonist BzATP has been covalently incorporated by UV light into P2X receptors to investigate the relationship between ligand occupancy and channel gating (Bhargava et al., 2012; Browne and North, 2013). Although these studies did not identify labeled residues, they provided valuable information on the mechanism of ATP binding.

X-RAY CRISTALLOGRAPHY: THE CONFIRMATION OF FUNCTIONAL AND BIOCHEMICAL STUDIES

As previously indicated, the recent determination of X-ray structures of the zfP2X4 receptor (Kawate et al., 2009; Hattori and

Gouaux, 2012) resolved in two states, the resting closed and open channel states, finally provided an unprecedented information not only regarding the three-dimensional shape of the receptor, but also on putative conformational modifications that couple ATP binding to channel opening. These data also provide a structural template for interpreting the huge amount of functional, mutagenesis, and biochemical data. In particular, as already reviewed, many key features of the binding site, including its interfacial location and ATP orientation as well as the identity of residues involved in ATP recognition, had been successfully anticipated from the biochemical and functional methods. These structural data thus represent a firm basis for understanding the mechanism by which agonists induce the opening of the ion channel.

COUPLING ATP BINDING TO CHANNEL GATING

Three ATP molecules bound to the trimeric receptor were resolved in the crystal structure (Hattori and Gouaux, 2012). Given that crystal structures represent snapshots among the multiple conformational states, this raises the question of whether P2X receptor channel opening involves the occupancy of one, two, or three binding sites. Early work based on single-channel recordings suggested that channel activation proceeds through three ATP binding steps before opening and partially liganded channels do not appear to open (Ding and Sachs, 1999). Thus, channels only open after being fully liganded. However, more recent studies using concatenated subunits or kinetic models suggest that two ATP molecules are sufficient to activate P2X receptors (Yan et al., 2010; Stelmashenko et al., 2012). This conclusion supports previous study suggesting that heteromeric P2X2/3 receptors are also activated by fewer than three agonist molecules (in this case αβ-methylene-5′-ATP) (Jiang et al., 2003). Interestingly, there is now evidence that occupancy of one binding site of P2X2 receptors does not produce detectable openings, but a conformational change that is spread to the second and third binding sites leading eventually to channel gating (Ding and Sachs, 1999; Jiang et al., 2011, 2012b; Browne et al., 2013). A functional significance of these results is that binding of the second and third ligand is strongly influenced by the binding of the first ligand revealing positive cooperativity.

The conformational change that follows binding of the first ligand suggests the existence of a transient, intermediate or primed closed state that precedes channel activation (Moffatt and Hume, 2007; Jiang et al., 2012b; Browne and North, 2013). The physiological relevance of this intermediate state is unclear, however, the fact that pyrimidine and diphosphate nucleotide analogs, which are not effective at P2X receptors, become effective following binding of a low concentration of ATP implied that mixtures of nucleotides present in the extracellular milieu of the nervous system may have functional roles (Browne and North, 2013).

The ATP binding site is ∼40 Å from the membrane-spanning segments, which constitute the ionic pore of P2X receptors. The recent ATP-bound crystal structure, and previous studies utilizing normal mode analysis (Du et al., 2012; Jiang et al., 2012a), metal-bridging experiments (Jiang et al., 2012a), electron microscopy (Roberts et al., 2012), and voltage-clamp fluorometry (Lorinczi et al., 2012), have now revealed a plausible activation mechanism that can be dissected into five steps (**Figure 4**): binding of ATP^{4-} (Li et al., 2013) to a pocket located at the interface between each subunit (first step) leads to the tightening of the head domain relative to the dorsal fin (second step). Because the ribose and adenine base interact hydrophobically with L217 and I232 (chain B), which are part of the dorsal fin, closure of the binding "jaw" induces the upward movement of the dorsal fin. Subsequently, the lower body, which is structurally coupled to the dorsal fin moves outward (third step), causing large expansion of the three lateral portals defined as "fenestrations" (fourth step). Finally, because the rigid β-sheet-folded lower body domain is directly coupled to the transmembrane helices 1 and 2, its outward flexing movement directly promotes channel gating by inducing the helices to

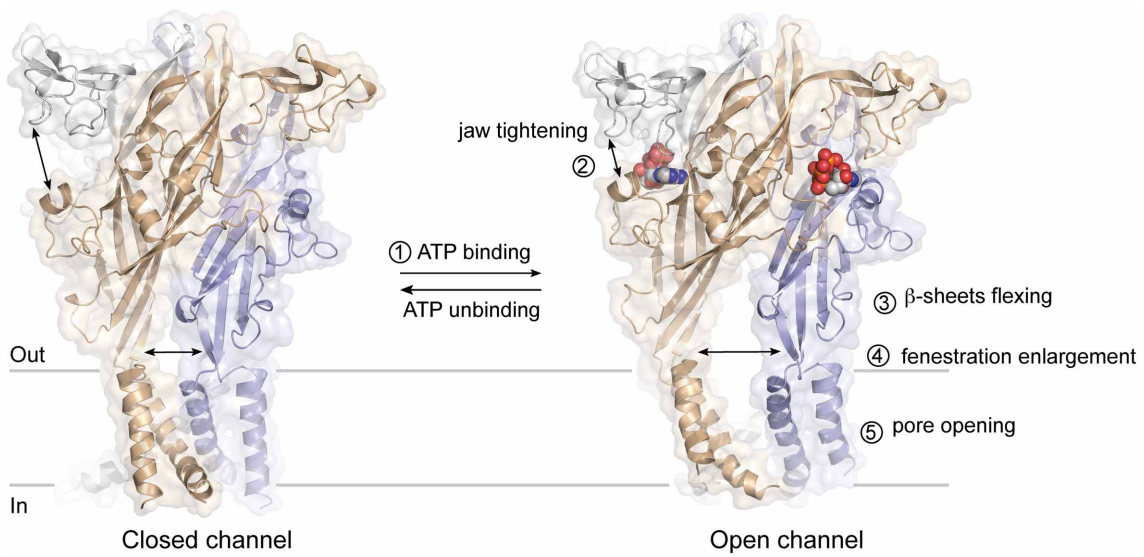

FIGURE 4 | Plausible mechanism of ATP-gated P2X receptors activation. The sequential steps leading to pore opening are indicated.

expand the pore by ∼3 Å (fifth step). This widening allows ions to cross the channel.

In this mechanism, major conformational changes take place at the subunit interface. Early studies have successfully anticipated the importance of these boundary contacts (Jiang et al., 2003, 2010; Nagaya et al., 2005; Marquez-Klaka et al., 2007), by restricting the relative movement of adjacent subunits by engineered disulfide bonds [for review see Jiang et al. (2013)]. These interfaces may be interesting targets for allosteric regulation of P2X receptors.

CONCLUDING REMARKS

Extensive experimental works, including mutagenesis coupled to functional essays, engineered site-directed chemical labeling, fluorescence measurements, and resolution of the ATP-bound crystal structure, have provided a more precise vision of the ATP binding process and channel gating. This is probably a crucial step for the design of new competitive antagonists with therapeutic properties. Molecules that can selectively modulate P2X receptors are needed because these receptors are involved in major neurological disorders. However, upon examining the ATP-binding site of P2X receptors, it seems that an apparent conserved nature of the orthosteric sites may pose some difficulties to achieve strong subtype selectivity. The search for allosteric modulators would provide an alternative issue to this challenge.

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ATP P2X3 receptors and neuronal sensitization

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Increasing evidence indicates the importance of extracellular adenosine triphosphate (ATP) in the modulation of neuronal function. In particular, fine control of ATP release and the selective and discrete ATP receptor operation are crucial elements of the crosstalk between neuronal and non-neuronal cells in the peripheral and central nervous systems. In peripheral neurons, ATP signaling gives an important contribution to neuronal sensitization, especially that involved in neuropathic pain. Among other subtypes, P2X3 receptors expressed on sensory neurons are sensitive even to nanomolar concentrations of extracellular ATP, and therefore are important transducers of pain stimuli. P2X3 receptor function is highly sensitive to soluble factors like neuropeptides and neurotrophins, and is controlled by transduction mechanisms, protein-protein interactions and discrete membrane compartmentalization. More recent findings have demonstrated that P2X3 receptors interact with the synaptic scaffold protein calcium/calmodulin-dependent serine protein kinase (CASK) in a state dependent fashion, indicating that CASK plays a crucial role in the modulation of P2X3 receptor stability and efficiency. Activation of P2X3 receptors within CASK/P2X3 complex has important consequences for neuronal plasticity and possibly for the release of neuromodulators and neurotransmitters. Better understanding of the interactome machinery of P2X3 receptors and their integration with other receptors and channels on neuronal surface membranes, is proposed to be essential to unveil the process of neuronal sensitization and related, abnormal pain signaling.

Keywords: trigeminal neurons, pain, receptor plasticity, purinergic signaling, migraine

INTRODUCTION

ADENOSINE TRIPHOSPHATE AS A CHEMICAL INDUCER OF SENSITIZATION

Sensitization is a process whereby primary sensory neuron afferents and central synapses become hyper-responsive to extracellular nociceptive stimuli so that they underlie neuropathic and chronic pain, including allodynia, hyperalgesia and spontaneous pain. Peripheral and central sensitization are thought to be supported by enhanced release of neurotransmitters and peptides, often co-released with adenosine triphosphate (ATP), from primary afferents to spinal synapses (Bardoni et al., 1997). Activation of the corresponding receptors in postsynaptic dorsal horn neurons induces central sensitization.

Fast conductive myelinated Aδ fibers and slow non-myelinated C-fibers sense different stimuli, in particular mechanical/chemical or tactile stimuli (Basbaum et al., 2009). Whether A- or C-fibers are more important for the generation of spontaneous firing in neuropathic pain, remains an unanswered question. One important priority for translational medicine is the identification of biomarkers for the functional role of distinct classes of C-fibers and Aδ fibers and for their transition from mono- to poly-modal function in chronic pain. It is not excluded that cellular crosstalk at ganglion level might also induce functional plasticity in non-nociceptive neurons to be recruited in persistent allodynia (Ueda, 2008). The recruitment of non-nociceptive sensory fibers generates an additional level of complexity that renders the sensitization incompletely understood in its complex molecular constituents and temporal evolution, with consequent slow development of new drugs to prevent/revert it.

One important consideration regards the differential contribution of sensory fibers in humans and rodents and, therefore, the difficulties to apply experimental data to clinically-useful models. Experiments performed with infrared diode laser stimulation on human subjects affected by painful neuropathies have demonstrated that pain conditions are associated with impaired function of Aδ fibers and low involvement of un-myelinated C-fibers (Tzabazis et al., 2011; Moeller-Bertram et al., 2013), while the opposite is found in rodents (Shields et al., 2010; Zhang et al., 2013). Nonetheless, a species-dependent difference in neural substrates of pain, as recently found in P2X3 receptor sequence (Serrano et al., 2012; Sundukova et al., 2012), does not exclude similar chemo-transduction mechanisms based on analogous mediators and modulators.

The molecular basis of transitions from acute sensitization to long-term hypersensitivity relies on complex temporal and spatial molecular mechanisms that are primed by exposure to soluble factors and intracellular neuronal and non-neuronal signaling. Gene expression and protein trafficking then strongly contribute to change pain receptor expression, supporting dysfunctional action potential firing into aberrant neurotransmitter release at the presynaptic terminal and, thus, inducing central sensitization of spinal and brainstem networks.

Among the soluble and cellular factors responsible for the early molecular signature of fiber sensitization and spontaneous

aberrant firing in a variety of pain-related diseases, one powerful candidate molecule is extracellular ATP (Hamilton and McMahon, 2000), co-released with other neurotransmitters and peptides or after mechanical stress by a number of different mechanisms (Corriden and Insel, 2010; Novak, 2011). Indeed, ATP acute injection activates C-nociceptors in healthy human skin without the involvement of mechano-responsive or mechano-insensitive C-fibers (Hilliges et al., 2002). ATP (whose extracellular concentration is limited in time and space by ectonucleotidases that generate active metabolites) binds to different subtypes of ligand-gated P2X channels or metabotropic P2Y receptors (Burnstock, 2008), amplifying the spectrum of reactive molecules in the extracellular space (Browne and North, 2013).

Combinatorial expression of ATP receptors with different affinity for ATP in distinct cell types allows modulation of purinergic signaling in different tissues. Primary sensory neurons widely express P2X3 receptors (Vulchanova et al., 1998) sensitive to nanomolar ATP concentrations (Sokolova et al., 2006) and implicated in the modulation of pain sensitivity as demonstrated using P2X3 knockout (KO) mice (Cockayne et al., 2000; Souslova et al., 2000; Zhong et al., 2001; Cockayne et al., 2005). Recent pharmacological research has been directed to discover new drugs capable of inhibiting P2X3 receptors because their pharmacological block could provide a significant contribution to reduce inflammatory and neuropathic pain (Ford, 2012; North and Jarvis, 2013). Nevertheless, only a few P2X3-selective antagonists have been reported to date (Jarvis et al., 2002; Ford, 2012) and are currently undergoing clinical trials (Fabbretti and Nistri, 2012).

While it is well known that changes in the activity of voltage-gated ion channels expressed by sensory neurons can contribute to chronic pain sensitization (McCleskey and Gold, 1999), the focus of the present review is on ATP-mediated signaling since it represents an early chemical signal that triggers pain in normal circumstances and that can predate the establishment of neuronal sensitization (Hamilton and McMahon, 2000). ATP, working through different (yet unknown) plasticity processes, eventually confers novel maladaptive activity to neurons and non-neuronal cells in the entire tissue. Together with ATP, several soluble factors and neuropeptides like nerve growth factor (NGF), calcitonin gene-related peptide (CGRP), cytokines and prostaglandins cooperate either to directly activate nociceptors (as well as to induce secondary long-lasting chain of genomic changes) or to evoke indirect paracrine responses after non-neuronal cells activation (Shu and Mendell, 2001; Giniatullin et al., 2008; Jakobsson, 2010; Kuner, 2010; Cady et al., 2011).

ROLE OF ATP-GATED P2X3 RECEPTORS IN NEUROGENIC INFLAMMATION AND NEURONAL SENSITIZATION

Inflammatory mediators influence neuronal expression of nociceptors and ion channels including ATP receptors, therefore contributing to spontaneous activity of sensory fibers and closing a vicious circle of pathological hyper-responsiveness (Ellis and Bennett, 2013).

Neuronal/non-neuronal cell crosstalk is highly modulated by neuronal ATP and its action not only on P2X3 receptors but also on low affinity ATP receptors (P2X4 or P2X7) known to give a strong contribution in inflammatory response (Toulme et al., 2010; Inoue and Tsuda, 2012). In addition, the reactivity of resident microglia-like cells (macrophages) in ganglia (Villa et al., 2010; Franceschini et al., 2013a) opens new vistas on the cellular mechanisms of regulation of neuronal sensitization at ganglion level.

The inflammatory components of neuropathic pain include activation of toll-like receptors (TLR) on neurons and non-neuronal cells (Christianson et al., 2011; Stokes et al., 2013). Experimental TLR stimulation with the component of the bacterial wall lipopolysaccharide (LPS) promotes significant up-regulation of P2X3 receptor function with faster recovery from desensitization (Franceschini et al., 2013b). This treatment also facilitates release of ATP (Franceschini et al., 2012) and tumor necrosis factor alpha (TNFα; Franceschini et al., 2013a). These data suggest that, in sensory ganglion culture, the development of a neuroinflammatory profile facilitates the release of endogenous mediators (including ATP and cytokines) to reinforce the activation of inflammatory cells and constitutively potentiates P2X3 receptors to amplify nociceptive signaling. Similar purinergic signaling likely occurs at central synapse, where block of ATP could represent a potential therapeutic target to limit microglia-mediated inflammatory responses associated with chronic pain sensitization (Ulmann et al., 2008; Jakobsson, 2010). The possibility of ATP-mediated crosstalk also within ganglia has recently been proposed (Ceruti et al., 2008; Ohara et al., 2009; Belzer et al., 2010; Ceruti et al., 2011; Huang et al., 2013), supporting the intrinsic role of satellite glial cells for adaptation mechanisms during chronic pain (Hanani, 2012; Kung et al., 2013) and their role as inflammatory cells (van Velzen et al., 2009).

These observations suggest that there is a complex sequence of cellular responses that exert chemical tissue priming to create the basal conditions permissive for sensitization. In analogy with adaptive immune responses, we expect that interleukin (IL-1β) priming causes amplification of antigen-presenting cells in ganglia, in particular satellite glial cells (Ben-Sasson et al., 2011).

INTRACELLULAR SIGNALING INDUCES SENSITIZATION VIA P2X3 RECEPTOR UPREGULATION

A major property of P2X3 receptors is the ability to rapidly adapt their function to changes in the extracellular milieu via receptor redistribution, trafficking, and phosphorylation. Our former studies have demonstrated that P2X3 receptors of trigeminal sensory neurons are tightly controlled by the fine balance between kinases and phosphatases, which regulate even the basal operational activity of these receptors (Giniatullin et al., 2008).

NGF is sufficient to directly sensitize nociceptive endings causing spontaneous pain (Bennett et al., 1998; Shu and Mendell, 2001; Rukwied et al., 2013), to sensitize P2X3 expressing nociceptors in mice (Ramer et al., 2001; D'Arco et al., 2007, 2009) and to induce acute sensitization of nociceptors in man (McKelvey et al., 2013; Silberstein, 2013). Manipulating NGF levels produces a major impact on ATP-mediated responses by altering intraneuronal signaling pathways (D'Arco et al., 2007; Giniatullin et al., 2008). Pharmacological blockade of protein kinase C (PKC) or Calcium/calmodulin-dependent protein kinase II (CamKII)

activation prevents NGF-induced sensitization (Bonnington and McNaughton, 2003), and NGF neutralization unleashes the Sarcoma tyrosine kinase (Src) kinase blocker C-terminal Src kinase (Csk) to limit P2X3 receptor function at membrane level (D'Arco et al., 2009) and to inhibit neuronal sensitization (Liu et al., 2008). cAMP response element binding protein (CREB)-mediated gene expression in dorsal horn neurons establishes peripheral and central sensitization (Fang et al., 2002) suppressed by extracellular signal-regulated kinase (ERK) blockers, and by protein kinase A (PKA), PKC or CaMK inhibitors (Kawasaki et al., 2004). In line with these data, CGRP signaling pathways activate CREB-mediated P2X3 receptor expression and function (Simonetti et al., 2008).

Using a transgenic knock-in (KI) mouse exhibiting $Ca_V2.1$ R192Q mutated voltage-gated calcium channels (P/Q-type) (Tottene et al., 2009), we previously identified multiple interactors (calcineurin, Cyclin-dependent kinase 5 (Cdk5) and CaMKII) associated to the gain of function of the mutated channel leading to larger intracellular calcium levels that modulate P2X3 receptor function in trigeminal sensory neurons. In particular, enhanced P2X3 receptor-mediated responses are found in KI neurons that depend on constitutive activation of CaMKII and are reversed by the selective $Ca_V2.1$ channel blocker ω-Agatoxin or by pharmacological block of CaMKII (Nair et al., 2010a). CaMKII sensitivity to intracellular calcium levels, is an important switcher of different intracellular pathways (i.e., Cdk5) that influence P2X3 receptor activity and function, as demonstrated in mice expressing $Ca_V2.1$ mutated channels (Nair et al., 2010a). CaMKII is also involved in P2X3 receptors export towards the surface membrane (Xu and Huang, 2004; Fabbretti et al., 2006; Hasegawa et al., 2009), a process that is largely dependent on ambient temperature (Pryazhnikov et al., 2011). Furthermore, the typical agonist-evoked desensitization of P2X3 receptors is associated to dynamic, calcium-sensitive redistribution of such receptors to lipid raft domains (Vacca et al., 2009; Gnanasekaran et al., 2011) and internalization (Vacca et al., 2011; Chen et al., 2012). Thus, the intracellular calcium homeostasis is important to modulate P2X3 receptor responses, as the calcium sensor neuronal Ca2+−sensor proteins (VILIP1) forms a signaling complex with P2X receptors and regulates P2X3 receptor sensitivity to ATP, and it even enhances the neuronal excitability of naive dorsal root ganglion (DRG) neurons (Chaumont et al., 2008; Liu et al., 2013).

In order to transduce ATP signals to downstream responses, we hypothesize that P2X3 receptors require discrete sorting to membrane compartments where, on a short term on-demand basis, all the molecular elements necessary for the correct signal transduction are anchored. Among many, calcium/calmodulin-dependent serine protein kinase (CASK) is a scaffold protein of emerging importance (Hsueh, 2011), sensitive to intracellular calcium, CamKII levels (Lu et al., 2003; Hodge et al., 2006; Malik et al., 2013) and Cdk5 (Samuels et al., 2007), all elements known to strongly modulate P2X3 receptors (Nair et al., 2010a,b).

CASK and P2X3 receptors are found within the same macromolecular complex: our data suggest that CASK acts like a docking point to stabilize P2X3 receptors expression at membrane level, as the CASK knockdown results in proteasome-dependent receptor disassembly and reduced P2X3 receptor current (**Figure 1**; Gnanasekaran et al., 2013). Interestingly, CASK is typically more directed to lipid rafts (Gnanasekeran and Fabbretti, unpublish data) and more strongly associated with P2X3 receptors in the voltage-dependent P/Q-type calcium channel subunit alpha-1A (CACNA 1A) KI mice (Pietrobon, 2010), characterized by altered calcium channel and CamKII activity (**Figure 2**; Gnanasekeran et al., under revison). In the KI model, CASK/P2X3 complex is uncoupled by ω-Agatoxin or the CaMKII inhibitor KN-93, reinforcing the role of intracellular calcium in the modulation of P2X3 receptors in sensory neurons.

One of the peculiar findings associated with the CASK/P2X3 complex is its dynamic nature that largely depends on the receptor functional activity (**Figure 3**; Gnanasekaran et al., 2013). In particular, nociceptive stimulation with NGF application strengthens P2X3/CASK co-purification, while P2X3 receptor function is sufficient to dissociate the complex (Gnanasekaran et al., 2013). It is, therefore, likely that both P2X3 receptor activity and CASK regulators (as CaMKII) control the CASK/P2X3 complex. In its role as scaffold protein, CASK links different adaptors and molecules (including other channels) to elicit further downstream signaling, like the stability and trafficking of receptors towards the membrane (Hsueh, 2011) and vesicle release (Spangler et al., 2013). In brain synapses, CASK has a negative role, as facilitated glutamatergic release was observed in KO CASK mice

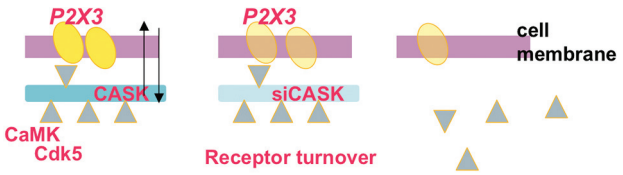

FIGURE 1 | Scheme of dynamic assembly of the CASK/P2X3 receptor complex at neuronal membrane level. The scaffold protein CASK (blue; left) and the P2X3 receptor (yellow) are associated in the same macromolecular complex. Note that adaptor molecules like CamKII and Cdk5 (triangles) are proposed to regulate the CASK/P2X3 receptor complex at membrane level and determine the strength of their interaction. Silencing CASK (middle and right panels) results in uncoupling of the CASK/P2X3 receptor complex followed by internalization of P2X3 receptors and their proteasomal degradation, suggesting that CASK is the anchor to maintain P2X3 receptor at membrane level.

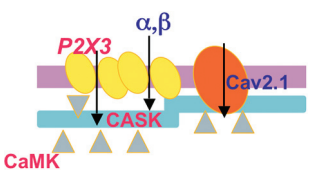

FIGURE 2 | Scheme of the CASK/P2X3 receptor complex in the R192Q mutation of the cacna1a gene. Both CASK and P2X3 receptors are more expressed in membrane lipid rafts of missense Cacna1a KI neurons, suggesting a role of CASK in creating larger P2X3 receptor clusters. $Ca_V2.1$ R192Q channel gain of function and enhanced CamKII activity produced by the increased influx of calcium are important for the formation of the CASK/P2X3 complex and receptor function.

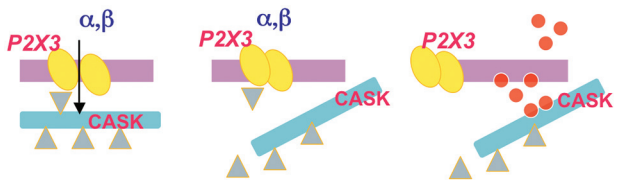

FIGURE 3 | Idealized diagram of the dynamic nature of the CASK/P2X3 complex. P2X3 receptor agonist application (α,β) induces an inward cationic current (left panel) that requires correct assembly of CASK/P2X3. When the agonist application is sustained to produce receptor desensitization, disassembly of the CASK/P2X3 complex occurs (middle). Thus, untethered CASK can be redirected to distinct downstream signaling (right panel) via multiple effectors (red dots).

(Atasoy et al., 2007), in agreement with the inhibitory effect of CASK over the P2X3 over-reactivity (Gnanasekaran et al., 2013).

Recent findings suggest that CASK, known to modulate export and trafficking of N-methyl-D-aspartate (NMDA) receptors (Jeyifous et al., 2009), induces synapse-associated protein 97 (SAP97) conformation changes to control the rate of glutamate receptor insertion into the synaptic compartment (Lin et al., 2013). Whether a similar process occurs in sensory ganglia or at central synapse, and if ATP has a role in this modulation remain a matter for future studies.

It is possible that altered CASK targeting in chronic pain states could impair communication between satellite cells and neurons via aberrant gap junction/hemichannels function (Márquez-Rosado et al., 2012) and with possible damage of neuro-satellite cell units. In particular, binding of CASK to neurexins and neuroligins in heterologous synapses (Fairless et al., 2008; Gokce and Südhof, 2013), indicates its potential involvement in a structural process to shape the extent and location even of neuron/non-neuronal cell communication.

Furthermore, at central level, it seems likely that presynaptic CASK/P2X3 functional interaction regulates synaptic strength in the spinal dorsal horn, reinforcing the interest for P2X3 receptors as key modulators of the fiber sensitivity in chronic pain.

CONCLUSIONS

In view of their high agonist affinity, P2X3 receptors appear as major candidates to sense even small changes in extracellular ATP and transduce them into downstream neuronal responses. Further potentiation of the ATP effects is determined by intracellular calcium signaling and subsequent kinase activation that mediates P2X3 receptor phosphorylation, expression and turn-over. Finally, membrane specialized domains could convey specific responses via dedicated signal transduction machinery whereby CASK could serve as a platform to orchestrate ATP signaling through sorting and redistribution of P2X3 receptors. ATP receptor stimulation could determine further release of neuromodulators (Gu and MacDermott, 1997), recruitment of inflammatory cells and progression to neuronal sensitization, thus contributing to fine regulatory mechanisms and strong plasticity of sensory neurons. Understanding the spatio-temporal scale of these processes is a priority to propose molecular targets useful for clinical applications to chronic pain.

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The therapeutic promise of ATP antagonism at P2X3 receptors in respiratory and urological disorders

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A sensory role for ATP was proposed long before general acceptance of its extracellular role. ATP activates and sensitizes signal transmission at multiple sites along the sensory axis, across multiple synapses. P2X and P2Y receptors mediate ATP modulation of sensory pathways and participate in dysregulation, where ATP action directly on primary afferent neurons (PANs), linking receptive field to CNS, has received much attention. Many PANs, especially C-fibers, are activated by ATP, via P2X3-containing trimers. P2X3 knock-out mice and knock-down in rats led to reduced nocifensive activity and visceral reflexes, suggesting that antagonism may offer benefit in sensory disorders. Recently, drug-like P2X3 antagonists, active in a many inflammatory and visceral pain models, have emerged. Significantly, these compounds have no overt CNS action and are inactive versus acute nociception. Selectively targeting ATP sensitization of PANs may lead to therapies that block inappropriate chronic signals at their source, decreasing drivers of peripheral and central wind-up, yet leaving defensive nociceptive and brain functions unperturbed. This article reviews this evidence, focusing on how ATP sensitization of PANs in visceral "hollow" organs primes them to chronic discomfort, irritation and pain (symptoms) as well as exacerbated autonomic reflexes (signs), and how the use of isolated organ-nerve preparations has revealed this mechanism. Urinary and airways systems share many features: dependence on continuous afferent traffic to brainstem centers to coordinate efferent autonomic outflow; loss of descending inhibitory influence in functional and sensory disorders; dependence on ATP in mediating sensory responses to diverse mechanical and chemical stimuli; a mechanistically overlapping array of existing medicines for pathological conditions. These similarities may also play out in terms of future treatment of signs and symptoms, in the potential for benefit of P2X3 antagonists.

Keywords: P2X3, AF-219, visceral disorders, afferent sensitization, airways hyperreactivity, cough, urinary symptoms

INTRODUCTION

ATP is an abundant, multifaceted molecule: the chemical capital stoking metabolism in every cell; ubiquitous transmitter and autocrine signal; extracellular herald of movement, distension, distress, ischemia, damage and inflammation (Burnstock, 2012). Moreover, ATP can foment irritation, pain and discomfort, provoking maladapted autonomic reactions (Burnstock, 2012; Ford, 2012; North and Jarvis, 2013). The mechanisms by which ATP is liberally and specifically discharged by cells during this process are now being delineated, with evidence for participation of processes akin to synaptic vesicle release, hemichannel efflux, and extrusion through ligand gated channels, in addition to the spillage of copious ATP during cell distress and rupture (Burnstock et al., 2012). In a variety of pathological settings, these release mechanisms may operate more aggressively, or alternatively enzymatic disposition mechanisms (nucleotidases) become weakened, leading to elevations in background and stimulated ATP concentrations in extracellular milieu. Such settings include inflammatory diseases (arthritis), ischemia, cancer, airways pathology, and bladder disorders, with the associated implication that excess ATP *per se* contributes to heightened sensations that attend these disorders (Ford, 2012).

This commentary focuses on the P2X regulation of primary afferent neurons (PAN), which link sites in the peripheral receptive field to the first synapse of the sensory pathway in the spinal dorsal horn and dorsal brainstem, and in particular how they process signals from hollow organs. PANs have their cells bodies in the dorsal root and cranial ganglia, their peripheral fibers en route to the receptive fields can be short or extremely long, and they exist in several distinctive types, with differential morphological properties, speeds of conduction and molecular markers and receptors. The diversity of sensorineuron types confers a wide range of functions from low threshold (non-nociceptive) proprioceptive, mechanosensitive and thermosensitive detection (mostly the faster fibers), to high threshold (nociceptive) fibers sensitive to noxious mechanical and/or chemical stimuli: some

which transmit signals rapidly (Aδ), and many others slowly (C). What is clear is that primary afferents are the first conduit for all sensory information, and thus the primary site that may undergo modulation and plasticity in chronic disease and injury, leading to persistently altered sensation or dysaesthesia (Basbaum et al., 2009; Burgess and Williams, 2010). ATP, acting via P2X3-containing receptors, is clearly able to modulate, perhaps even drive, some of these plasticity changes, and such findings may have ramifications in identifying novel therapeutics for a range of sensory maladies (Ford, 2012; North and Jarvis, 2013).

In considering the effects of P2X3 activation in visceral "tube and sac" (or "hollow-organ") systems, our focus is placed on the lower urinary tract (LUT) and airways. These systems share many traits from a morphological, functional and therapeutic perspective (as discussed in more detail below), and are notable in that the most common pathologies in either system are associated with a range of primary symptoms and signs that include persistent and heightened, inappropriate irritative sensations and exaggerated autonomic reflexes, as depicted in **Figure 1**. To date, the primary sensory causes of both hyperesthesia and hyperreflexia in these systems have remained unclear, and have been therapeutically intractable and/or underexplored. Our suspicion is that this situation may be on the threshold of a significant advance, with the arrival of selective P2X3 receptor antagonists. Signaling via ATP-P2X3 seems to be not just another participant in the extracellular "soup" of transmitters, autacoids and inflammatory cytokines that contribute to pathologically suppressed sensory thresholds, but is a key common aggravator in the receptive field: promoting sensitization of PANs, priming them to many forms of chemical and physical stimuli that drive afferent excitatory traffic. Thus primed with lowered thresholds for activation, stimuli that would normally be perceived as innocuous are able to trigger inappropriately heightened and unpleasant sensations (hyperesthesia) and untoward responses (hyperreflexia), as illustrated in **Figure 2**. The components of this include: a convergent process whereby abundant irritative stimuli elicit increases in extracellular ATP concentrations, especially in disease; increased expression and cell-surface trafficking of the P2X3-containing receptors on PAN endings (Giniatullin et al., 2008; Gnanasekaran et al., 2011); activation of key downstream excitatory pathways, such as CASK and PKC isoforms (especially PKCε), by these elevated ATP levels leading to reduced thresholds for activation of PANs by many other sensitizing stimuli (Parada et al., 2005; Gnanasekaran et al., 2013; Prado et al., 2013; Volonté and Burnstock, 2013). In focusing on the current topic of P2X3 participation in the sensitization of *peripheral* terminals of PANs in common sensory pathologies, it may be noted that we are potentially ignoring another possible key locus of sensitization: the central PAN terminals in spinal and brainstem dorsal horn. It is acknowledged that P2X3 receptors may participate in modulating the strength of synaptic communication with second order neurons (Gu and MacDermott, 1997) and that CNS penetrant antagonists may reduce sensory wind-up quite distal from the receptive field; however, for the focus of the current review these aspects will not be discussed further.

THE SENSITIZING PROPERTY OF ATP

Five decades ago it was reported that fluid from lysed red blood cells applied to exposed blister bases on the human forearm evoked pain and discomfort (Keele and Armstrong, 1964). In subsequent investigations (Collier et al., 1966; Bleehen et al., 1976; Bleehen and Keele, 1977), the candidate chemicals responsible were successively eliminated revealing that of the many chemicals

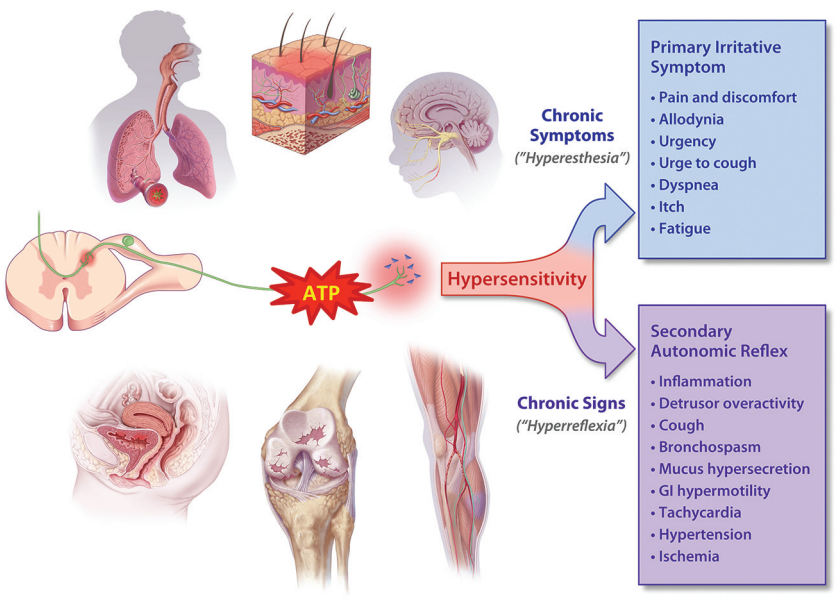

FIGURE 1 | ATP is released in heightened amounts in a variety of somatic and visceral tissue systems and may cause hyperexcitability ("sensitization") of PANs. Depending on the nature of the affected tissue, the elevated afferent discharge drives the increased perception of irritative symptoms (hyperesthesia) as well as lowering the threshold for activation of autonomic reflexes. These elevated reflexes (hyperreflexia) in turn give rise to many of the signs of chronic disorders, which can usually be easily observed or measured, if not perceived by the patient.

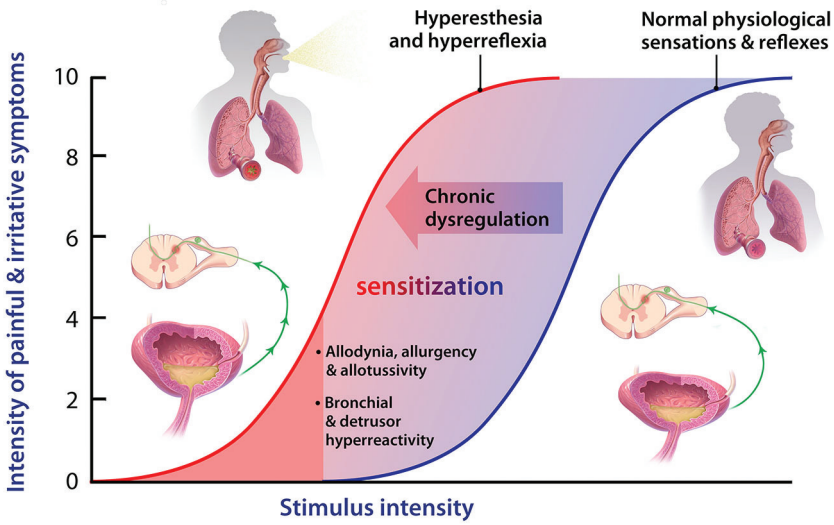

FIGURE 2 | Normal physiological sensory perception and reflexes are important defensive mechanisms, under conditions of acute stress or physical threat, when a high stimulus intensity (blue sigmoid) represents potential harm. During chronic dysregulation, afferent functions experience sensitization, wherein normally low threshold or sub-threshold stimulus intensities, posing little or no threat, now induce unpleasant sensations and inappropriate autonomic responses. Many mechanisms have been proposed to contribute to such sensitization, but the key priming autacoid remains elusive, though it could turn out to be ATP in some visceral systems such as LUT and airways.

discharged it is ATP itself that causes much of the pain. A generation later, in a reductionist version of the forearm studies, a co-culture of rat trigeminal afferents with skin cells was described (Cook and McCleskey, 2002): an electrode recorded activity of one neuron while a proximal keratinocyte was lysed yielding excitations following spillage of sensitizing cellular contents. The responsible chemical present in the lysate: ATP, acting—as was deduced in the interim—by opening P2X3-containing cation channels. These findings are consistent and profound: ATP is a key sensitizing autacoid.

The effects of ATP on blister bases can be mimicked by intradermal injection (Coutts et al., 1981), and by iontophoretic application to UV sensitized skin (Hamilton et al., 2000) in healthy human subjects. Intradermal ATP was also shown to sensitize cutaneous C-fibers (Hilliges et al., 2002). Subsequently, ATP was reported to produce moderate to strong pain and tenderness after intramuscular (trapezius) infusion in volunteers (Mork et al., 2003), which has recently been extended with a report of pain, ache and fatigue after injection into themar muscle (thumb pad; Pollak et al., 2013). Preclinical correlates of similar effects of ATP in rodents are also well described, based on "pain-related" or "nocifensive" responses evoked. For example, ATP (or $\alpha\beta$-MeATP) injection into the paw of rat evokes nocifensive responses (Bland-Ward and Humphrey, 1997; Hamilton et al., 1999, 2001; Tsuda et al., 2000). Similarly, ATP or $\alpha\beta$-MeATP injection into sensitized temporomandibular joint (TMJ) attenuates pressure thresholds (Shinoda et al., 2005), and into dental pulp sensitizes trigeminal afferents (Cherkas et al., 2012).

These effects of ATP on somatosensory systems form part of a significant body of evidence that P2X3 receptors contribute to increased nocifensive behaviors in many models of musculoskeletal and neuropathic pain, as extensively reviewed (see Khakh and North, 2006; Burnstock, 2013). The impact of selective antagonists to inhibit behaviors in these models is impressive allowing justified speculation about the potential for benefit in human musculoskeletal and neuropathic pain conditions (Jarvis et al., 2002; Ford, 2012; North and Jarvis, 2013). However, although somatic pain conditions seem to capture more attention, it is in the viscera, where sensory symptoms are so poorly addressed, that a greater breadth of evidence has evolved from a wide range of investigations that place ATP and the P2X3 receptor mechanism at the heart of pathological sensitization and where therapeutic potential may be most appealing.

In visceral systems, irritative direct effects of ATP have also been described: two reports have shown that inhalation of ATP can activate sensory responses in the airways driving the perception of symptoms (coughing, wheezing, dyspnea and chest-tightness; Basoglu et al., 2005) and bronchoconstriction (reduced FEV1; Pellegrino et al., 1996; Basoglu et al., 2005), with asthmatic subjects being more sensitive than healthy control subjects; it should be noted that on a molar basis ATP was found to be more potent than AMP, indicating that the effects were due to the inhaled ATP and not its dephosphorylated metabolite (adenosine). It has also been reported from several studies that intravenous infusions of ATP in pre-terminal cancer patients produces a common adverse effect in a large proportion of patients: chest discomfort/pain, dyspnea and the urge to take a deep breath (Haskell et al., 1998; Beijer et al., 2007). Some of these effects had been predicted based on a small number of preclinical evaluations examining ATP activation of canine pulmonary vagal afferents (Hurt et al., 1994) as well as participation in induction of cough in guinea pigs in response to tussive agents (Kamei et al., 2005; Kamei and Takahashi, 2006).

Unlike the situation in airways and somatic systems, no clinical reports indicate that ATP has been studied after its direct intravesical infusion, and thus it remains to be determined whether activation of sensation within urinary bladder could be so elicited. In animals, it clearly does produce marked local effects on afferent function after instillation (as described later), and this has been widely studied as a model of bladder irritability (Pandita and Andersson, 2002; Yu and de Groat, 2008; Ford and Cockayne, 2011). One thing that is consistently notable in all of the studies looking at ATP application in clinical and preclinical settings is that the effects of ATP on sensation (or afferent traffic) are greater when there is underlying irritation (a blister, UV and chemical insult) or pathology (asthma, bladder pain syndrome (BPS)).

MORPHOLOGICAL AND WIRING SIMILARITIES OF URINARY TRACT AND AIRWAYS

The urinary bladder and airways walls show a significant organizational similarity, somewhat superficially reflected by the cartoon diagrams in **Figure 3**. In both tissues, a smooth muscle layer (more extensive in the case of the detrusor), under phasic excitatory control of parasympathetic efferent nerves, provides for compliance and tone during distension and constriction, and may become tonically activated in compliance disorders such as asthma and overactive bladder (OAB). This muscularis is anchored by a layer of cartilage in the airways (of decreasing presence with narrowing of the airway branches) and by serosal fat, fibrous connective tissue and peritoneum in the LUT. The smooth muscle itself is layered by a submucosa, or lamina propria, which harbors many connective tissues, vascular plexuses, inflammatory, intrinsic modulatory (myofibroblasts) and secretory cells. The most luminal layer has its margin as the basement membrane, and supports the epithelium, which in the airways is a pseudostratified columnar epithelium with many cells ciliated on the apical surface; and in urinary bladder is a transitional epithelium, comprising a basal cell, 3–4 transitional cells, and a highly specialized apical "umbrella" cell that is coated with unique proteins call uroplakins. Clearly the apical epithelial differentiation contrasts sharply between airways and bladder, reflecting the starkly different physiological needs regarding permeance: airways are obligatorily designed for chemical and fluid exchange, whereas the LUT uses its apical, uroplakin-decorated surface as a primary barrier to limit permeance from lumen to parenchyma (although this barrier function is often undermined by infections and in chronic diseases such as BPS; see Wang et al., 2005).

What appears to be intriguing in each tissue system is that the epithelium plays a key role not only in protecting the tissue from "the outside world", providing resistance to pathogens and defense against chemicals, but also in sensing changes (pressure, movement, irritation) and signaling to adjacent afferent nerve endings. In this latter respect, it is well established that copious release of ATP accompanies such environmental changes, and this ATP communicates with the afferents via P2X3 receptor activation.

On a simplified level, the wiring control of these organ systems also exhibits similarities. In the LUT, sensory afferents of the pelvic, pudendal and splanchnic (hypogastric) nerves, with the unmyelinated C-fibers in significant numerical predominance

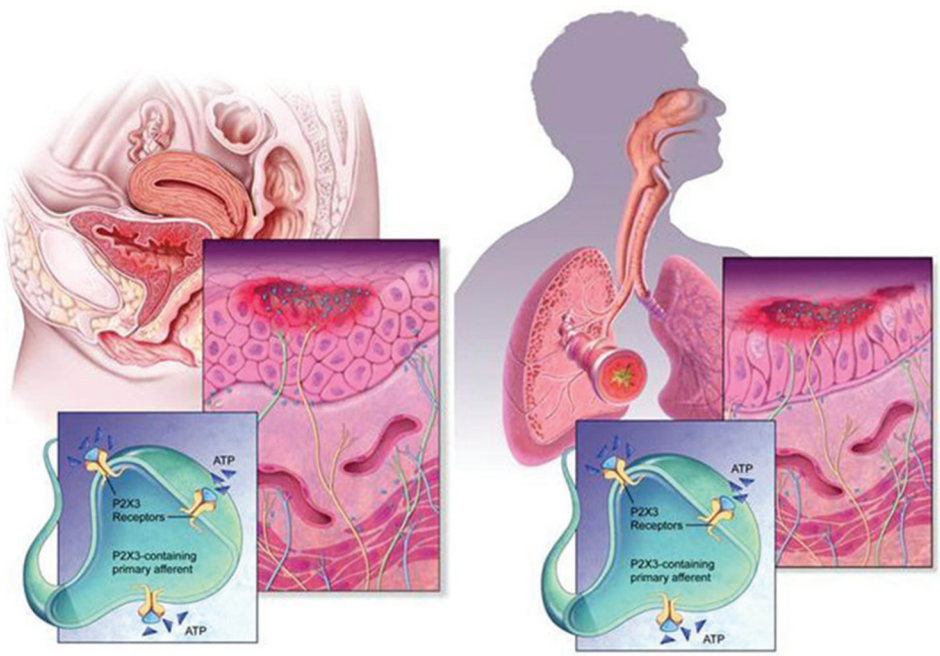

FIGURE 3 | Morphology and wiring of LUT and airways. The urinary tract and airways walls show a similar overall morphology, despite quite distinct structural differentiation in the epithelial layer. In both systems, ATP (shown as blue triangles) is present in large extracellular concentrations, released by various cells including epithelia, fibroblasts and smooth muscles, and can activate C-fiber afferent and promote sensitization. Release of ATP is augmented in conditions of stress, injury, inflammation and infection.

over Aδ, convey much of the ongoing information to the CNS, where primary coordination is modulated at the pontine level. Efferent excitatory function to support voiding is largely carried by pelvic nerve parasympathetic fibers, with sympathetic innervation of the bladder neck and urethra providing the autonomic support during the long periods of continence, coupled with suspension of parasympathetic drive and maybe some sympathetic activity maintaining detrusor muscle compliance during filling. A large proportion of C-fibers and some Aδ-fibers from the LUT express P2X3 subunits and respond to ATP.

The upper and lower airways are densely innervated by sensory and autonomic fibers. Although a small percentage of fibers originate from dorsal root ganglia (DRG), most afferent fibers are carried by the vagus nerve, with cell bodies contained in the nodose (epibranchial placode derived) and jugular (neural crest derived) cranial ganglia (Undem et al., 2004). These embryological derivations lead to differentiation also in the properties of the numerically predominant populations of afferents, the C-fibers. For example, the proportion of fibers containing neuropeptides is high in DRG and jugular and is low in nodose. Similarly, there is differentiation in terms of P2X3 containing receptors, such that nodose fibers express P2X2 and P2X3 subunits, and likely carry responses by P2X2/3 heterotrimers, whereas neural crest derived afferents express P2X3 and little P2X2, and respond via the P2X3 homotrimer, at least in rodents (Undem et al., 2004; Kwong et al., 2008; Nassenstein et al., 2010).

How all these elements militate together and integrate their participation in organ function has been studied extensively over decades, using all sorts of *in vivo* and deconstructed systems; of these, one type of approach has offered greatest perspicacity: the isolated tissue-nerve preparation, as further discussed below.

TUBE AND SAC FUNCTION AND DYSFUNCTION: LOWER URINARY TRACT

The functions of the LUT, dominated by the passive, low pressure storage of volumes of urine, irregularly interrupted by brief episodes of coordinated micturition, operate by what seems a simple switch operated process: long periods of detrusor compliance and expansion coupled with sympathetically maintained bladder neck closure and urethral coaptation are conveniently—and preferably consciously—awoken by a parasympathetically driven coordinated detrusor muscle contraction and urethral/sphincteric relaxation with parallel suspension of sympathetic and somatic motor drive. Outflow of these efferent autonomic signals are regulated by pontine storage/micturition centers (Barrington's nuclei), in turn under descending cortical control (Fowler et al., 2008; de Groat and Wickens, 2013). This latter factor is somewhat unique for a visceral organ system.

The afferent limb of urine storage and elimination displays a dualism, both structurally and functionally. Two operationally and neurally distinct paths appear to sense the condition of the LUT, and relay this information to spinal and supraspinal circuits, via the periaqueductal gray (PAG) to pontine control centers (de Groat and Wickens, 2013). One of these uses in particular thinly myelinated Aδ fibers to detect volume expansion at high thresholds (via stretch and chemical receptors) in the bladder wall and conveys this information to the pontine centers and beyond, to inform conscious and graded perception (sense of fullness, developing urge to void, extreme urge to void) and prepare consciously integrated autonomic reflex coordination. The second system, perhaps a more primitive one, engages unmyelinated C-fibers and detects filling at a broader range of volume thresholds, as well as signals of local distress (infection, inflammation), and can elicit local (spinal segmental) initiation of efferent autonomic responses that lead to increased detrusor activity. de Groat (1997) has described this C-fiber system as the "reflex bladder" circuit, in that it operates without conscious control. In a healthy adult these latter afferent signals are considered to be under considerable descending inhibition that is established purposely during early development of conscious voiding control (de Groat, 1997). Accordingly, a healthy adult relies mostly on the first (Aδ) pathway to ascertain bladder status and desire to void, with C-fiber signals failing to escape tonic suppression except when local pathological conditions arise (such as in infection). The "reflex bladder" (**Figure 4**) may represent the state dominant in the infant, before descending control emerges, and that can abruptly return during urinary tract infections (where the discharge to segmental circuits is too great to suppress), as well as after the erosion of the descending inhibitory influences that can occur abruptly (spinal injury, stroke) or gradually, as in neurodegenerative disease, or even aging (de Groat, 1997).

It has been suggested that the common urological conditions associated with irritative LUT symptoms (LUTS): urgency, frequency, nocturia, incontinence, discomfort and even pain are mediated by the unsilencing of these C-fiber pathways and emergence of the reflex bladder. Thus, an optimal approach would be to target selectively these afferents for suppression, to provide relief for countless numbers of LUTS sufferers with conditions such as OAB, benign prostatic hyperplasia (BPH), BPS and chronic pelvic pain syndrome (CPPS): all representing forms of pelvic hypersensitivity, typified by inappropriate urgent sensations at modest filling that trigger unwelcome efferent responses (hyperreflexia). Ideally, an understanding of what substances (cytokines, trophic factors) cause the unsilencing of the reflex bladder would be additionally helpful.

TUBE AND SAC FUNCTION AND DYSFUNCTION: THE AIRWAYS

As was described for the LUT, the airways depend upon the coordination of afferent inputs to central (in this case medullary) nuclei to regulate the critical and extensively rhythmic efferent autonomic influences upon upper and lower respiratory tissues. Given the functional complexity of airways and the second by second need to modulate and tune functions, exquisite surveillance of the tissues (and the blood gases) and timely modulation of efferent responses are all the more critical to homeostatic wellbeing, and thus the complexity of afferent inputs is amplified.

What is clear is that the airways need to respond appropriately to subjectively perceived irritations (particulate or chemical) as well as the perception of "air hunger". Likewise, they must respond to afferent signals with well-regulated autonomic reflexes that may variously govern airways caliber and conductance

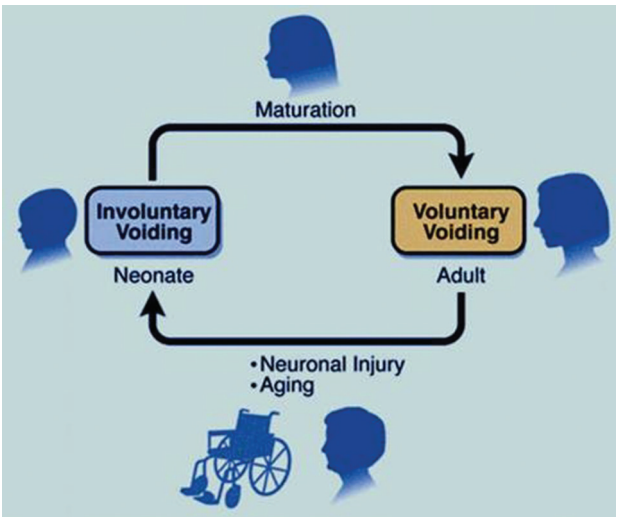

FIGURE 4 | The reflex bladder. In the neonate, C-fibers carry bladder filling signals to activate spinal segmental reflexes that regulate involuntary excitatory responses. Overlaying and—in a healthy person—overriding this reflex bladder is a voluntary control system, that is laid down during the early post-natal years. Here, Aδ fibers play a dominant role, impacting with second order neurons that send signals up to the brain. In neurogenic-bladder patients (typified following spinal injury), a rapid deterioration of this descending inhibitory control occurs, "unmasking" the C-fiber reflex beneath. The more gradual emergence of this reflex, due to idiopathic loss of descending C-fiber inhibition, may account for the development of many LUT symptoms (as conceived by WC de Groat).

(bronchoconstriction and dilation), fluid and mucus secretion and ciliary clearance. As described for the LUT, very common airways pathologies arise that are associated with poorly coordinated afferent-efferent communication, inappropriate sensations (hypertussive perceptions and dyspnea) and markedly reduced sensory thresholds that lead to maladaptive reflexes (airways hyperreactivity and hypersecretion). Variously, these signs and symptoms are common in chronic cough, asthma, COPD and interstitial disease, with hypersensitivity and hyperreflexia being key manifestations of widespread diseases. Although these diseases may have etiological bases in immune dysregulation and chemical exposure, it remains clear that current therapeutics offer merely patchy resolution of some but not all signs and symptoms. Though the underlying causes may largely drive changes through inflammatory pathways and factors, it remains clear that afferent targets in the respiratory system could offer important opportunities for therapeutic intervention.

TISSUE-NERVE PREPARATIONS

An approach that has been employed to impressive effect in the study of both LUT and airways to elucidate the origination and composition of afferent nerve responses to mechanical and chemical stimulation signals is the intact "tissue-nerve" preparation from rodents. The bladder-nerve preparations (as originally defined by Morrison and colleagues: Namasivayam et al., 1998; Morrison, 1999) and the airways-nerve preparations (in various forms, as described by Undem and Colleagues: Myers et al., 1991; Riccio et al., 1996; McAlexander et al., 1999; and by Fox et al., 1993, 1995) have provided interesting insights to the types of sensory fibers activated by distinct stimuli and what chemical messengers, receptors and channels mediate them. The advantages of these in vitro preparations are nicely described in the respective reports: they allow for study of chemical application and sensitization, and controlled mechanical force and volume displacement at the level of the airway or urinary tract "receptive" nerve endings, but where such variables can be isolated from the impact of descending nervous modulation and hemodynamic or inflammatory factors. They also allow for more precise direction of the nature and location of stimuli, and allow control over concentrations of exogenous irritants and modulators. The results from these studies have been very revealing. Most particularly, these preparations allow for study of maneuvers and chemical stimuli that directly effect changes in afferent discharge as well as those that may act directly but function to modulate or sensitize afferents to other stimuli (e.g., sensitizing autacoids; Myers et al., 1991). In the context of the ATP-P2X3 mechanism, the findings have been insightful.

In the LUT preparations (mostly bladder/urethra—pelvic nerve, but also ureter—pelvic/hypogastric nerve), Namasivayam et al. (1999) were the first to follow the bold proposal (from Ferguson et al., 1997) that the distension induced release of copious ATP from urothelial cells in bladder reflected a role for local release of the nucleotide in signaling to primary afferents on the status of bladder filling. They showed quite clearly that the multi-unit recordings from pelvic nerve during distension were largely (> 50%) dependent on ATP receptors, by showing that treatment of the distending bladder with the nonselective P2 antagonist suramin reduced firing by a half, and that $\alpha\beta$-MeATP desensitization led to 65–75% reduction of nerve firing. Subsequently, it was shown that bladders from P2X3 gene knockout and P2X2–P2X3 double knockout mice would distend with much larger volumes of fluid before significant response was observed in pelvic nerve recordings (Vlaskovska et al., 2001;

Cockayne et al., 2005). The role of ATP mediating pelvic nerve firing during filling has been explored by many groups, and it is clear now that certain subpopulations of afferent nerves may mediate this specific response, that the effects are sensitive to blockade with P2X3 selective antagonists (Rong et al., 2002; Zagorodnyuk et al., 2007, 2009; Sun et al., 2012) and in particular that the contribution of this receptor function is upregulated in disease models (Yu and de Groat, 2008, 2010; Sun et al., 2012); this occurs possibly due to increased receptor expression and coupling, and/or an elevation in the amount of ATP that is discharged by distension. Most recently, the beneficial impact of botulinus toxin (onabotulinumA) on sensory symptoms in OAB have been modeled in a mouse bladder-nerve preparation illustrating that intravesical exposure of the toxin reduces both ATP release during distension and afferent nerve discharge (pelvic and hypogastric; Collins et al., 2013). Given this seemingly unique role of the ATP-P2X3 mechanism in the LUT, it is not surprising that selective antagonism of this receptor population represents a breakthrough opportunity for management of conditions where sensory symptoms (urgency, frequency, nocturia, pain) and attenuated thresholds for initiation of reflex detrusor excitability are common.

In the airways, focus on ATP as a sensitizing autacoid or transmitter has been limited, indeed more focus has been placed on its potential roles in inflammation, ciliary motility and mucus clearance (Idzko et al., 2007; Cicko et al., 2010; Koeppen et al., 2011). However, our recent efforts to examine the contribution of ATP-P2X3 signals to afferent excitation generated evidence for significant convergence of stimuli onto this target. Using an isolated perfused lung-vagus nerve preparation (Weigand et al., 2012), it was seen that, consistent with previous reports, methacholine and histamine were both able to produce bronchoconstriction and action potential discharge in nodose derived vagal fibers. ATP was also able to activate nodose fibers, but without eliciting changes to perfusion resistance, indicating a lack of direct effect on smooth muscle tone. The effects of ATP were inhibited by two chemically distinct P2X3-P2X2/3 antagonists, TNP-ATP and AF-353, which also were both able to inhibit the excitatory effects of methacholine and histamine on nodose neurons, though leaving the constrictor responses unaffected. The implication, that ATP is the mediator of the indirect neural responses to the two spasmogens, was also in evidence by the loss of neural responses if apyrase was present. Thus, ATP is a necessary intermediate for bronchoconstriction-induced nodose C-fiber excitation; the fact that ATP is unable to elicit action potentials of jugular-derived vagal afferents also explains why jugular C-fibers are not activated by histamine (Undem et al., 2004; Kwong et al., 2008). These dramatic findings put ATP in the spotlight as a key sensitizer of airways afferents under a variety of physiological circumstances, and raise the possibility that specific antagonism of P2X3-containing receptors may have a potential in several respiratory conditions where afferent activation and hyperreflexia drives both bronchial hyperreactivity and abundant sensory symptoms that are so poorly managed.

It is of significant note that the use of the LUT and airways "organ-nerve preparations" has allowed for the identification in each system of a major and shared pathway, that so crucially coordinates integrated signaling, and that might have been more difficult to reveal so clearly in the intact organism or by using a more reductionist approach. These isolated preparations allow for experimenter designed degrees of de-construction and signal isolation that can be examined and tested, then be re-constructed to understand their place in physiology and pathobiology.

CURRENT AND FUTURE MANAGEMENT OF SIGNS AND SYMPTOMS IN LOWER URINARY TRACT AND AIRWAYS

Given the morphological and neurophysiological similarities between these organ systems, their dependencies on movement and compliance coupled with finely tuned parasympathetic coordination, it is perhaps not surprising that the current treatment options for millions of patients with signs and symptoms of disorder in either system share so many mechanistic features. Ironically, when one thinks about the pathophysiological underpinnings that have motivated decades of pharmaceutical discovery in these systems in the quest of novel, transformative therapies, the approaches could hardly be more different: in the airways, the immune system has received preponderant focus, with an abundance of enzyme, chemokine and cytokine targets and various immune and inflammatory cell types pursued as the cause du jour; in the LUT, it is the efferent neuroeffector influences and the apparently overexcitable smooth muscles that have historically been the focus. In neither case has decades of effort revealed a genuinely robust return in terms of transformational therapeutic attenuation of unmet need. Similarly, in both systems, the attention paid to the afferent circuits has been relatively insubstantial, especially given that the presenting disease burden represents such profoundly disturbing sensory experiences for so many of the patients: labored breathing, wheeziness, chest tightness, air hunger, urge to cough and cough itself; urinary frequency, persistent urgency, discomfort and pelvic pain, disturbed sleep (nocturia) and continence failure.

In the face of this significant sensory plight, the pulmonary and urological patients both have access to two mechanistic classes in common that aim to reduce the parasympathetic excitation of smooth muscles and improve compliance: antimuscarinics (for example: ipratropium, tiotropium and aclidinium for airways symptoms; oxybutynin, tolterodine and solifenacin for LUT symptoms) and β agonists (for example: albuterol, salmeterol and formoterol for airways symptoms; the newly developed mirabegron for LUT symptoms). The measure of clinical effectiveness of such agents within these systems is difficult to compare; however, the accessibility of the airways for inhalation therapy allows local delivery of drug concentrations imparting more meaningful clinical response than seems to have been achieved so far in LUT, where intravesical delivery is much less convenient or well-tolerated. Currently, systemic adverse events (AEs) greatly impair dosing to therapeutic effectiveness and frequently discourage LUTS patients from persisting with treatment. Whether this latter situation is improved with the new $\beta 3$ adrenoceptor agonist will be followed closely.

Beyond these classes, airways patients also derive significant benefit from the local delivery of corticosteroids which go some way to reduce the inflammation and severity of some of the common symptoms (and indeed may even blunt afferent discharge).

Such an option has been examined in the LUT, but here local approaches are not so feasible or convenient, and safety concerns with chronic systemic exposure would preclude routine steroid usage even if some benefit might be afforded (as has been reported for severe ulcerative forms of interstitial cystitis). In both systems, direct pharmacological targeting of sensory targets has been of limited value so far, except in some exceptional situations such as using inhalation delivery of local anesthetics (e.g., lidocaine) for intractable dyspnea in cardiac patients or their intravesical instillation for pain in BPS.

Thus, the current therapeutic options for patients whose lives are seriously impacted by abundant sensory symptoms from both organ systems do not directly target the C-fibers and their triggered reflexes; rather they focus on the efferent limb on the reflex: block the parasympathetic drive to smooth muscle excitability, or increase detrusor compliance by activating β-adrenoceptors receptors that are present (though in neither case likely well innervated). Overall, these approaches offer benefit but are potentially undermined by on-target AEs (especially, in the case of antimuscarinics, dry mouth and CNS effects). Clearly, there is a great need for something directed at quelling the sensory pandemonium.

P2X3 ANTAGONISTS—PROGRESSION TO CLINIC

So far, despite the efforts of a several pharmaceutical organizations (see Gum et al., 2012), only one medicinal candidate P2X3 antagonist has progressed into human studies (Ford, 2012). The aryloxy-pyrimidinediamine, AF-219 (Ford et al., 2013; Smith et al., 2013) is an orally active small molecule (Mol Wt. ∼350 Daltons) antagonist at human P2X3-containing receptors. The inhibitory potency (IC_{50}) of AF-219 has been reported as ∼30 nM versus recombinant hP2X3 homotrimers and 100–250 nM at hP2X2/3 heterotrimeric receptors, potencies very similar to those reported for recombinant rat receptors, and it displays no inhibitory impact on any non-P2X3 subunit containing receptors (IC_{50} values $\gg$ 10,000 nM at recombinant homotrimeric hP2X1, hP2X2, hP2X4, rP2X5 and hP2X7 channels). Reports from other related chemical members of this P2X3 selective pyrimidinediamine class have shown that the mechanism of inhibition is non-competitive (allosteric) and have been mixed regarding species-independency of P2X3 receptor potency estimates: AF-353 (Gever et al., 2010) shows remarkable potency congruency between human and rat recombinant P2X3 homotrimers (IC_{50} values of 8.7 and 8.9 nM, respectively) whereas the more potent analog AF-792 (also referred to as RO-51; developed initially as a potential prodrug for AF-353) was shown to be less potent at human versus rat P2X3 receptors in one report (Serrano et al., 2012) and yet species-independent in another (Jahangir et al., 2009). It is important to note that some selectivity for P2X3 versus P2X2/3 channels has been a common claim across several chemical classes of inhibitors (see Gum et al., 2012: e.g., AF-219 analogs, nucleotides such as TNP-ATP, benzenetricarboxylic acids such as A-317491), although in most studies values reported are not affinity determinations but IC_{50} estimates. Under such circumstances true selectivity cannot be categorically inferred, especially for the competitive antagonists (such as TNP-ATP and A-317491) as the IC_{50} is a parameter that will change with agonist concentration used and depends on agonist potency at the different trimers.

To date, AF-219 has completed four Phase 1 (safety, tolerability and pharmacokinetic studies in normal healthy volunteers) and one Phase 2 (patient) studies, with 3 additional Phase 2 studies in progress.[1] The three ongoing study are in osteoarthritic joint pain, BPS/interstitial cystitis and in asthmatic patients with results to be reported in mid-2014. The completed patient study was undertaken in patients with chronic, treatment-refractory cough, and was disclosed at the European Respiratory Society congress in September 2013 (Abdulqawi et al., 2013). In this 24 patient two (14-day) period, placebo controlled crossover study, AF-219 markedly and significantly reduced objective cough frequency: daytime cough rate –84% (95% CI –94 to –60; $p < 0.001$; per protocol analysis), and comparably reduced patients subjective cough related symptoms.

This was a relatively small pilot study, with a single, high daily dose level compared with placebo in patients with considerable cough burden. Nevertheless, the unprecedented magnitude of efficacy observed and its objective nature, coupled with the apparent absence of benefit on placebo, would appear to offer strong initial validation of the P2X3 target in a patient group with a significant "hollow-organ" sensory disorder, in keeping with the potential identified from preclinical in vitro and organ-nerve preparations. These findings also augur well for other signs and symptoms of airways disease that are impacted by afferent hyperexcitability, as well as important and bothersome conditions emanating from other tube and sac systems, including LUT. We await their outcomes with anticipation and excitement.

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Primitive ATP-activated P2X receptors: discovery, function and pharmacology

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Adenosine 5-triphosphate (ATP) is omnipresent in biology. It is therefore no surprise that organisms have evolved multifaceted roles for ATP, exploiting its abundance and restriction of passive diffusion across biological membranes. A striking role is the emergence of ATP as a *bona fide* transmitter molecule, whereby the movement of ATP across membranes serves as a chemical message through a direct ligand-receptor interaction. P2X receptors are ligand-gated ion channels that mediate fast responses to the transmitter ATP in mammalian cells including central and sensory neurons, vascular smooth muscle, endothelium, and leukocytes. Molecular cloning of P2X receptors and our understanding of structure-function relationships has provided sequence information with which to query an exponentially expanding wealth of genome sequence information including protist, early animal and human pathogen genomes. P2X receptors have now been cloned and characterized from a number of simple organisms. Such work has led to surprising new cellular roles for the P2X receptors family and an unusual phylogeny, with organisms such as *Drosophila* and *C. elegans* notably lacking P2X receptors despite retaining ionotropic receptors for other common transmitters that are present in mammals. This review will summarize current work on the evolutionary biology of P2X receptors and ATP as a signaling molecule, discuss what can be drawn from such studies when considering the action of ATP in higher animals and plants, and outline how simple organisms may be exploited experimentally to inform P2X receptor function in a wider context.

Keywords: P2X receptor, evolution, molecular, pharmacology, structure-activity relationship, ion channels

INTRODUCTION

Seminal early discoveries identified the role of acetylcholine and norepinephrine in chemical transmission at neuronal synapse. However, the concept of non-adrenergic non-cholinergic (NANC) chemical transmission mediated by adenosine 5-triphosphate (ATP) emerged much later and was met initially with sizable resistance amongst the pharmacology and neuroscience communities (Burnstock, 2012). The development of the hypothesis that ATP can act as a transmitter molecule also came to challenge "Dales Principle" which suggested that a neuron only utilizes one transmitter molecule to communicate. It is now well accepted that neurons of the central and peripheral nervous systems release ATP as a co-transmitter. Indeed ATP is the sole transmitter molecule to act post-synaptically in some instances, for example during sympathetic innervation of submucosal arterioles of the intestine (Evans and Surprenant, 1992). Some molecules that are associated with neurotransmission such as GABA and glutamate are utilized by primitive organisms, which lack nervous systems, for the purpose of cellular signaling (Fountain, 2010).

The human genome encodes a family of cell surface receptors that are activated by extracellular adenine and uridine signaling nucleotides. The metabotropic arm of this family are the G protein-coupled P2Y (mammalian $P2Y_{1,2,4,6,11,12,13,14}$) receptors that respond to ATP, ADP, UTP, UDP, and UDP-glucose with receptor subtype selectivity, and typically mediate slow responses to nucleotides. P2X receptors (mammalian $P2X_{1-7}$) are ligand-gated ion channels and mediate fast responses solely to ATP. In mammals, P2X receptor activation is associated with diverse physiological and pathophysiological processes including pain, inflammation, taste and smooth muscle contraction (Khakh and North, 2006). P2X receptors are non-selective cation channels, consequently receptor activation leads to membrane depolarization and cellular calcium influx, both by direct receptor permeation of calcium and voltage-gated calcium channel activation (North, 2002).

Adenosine 5-triphosphate is omnipresent in biology and plays many important roles including energy transfer and as a phosphate donor in enzymatic reactions, though its acceptance as a certified signaling ligand was slow despite early reports of potent physiological action in mammalian systems. The effects of exogenous ATP on insects and invertebrates has been known for some time (for a review see Burnstock and Verkhratsky, 2009), though the identification and cloning of P2X receptors from invertebrates and primitive single-celled organisms has been far less widespread. The identification of genes encoding putative P2X receptors in primitive organisms has been aided by a wealth of structure-function information for mammalian receptors, and an ever-expanding library of curated genomes for single-celled organisms including algae, amoeba and basal fungi. For example, the $P2X_A$ receptor of *Dictyostelium discoideum* shares only very low primary sequence homology with mammalian P2X receptors (Fountain et al., 2007). A translated BLAST search using the full-length 378 amino acid

sequence of the *Dictyostelium* as a query, provide no homologous mammalian P2X receptor sequences when using an expect value (*E* value) of 10. However, using the second transmembrane (TM2) domain as a search term returns hits of mammalian homologous, giving confidence of authenticity based on the conserved pattern of TM2 residues known to be critical to the function of mammalian P2X receptors. Bioinformatics is a powerful approach for expanding the phylogeny of primitive P2X receptors, however, caution must be applied when making structural or functional inferences to mammalian P2X orthologues without cloning and definitive demonstration of functionality. This is because several cloned P2X receptors from primitive species fail to form functional ATP-activated channels (Fountain et al., 2008; Ludlow et al., 2009). This review will focus on the P2X receptor homologues cloned from invertebrate and primitive non-vertebrate organisms that have been shown definitively to be ATP activated ion channels through experimentation.

CLONED NON-VERTEBRATE AND PRIMITIVE P2X RECEPTORS

The pharmacological properties of cloned P2X receptors are summarized in **Tables 1** and **2**.

Schistosoma mansoni (TREMATODE)

The first non-vertebrate P2X receptor was cloned from the human pathogen *S. mansoni*. *Schistosoma* are parasitic blood fluke and are trematodes belonging to the platyhelminthe genus. *S. mansoni* infection in humans causes schistodomiasis, a chronic illness which can lead to severe damage of multiple organs. *S. mansoni* encodes a protein that has 26–37% sequence homology with human $P2X_1$–$P2X_7$. Expression in *Xenopus oocytes* produces an ATP activated ion channel (*Sch*P2X) that responds to ATP with an EC_{50} of 22 μM (Agboh et al., 2004). BzATP, a full agonist at mammalian $P2X_7$ receptors, acts as a partial agonist at *Sch*P2X evoking a maximal response 75% that of ATP. Though less efficacious BzATP is more potent than ATP, evoking half-maximal responses at 4 μM. ATP evoked responses at *Sch*P2X are inhibited by classical purinergic receptor antagonists PPADS and suramin with IC_{50} values of 4 and 10 μM, respectively. Permeability studies in HEK293 cells expressing *Sch*P2X reveal a high permeability to calcium ($P_{Ca}/P_{Na} = 3.8$) that is comparable to mammalian P2X receptors. Cation substitution experiments reveal *Sch*P2X and mammalian P2X receptors have conserved ionic pore diameters. Praziquantel is a drug used in the treatment of schistodomiasis. Though the action of praziquantel is dependent upon affecting calcium homeostasis in worms (Kohn et al., 2001), praziquantel does not inhibit *Sch*P2X (Agboh et al., 2004).

Table 1 | Agonist sensitivity of cloned P2X receptors.

	ATP	BzATP	αβmeATP	βγimidoATP
*Ot*P2X	250	insensitive (1 mM)	> 5mM	insensitive (1 mM)
Dd$P2X_A$	97	(3 mM, 25%)	95	15
Dd$P2X_B$	266	ND	ND	85
Dd$P2X_E$	511	ND	ND	(3 mM, 22%)
*Hd*P2X	45	12 (65%)	(100 μM, 50%)	ND
*Bm*P2X	70	ND	ND	ND
*Sch*P2X	22	4 (75%)	ND	ND
*Lym*P2X	6	2 (33%)	(100 μM, 37%)	ND

Values are given as approximates of reported EC_{50} concentrations. EC_{50} values are given as μM. ND indicates antagonist sensitivity not determined. Maximum responses for partial agonist are given in parenthesis as % maximum ATP response. Where EC_{50} values have not been determined experimentally maximum concentrations tested are given in parenthesis with response as % maximum ATP response.

Dictyostelium discoideum (AMOEBA)

Dictyostelium discoideum, a soil-living amoeba, transitions from a community of unicellular amoebae into a multicellular fruiting body during its developmental lifecycle. This eukaryote belongs to the phylum Mycetozoa, emerging after plants and a common ancestor to fungi and animals. *Dictyostelium* amoeba display many animal cells traits including chemotaxis and phagocytosis. The chemical stimuli that govern the social behavior and complex development of *Dictyostelium* remain a subject of intense study. Prior to the discovery of ATP activated P2X receptors in *Dictyostelium* it was known that the neurotransmitters glutamate and GABA are important signaling cues governing cell fate and development in the organism (Fountain, 2010). The genome of *Dictyostelium* encodes five P2X receptor homologues (*Dd*$P2X_{A-E}$) that display low primary sequence homology with human P2X receptors. The family of *Dictyostelium* P2X receptors are the most extensively studied of the primitive P2X receptor paralogues in terms of biophysics, structure-function and physiology. *Dd*$P2X_A$, *Dd*$P2X_B$, and *Dd*$P2X_E$ form functional ATP-activated ion channels when expressed in HEK293 cells or *Xenopus* oocytes (Fountain et al., 2007; Ludlow et al., 2009; Baines et al., 2013). ATP evoked currents can be detected in HEK293 cells expressing *Dd*$P2X_D$ under experimental conditions of low extracellular Na^+ (Baines et al., 2013), but *Dd*$P2X_C$ expression fails to produce functional ion channels in either HEK293 or *Xenopus* oocytes (Ludlow et al., 2009; Baines et al., 2013). ATP is a full agonist at *Dictyostelium* P2X receptors with EC_{50} values in the range 100–500 μM. *Dd*$P2X_A$ receptors are most sensitive to ATP and *Dd*$P2X_E$ receptors least sensitive (Fountain et al., 2007; Ludlow et al., 2009; Baines et al., 2013). αβme-ATP is a full agonist of equal potency to ATP at $P2X_A$ and BzATP act as a weak partial agonist (Fountain et al., 2007). Interestingly the hydrolysis resistant ATP analog βγimido-ATP is a full agonist at *Dd*PX_A and *Dd*$P2X_B$ receptors with 3–10-fold greater potency than ATP (Fountain et al., 2007; Ludlow et al., 2009). βγimido-ATP was found to act as a very weak agonist at *Dd*$P2X_E$ receptors. *Dictyostelium* P2X receptors are freely permeable to Na^+, Ca^{2+} ($P_{Ca}/P_{Na} = 1.5$; Fountain et al., 2007), K^+ ($P_K/P_{Ca} = 1.8$–2.0; Ludlow et al., 2009), NH_4^+ ($P_{NH4}/P_{Ca} = 1.8$–2.0; Ludlow et al., 2009) and choline ($P_K/P_{Ca} = 0.5$–0.6; Fountain et al., 2007; Ludlow et al., 2009). In addition to a range of cations, *Dd*$P2X_A$ and *Dd*$P2X_B$ also freely permeate Cl^- when expressed in *Xenopus* oocytes (Ludlow et al., 2009). Permeability to Cl^- is an usual feature amongst P2X receptors, however, not unique. Chick and human $P2X_5$ receptors are also reported to permeate Cl^- (Ruppelt et al., 1999; Bo et al., 2003). Unlike mammalian P2X all *Dictyostelium* P2X receptors are insensitive to antagonism by suramin, PPADS or TNP-ATP (Fountain

Table 2 | Antagonist sensitivity of cloned P2X receptors.

	Suramin	PPADS	TNP-ATP	Cu^{2+}	Zn^{2+}
*Ot*P2X	No block (100 μM)	No block (100 μM)	No block (100 μM)	No block (100 μM)	ND
Dd$P2X_A$	No block (100 μM)	No block (100 μM)	No block (100 μM)	0.04	ND
Dd$P2X_B$	No block (100 μM)	No block (100 μM)	ND	(100 nM, 85% block)	ND
Dd$P2X_D$	ND	ND	ND	(100 nM, 30% block)	ND
Dd$P2X_E$	No block (100 μM)	No block (100 μM)	ND	(100 nM, 70% block)	ND
*Hd*P2X	23	15	ND	20	63
*Bm*P2X	5 (partial > 300 μM)	ND	ND	ND	ND
*Sch*P2X	10	0.5 (partial > 100 μM)	ND	ND	ND
*Lym*P2X	27 (partial > 300 μM)	8	ND	(1 mM, 63%)	(1 mM, 66%)

Values are given as approximates of reported IC_{50} concentrations. IC_{50} values are given as μM. ND indicates antagonist sensitivity not determined. % block is given where an IC_{50} value has not been determined.

et al., 2007; Ludlow et al., 2009). This makes *Dictyostelium* P2X receptors very useful tools for understanding antagonist action at P2X receptors as the structural determinants of drug binding in P2X receptors are poorly defined. A common feature shared by both *Dictyostelium* and mammalian P2X receptors is modulation by divalent metal ions (Virginio et al., 1997; Coddou et al., 2003). Cu^{2+} potently blocks *Dd*$P2X_A$ with a half-maximal inhibitory of 40 nM (Fountain et al., 2007). *Dd*$P2X_B$, *Dd*$P2X_D$ and *Dd*$P2X_E$ are blocked to a varying degree (30–85%) by 100 nM Cu^{2+}. Ni^{2+} is less potent at blocking *Dd*$P2X_A$ currents (IC_{50} 60 μM; Fountain et al., 2007).

The most striking feature of *Dictyostelium* P2X receptor functionality is their exclusive intracellular residence. Although some mammalian P2X receptors exist between intracellular compartments (Qureshi et al., 2007) and the plasma membrane *Dictyostelium* P2X receptors are targeted inside the cell. Several reports confirm that *Dictyostelium* P2X receptors reside on the contractile vacuole (Fountain et al., 2007; Ludlow et al., 2009), an osmoregulatory organelle and acidic calcium store (Heuser et al., 1993; Malchow et al., 2006; Sivaramakrishnan and Fountain, 2012). In a study by Fountain et al. (2007) *Dictyostelium* cells lacking *Dd*$P2X_A$ through genetic disruption were found to swell in response to hypotonic stress but lack any regulatory cell volume decrease, suggesting a severe impairment of contractile vacuole function. The phenotype was reconfirmed in a later study by Baines et al. (2013) who demonstrated that regulatory cell volume decrease could be rescued, or partially rescued, in *Dd*$P2X_A$ knockout cells by overexpression of *Dd*$P2X_A$, *Dd*$P2X_B$, *Dd*$P2X_D$, or *Dd*$P2X_E$, but not *Dd*$P2X_C$ which fail to form functional ion channels when expressed in HEK293 or *Xenopus oocytes* (Ludlow et al., 2009; Baines et al., 2013). These data indicate a requirement for ATP activation of P2X receptors for normal contractile vacuole function and osmoregulation. These findings are not in agreement with a study by Ludlow et al. (2009) who demonstrate that *Dictyostelium* lacking all five P2X receptors still undergo regulatory cell volume decrease, despite a delay in recovery. The differences in phenotype reported can be explained by strain variance. In a recent side-by-side examination (Sivaramakrishnan and Fountain, 2013) of AX2 (used by Ludlow et al., 2009) and AX4 (used by Fountain et al., 2007; Baines et al., 2013) laboratory strains of *Dictyostelium*, it was found that wild-type AX2 and AX4 vary in the degree of volume recovery following hypotonic swelling and that AX2 but not AX4 can tolerate loss of *Dd*$P2X_A$. Within the vacuolar membrane P2X receptors are orientated such that the ATP binding site (ectodomain) faces the vacuole lumen, suggesting that the P2X receptors are positioned to sense changes in luminal ATP. Experiments using purified vacuoles demonstrate that ATP can be translocated into the vacuole lumen, representing a possible mechanism of ATP accumulation (Sivaramakrishnan and Fountain, 2013). Addition of ATP to intact vacuole preparations causes release of stored calcium. The magnitude of calcium release is reduced in *Dd*$P2X_A$ knockout vacuoles and ablated in vacuoles lacking all five P2X receptors. These data suggest that vacuoles respond to luminal ATP accumulation by releasing stored calcium via intracellular P2X receptor activation (Sivaramakrishnan and Fountain, 2013). It remains unclear how P2X receptor dependent calcium release contributes to contractile vacuole function though possibilities include facilitation of docking or vacuole fusion. Vesicular $P2X_4$ receptor activation has been shown recently to facilitate vesicle fusion in mammalian cells in a calcium-dependent fashion (Miklavc et al., 2011; Thompson et al., 2013). Vacuoles isolated from AX2 amoeba release substantially less calcium in response to ATP in comparison to AX4 vacuoles, which may provide some mechanistic insight into the difference in P2X receptor dependency for osmoregulation between the two strains (Sivaramakrishnan and Fountain, 2013).

Extracellular ATP is detectable in suspensions of *Dictyostelium* (Parish and Weibel, 1980). Early work demonstrated that application of extracellular ATP stimulates Ca^{2+} influx in *Dictyostelium*which was sensitive to the purinergic receptor antagonist suramin (Parish and Weibel, 1980). More recently this has been demonstrated using aqueorin expressing strains of *Dictyostelium* (Ludlow et al., 2008), though in this study the ATP evoked Ca^{2+} responses were insensitive to the P2 receptor antagonists suramin and PPADS but did display sensitivity to block by low micromolar Cu^{2+} as for the *Dictyostelium* $P2X_A$ receptor (Fountain et al., 2007; Ludlow et al., 2009). Despite this, the ATP evoked calcium response remains intact following genetic knockout of all five P2X receptors

($P2X_{A-E}$; Ludlow et al., 2009) suggesting the *Dictyostelium* P2X receptors do not mediate responses to extracellular ATP and supports their intracellular residency (Fountain et al., 2007; Ludlow et al., 2009; Sivaramakrishnan and Fountain, 2012).

Ostreococcus tauri (ALGAE)

Ostreococcus are primitive single celled algae and the smallest free-living eukaryotes. They belong to the *Prasinophyceae* class of unicellular green algae that mainly includes marine planktonic species, and are close to the evolutionary origins of photosynthesis. *O. tauri* encodes a protein of 387 amino acids termed *Ot*P2X that shares 23% primary sequence identity with the *Dictyostelium* $P2X_A$ receptor (Fountain et al., 2007) and around 28% identity with human P2X receptors. Expression of *Ot*P2X-*myc* in HEK293 cells produces a 50-kDa protein (Fountain et al., 2008). The receptor contains many of the residues considering important for mammalian P2X receptor function, including conservation of ectodomain lysine residues are positions equivalent to Lys^{69} and Lys^{308} of rat $P2X_2$, though overall the ectodomain is poorly conserved. The N-terminal YXTXK/R sequence is retained, however, the C-terminal YXXXK motif shown to promote membrane retention in mammalian receptors (Chaumont et al., 2004) is replaced with a YESWL sequence.

ATP evokes *Ot*P2X channel opening with a half maximal concentration around 250 μM and activation threshold of around 30 μM. Whole-cell currents display modest desensitization in the presence of ligand. Single channel analysis revealed that *Ot*P2X open channel properties are flickery in nature (Fountain et al., 2008). α,β-methylene-ATP evokes very small currents though bzATP, β,γ-imido-ATP or other nucleotides triphosphates elicit no response (Fountain et al., 2008). The antagonist profile of *Ot*P2X is similar to that of the *Dictyostelium* P2X receptors with suramin, PPADS and TNP-ATP all failing to cause block up to 100 μM. Unlike the *Dictyostelium* P2X receptor, *Ot*P2X receptor currents are unaffected by Cu^{2+} upto 100 μM.

In contrast to other P2X receptors, *Ot*P2X displays poor calcium permeability ($P_{Ca}/P_{Na} = 0.4$). The poor permeation of calcium is a result of a major structural difference between *Ot*P2X and mammalian receptors, i.e., the absence of an aspartate residue that is highly conserved amongst other primitive and mammalian P2X receptors in the second transmembrane domain, the conserved aspartate is replaced by an asparagine residue. Asn^{353} in *Ot*P2X is equivalent to Asp^{349} in rat $P2X_2$. Though a switch from an acidic to a basic moiety can clearly be tolerated at this position in *Ot*P2X, a [N353A] mutation renders the receptor non-functional (Fountain et al., 2008). *Ot*P2X[N353R] enhances the calcium permeability ($P_{Ca}/P_{Na} = 0.64$; Fountain et al., 2008) but not back to the level of mammalian P2X receptors. This suggests other residues in mammalian P2X receptors also contribute to high calcium permeability (Migita et al., 2001; Samways and Egan, 2007). The pore diameter of *Ot*P2X receptors estimated from the relative permeability of a range of cations suggests a permeability cut-off of 1 nm. This broadly agrees with estimates of mammalian and *Dictyostelium* P2X receptor pore sizes (Evans et al., 1996; Fountain et al., 2007) and suggests architectural conservation of the selectivity filter between very early P2X receptor proteins and mammalian P2X receptors.

Though *Ot*P2X is clearly expressed at the plasma membrane when overexpressed in HEK293 cells, the subcellular localization of *Ot*P2X in *O. tauri* has not been confirmed. Such experiments are hampered but the lack of selective antibodies to *Ot*P2X and the size of the organism (around 1 μM in diameter) not being amenable to conventional immunocytochemical studies. However, experiments to determine whether ATP can induce calcium entry in *O. tauri* suspended in artificial sea water provide some indirect data supporting a lack of cell surface expression of P2X receptors. Though ATP does not stimulate calcium influx micromolar capsaicin, the TRPV1 channel agonist, did stimulate influx. A TRPV1 homologue is encoded by this primitive algae.

Monosiga brevicollis (Choanoflagelate)

Choanoflagelates are free living unicellular and colonial flagellate eukaryotes considered the closest living relative of animal cells. Fountain et al. (2008) reported cloning of a P2X receptor homologue from *M. brevicollis*. The *Mb*P2X receptor formed functional ATP activated ion channels when expressed in HEK293, though a pharmacological and biophysical characterization has not yet been published.

TARDIGRADE (*Hypsibius dujardini*)

Hypsibius dujardini belongs to the phylum Tardigrade that shares features common to nematodes and arthropods. Around 400 μM in length, these multicellular organisms inhabit moss and freshwater environments, and are capable of lowering their metabolism enough to survive desiccating environments for long periods. Bavan et al. (2009) identified a 330 bp EST from *H. dujardini* which when translated shared homology with mammalian P2X receptors. The full-length coding region translates to a 480 amino acid protein (*Hd*P2X) that shares between 36 and 38% sequence homology with human $P2X_1$, $P2X_3$, and $P2X_4$. Phylogenic analysis suggests *Hd*P2X is an ancestor of vertebrate P2X receptors and orthologous to other non-vertebrate receptors including *Dictyostelium*, *S. Mansoni* and *O. tauri*. Expression of *Hd*P2X cRNA in *Xenopus* oocytes produces ATP (EC_{50} 45 μM) activated ion channels that mediate transient inward currents that rapidly desensitizes in the presence of ligand. Despite this rapid desensitization and in contrast to the human $P2X_1$ receptor that also displays rapid desensitization during ATP application, *Hd*P2X currents recover. Current amplitude is fully recovered following 5 min agonist wash-off. *Hd*P2X can be activated by both BzATP and αβ-methylene-ATP. BzATP acts as a partial agonist at *Hd*P2X with maximal concentrations producing around 65% of ATP. Though BzATP is less efficacious it acts more potently than ATP with an EC_{50} around 12 μM. α,β-methylene-ATP is less efficacious than ATP and evokes current amplitudes that are 50% of ATP responses. BzATP potency and efficacy with regard to ATP at *Hd*P2X mirror that of BzATP properties at *Sch*P2X receptors (Agboh et al., 2004).

*Hd*P2X is blocked by the broad-spectrum purinergic receptor antagonists PPADs (IC_{50} 15 μM) and Suramin (IC_{50} 23 μM), which is in contrast to other primitive P2X receptors that are insensitive to classical antagonists. Micromolar Cu^{2+} and Zn^{2+} block *Hd*P2X. Divalent metal ions modulate mammalian P2X receptor function and the *Dictyostelium* $P2X_A$ receptor. Cu^{2+} and Zn^{2+} inhibit ATP-evoked responses with an IC_{50} of 20 and

63 μM, respectively. In mammalian $P2X_2$ and $P2X_7$ the modulatory activity of Cu^{2+} is mediated through interaction with histidine residues in the ectodomain. Bavan et al. (2009) produced histidine-to-alanine mutant *Hd*P2X receptors to investigate a role in divalent metal ion action. Though *Hd*P2X inhibition by Zn^{2+} was unaffected by alanine substitution of ectodomain histidine residues, the effectiveness of Cu^{2+} block was limited in *Hd*P2X[H252A/H306A] and [H232A/H306A] double mutants suggesting a role for these residues in coordinating Cu^{2+} action. ATP evoked currents mediated by *Hd*2X are potentiated by the macrocyclic lactone ivermectin which also potentiates mammalian $P2X_4$ and *S. mansoni* receptors.

Lymnaea stagnalis (POND SNAIL)

The pond snail *Lymnaea stagnalis* has proven a useful model to study fundamental aspects of the CNS. Its relatively simple CNS contains <22,000 neurons making it amenable to study processes of associative memory and taste. ATP release in molluscan CNS has been studied in real-time (Gruenhagen et al., 2004) highlighting a potential for purinergic receptors in invertebrate CNS function. A full-length P2X receptor orthologue has been cloned from *L. stagnalis* CNS (*Lym*P2X). *Lym*P2X is 435 amino acids in length and shares 31–46% identity with human $P2X_1$–$P2X_7$, sharing most similarity with the human $P2X_4$ receptor. *Lym*P2X expressed in *Xenopus oocytes* mediates inward currents that can be activated by ATP, BzATP and α,β-methylene-ATP. ATP is a full agonist that produces half-maximal responses at 6 μM. BzATP is 3-fold more potent (EC_{50} 2 μM) than ATP at *Lym*P2X but acts as a partial agonist, producing a maximal response 66% that of ATP maximal response. α,β-methylene-ATP acts as a weak agonist. *Lym*P2X is blocked by both PPADS and suramin. PPADS can completely block ATP evoked currents above 100 μM and has a half-maximal inhibitory concentration of 8 μM. Suramin is less effective with an IC_{50} of 27 μM and produces incomplete channel block even at a concentration of 300 μM. The suramin resistant component accounts for around 40% of maximum currant (Bavan et al., 2012). *Lym*P2X currents are not potentiated by ivermectin but are potentiated by 100 μM Cu^{2+} or Zn^{2+}. The level of potentiation is between 45 and 75%. However, the effect of divalent metal ions is biphasic as 1 mM Cu^{2+} or Zn^{2+} inhibits the receptor by around 65%. The CNS of *Lymnaea* has several discernable ganglia including buccal, cerebral, pedal, pleural, left parietal, right parietal, and visceral ganglia. In situ hybridization reveals widespread expression of *Lym*P2X in neurons of all ganglia, though quantitation of *Lym*P2X mRNA transcripts reveals highest expression in neurons of pedal ganglia and the lowest levels in pleural neurons (Bavan et al., 2012). Though a physiological role of *Lym*P2X is yet to be assigned, it is highly likely that the receptor is placed to respond to ATP secreted by neurons or supporting cells of the mollusc CNS.

Boophilus microplus (TICK)

The *B. microplus* tick causes detrimental effects to cattle wellbeing through blood feeding and transmission of disease. The tick P2X receptor homologue *Bm*P2X forms a functional ATP activated ion channel when expressed in *xenoupus* oocytes (Bavan et al., 2011). The 414 amino acid long receptor shares between 30 and 44% sequence identity with human receptors, sharing the most identity with human $P2X_4$ and least with $P2X_7$. The receptor contains many structural motifs common to mammalian P2X receptors including 10 conserved ectodomain cysteines, positive and aromatic residues implicated in ATP binding and N-terminal putative protein kinase C phosphorylation site. Currents passed by *Bm*P2X exhibit extremely slow kinetics. ATP evoked currents reach peak after almost 5 s, which is in stark contrast to the millisecond activation kinetics of mammalian P2X receptors (North, 2002). Current decay in the presence of agonist are also markedly slow. *Bm*P2X currents decay by around 10% after 20 s exposure to ATP with 50% decay occurring after prolonged (>5 min) agonist application. Despite limited current decay in the presence of agonist, rundown in peak responses is marked. Consecutive ATP applications cause a 12% reduction in peak currents. Bavan et al. (2011) identified sequences positively charged residues in the C-terminus responsible for controlling receptor desensitization. Basic residues in the receptor C-terminus also control the desensitization kinetics of human P2X receptors (Fountain and North, 2006). However, the C-terminus does not contribute to receptor rundown properties. ATP activates *Bm*P2X with an EC_{50} value of 70 μM, though adenosine, ADP or UTP (all up to 1 mM) do not evoke currents. Suramin antagonizes *Bm*P2X ($IC_{50} = 5$ μM) but produces an incomplete block with currents persisting up to 300 μM. Ivermectin potentiates ATP evoked currents at mammalian $P2X_4$ receptors (Priel and Silberberg, 2004), *S. mansoni* P2X (*Sm*P2X) and *H. dujarini* (*Hd*P2X) receptors. Despite its broad-spectrum anti-parasitic activity ivermectin does not potentiation *Bm*P2X currents. However, currents are potentiated by amitraz, a triazapentadine compound used widely in the treatment of tick infestation in cattle. Peak currents are potentiated by 23 and 94% by 1 and 100 μM amitraz, respectively. The identification of *Bm*P2X is of major interest, not only as a target for potential new anti-parasitic drugs, but also as an example on an arthropod P2X receptor. Genomic information reveals that other arthropods including *Drosophila melanogaster*, *Apis mellifera* and *Anopheles gambiae* lack P2X receptors (Fountain and Burnstock, 2009). The existence of P2X receptors in the arthropod phylum suggests selective loss of P2X receptors in some, and likely the majority, of insect species.

GENERAL STRUCTURAL CONSERVATION WITH MAMMALIAN RECEPTORS

Functional mammalian P2X receptors assemble as oligomers of three pore-forming subunits (Young et al., 2008; Kawate et al., 2009). This trimeric oligomeric state is unusual amongst other ligand-gated and voltage-gated ion channel families, including ionotropic glutamate and nicotinic acetylcholine receptors (Cys-loop superfamily), but shared by ASIC and intracellular TRIC channels. Our previous work demonstrates that *Dictyostelium* P2X assemble as trimers, at least when expressed as recombinant receptors in mammalian cells (Fountain et al., 2007), suggesting a conservation of trimer formation by primitive P2X receptors. Expression of *Dictyostelium* P2X receptors in Sf9 insect cells also results in strong trimer formation, and the *Dictyostelium* receptor trimers are a similar size to that of vertebrate receptor trimers (Valente et al., 2011). The *Dictyostelium* P2X receptors remain the best structurally characterized primitive P2X receptors. The

low primary sequence homology with mammalian P2X receptors (Fountain et al., 2007) and significantly different 3D structure (Valente et al., 2011) make them interesting candidates for future structural studies.

Ten ectodomain cysteine residues are highly conserved in mammalian P2X receptors and interact to form five disulphide bonds. The cysteines are positioned at residues C177, C126, C132, C149, C159, C165, C217, C227, C261, and C270 based on human $P2X_1$ numbering. These disulphide bonds are resolved in the zebrafish P2X4 crystal structure (Kawate et al., 2009) and influence the structure of the ATP binding pocket, channel gating properties, and trafficking of mammalian receptors (Rokic et al., 2010; Jindrichova et al., 2012). The degree of ectodomain cysteine conservation in cloned primitive P2X receptors varies greatly and shows no correlation with species phylogeny. Trematode (*S. mansoni*) and tarigrade (*H. dujardini*) receptors retain all equivalent cysteine residues, whereas algae (*O. tauri*) and choanoflagellate (*M. brevicollis*) receptors lack C217, C227, and C117, C165 equivalents, respectively. Based on the prediction of cysteine–cysteine pairing for disulphide bond formation in the human $P2X_1$ receptor (Ennion and Evans, 2002), this would predict both the *O. tauri* and *M. brevicollis* receptor lack a single ectodomain disulphide bond, though at different positions. Strikingly, the *Dictyostelium* $P2X_A$ receptors lacks cysteines at all equivalent position yet is a functional ATP activated ion channel (Fountain et al., 2007), suggesting a marked difference in ectodomain tertiary structure despite an ability to bind micromolar ATP (Valente et al., 2011).

FUTURE PERSPECTIVES AND EXPERIMENTAL ADVANTAGES

A growing wealth of genomic information for single celled and non-vertebrate species makes it highly likely that our knowledge of P2X receptor phylogeny is set to expand rapidly. Recently several putative P2X receptor sequences were reported from sea sponge (*Amphimedon queenslandica*), amoeboid holozoan (*Capsaspora owczarzaki*) and nematode (*Xiphinema index*; Cai, 2012). Interestingly the same report identifies P2X orthologues in three species of basal fungi, namely *Allomyces macrogynus*, *Spizellomyces punctatus,* and *Batrachochytrium dendrobatidis* (Cai and Clapham, 2012). Though the function of these newly identified P2X receptors is yet to be demonstrated experimentally, these putative receptors share many of the structural hallmarks associated with P2X receptor function (Cai, 2012). Their existence suggests some phyla initially thought to lack P2X receptors, such as nematode and fungi (Fountain and Burnstock, 2009), may contain some species that do posses ATP activated ion channels. Identification of P2X receptors in the single celled green algae species *Ostreococcus tauri* demonstrate that the existence of P2X receptors predates the origins of multicellularity, and that the evolution of these receptor class occurred more than 1 billion years ago. Similar sequences are also present in the genome of *Osteococcus lucimarinus* (Palenik et al., 2007). Though *Ot*P2X share poor primary structure homology with mammalian P2X receptors the proteins assembly to fully functional ATP activated ion channels. Elucidating the physiological role of P2X receptors in such small organisms will be technically challenging but of immense interest. *O. tauri* are photosynthetic organisms yet to date the has been no functional or genomic evidence presented for the existence of P2X receptors in higher plants. P2X receptors are notably absent from some species used extensively in neuroscience as model organisms including *Caenorhabditis elegans* and *Drosophila melanogaster* (Fountain and Burnstock, 2009). The absence of functional P2X receptors in *Drosophila* has been used as an experimental advantage for the study of neural circuits and behavior in this genetically amenable model organism. Ectopic expression of rat $P2X_2$ in *Drosophila* neurons allows for channel activation by laser-stimulated uncaging of caged ATP injected into specific fly brain areas. This allows pair activation of a specific set of neurons with exposure to a second stimulus such as odor (Zemelman et al., 2003).

SUMMARY

In summary, the phylogenetic distribution of P2X receptors is incomplete but demonstration of functional receptors in simple unicellular organisms suggests evolution of this receptor class occurred over one billion years ago. The fact that many primitive P2X receptors share very low sequence homology with mammalian P2X receptors, including absence of key motifs, yet still retain micromolar sensitivity to ATP and common permeability properties is intriguing. Some low homology receptors which lack sensitivity to common P2X receptors are likely to be useful tools in the future with which to delineate the residues that coordinate drug binding at P2X receptors. Though we have gathered much structural information from cloning and characterization of primitive P2X receptor our understanding of their cell biology and physiology is restricted, but likely to provide fundamental information about why and how the P2X receptor class of ligand-gated ion channels evolved.

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Desensitization properties of P2X3 receptors shaping pain signaling

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ATP-gated P2X3 receptors are mostly expressed by nociceptive sensory neurons and participate in transduction of pain signals. P2X3 receptors show a combination of fast desensitization onset and slow recovery. Moreover, even low nanomolar agonist concentrations unable to evoke a response, can induce desensitization via a phenomenon called "high affinity desensitization." We have also observed that recovery from desensitization is agonist-specific and can range from seconds to minutes. The recovery process displays unusually high temperature dependence. Likewise, recycling of P2X3 receptors in perimembrane regions shows unexpectedly large temperature sensitivity. By applying kinetic modeling, we have previously shown that desensitization characteristics of P2X3 receptor are best explained with a cyclic model of receptor operation involving three agonist molecules binding a single receptor and that desensitization is primarily developing from the open receptor state. Mutagenesis experiments suggested that desensitization depends on a certain conformation of the ATP binding pocket and on the structure of the transmembrane domains forming the ion pore. Further molecular determinants of desensitization have been identified by mutating the intracellular N- and C-termini of P2X3 receptor. Unlike other P2X receptors, the P2X3 subtype is facilitated by extracellular calcium that acts via specific sites in the ectodomain neighboring the ATP binding pocket. Thus, substitution of serine275 in this region (called "left flipper") converts the natural facilitation induced by extracellular calcium to receptor inhibition. Given their strategic location in nociceptive neurons and unique desensitization properties, P2X3 receptors represent an attractive target for development of new analgesic drugs via promotion of desensitization aimed at suppressing chronic pain.

Keywords: desensitization, extracellular ATP, P2X3 receptor, sensory neuron, pain

DESENSITIZATION OF INOTROPIC RECEPTORS

Desensitization is a loss of receptor responsiveness which develops with continuous presence of agonist. Desensitization is a general phenomenon which can be observed in most membrane receptor types, either metabotropic or ionotropic. Unlike metabotropic receptors which desensitize mainly via receptor internalization, thereby temporarily subtracting responsive elements to the extracellular stimulus (Ferguson, 2001), ionotropic receptor desensitization involves structural and functional changes in membrane residing receptors. The fundamental properties of acetylcholine (ACh) ionotropic receptor desensitization were originally described by Katz and Thesleff (1957) working on the frog neuromuscular junction (for review see Giniatullin et al., 2005) and can still be used to supply important principles to understand desensitization of many ionotropic receptors including those activated by extracellular ATP.

Ionotropic ATP receptors comprise P2X1 – P2X7 subtypes widely expressed in various tissues (Burnstock, 2013). The rate of desensitization and recovery processes vary widely within the family of P2X receptors (North, 2002). Thus, desensitization is developing very fast up to complete, yet reversible, loss of response in homotrimeric receptors composed of P2X1 and P2X3 subunits (North, 2002; Coddou et al., 2011). In contrast, the other P2X subtypes are less prone to desensitization. One goal of the present review is to discuss how the strong desensitization process of P2X3 receptors can coexist with the function of P2X3 receptor activation in pain signaling and whether facilitating desensitization might actually be exploited for analgesic purposes. We aim at resolving this conundrum by taking into account the receptor kinetic properties and dynamics.

P2X3 RECEPTORS UNIQUE PROPERTIES

P2X3 receptors are similar in some desensitization properties (fast onset and slow recovery) to the P2X1 subtype. However, their distinctive characteristics are listed below.

- Fast desensitization onset (ms range; similar time-course for P2X1 subtype)
- Very slow (min range) recovery from desensitization (shared with P2X1 subtype)
- High affinity desensitization (HAD) by very low agonist concentrations without generating a macroscopic current
- Heteromerization of P2X3 with P2X2 subunits to generate sustained non-desensitizing responses

- Remarkable resistance of desensitization onset to changes in temperature and divalent cation concentration
- Strong acceleration of recovery (resensitization) by increases in extracellular Ca^{2+} levels and inhibition by extracellular Mg^{2+}
- Speed of recovery highly dependent on temperature (higher temperature, faster recovery)
- Recovery sensitive to signal transduction mechanisms and controlled by the specific modulators

These properties are described in more detail in the following sections of this review.

DESENSITIZATION ONSET OF P2X3 RECEPTORS

P2X3 receptors can be activated by different agonists such as ATP, α,β-meATP, ATP-γ-S, β,γ-meATP, CTP, 2MeS-ATP and Bz-ATP which can all operate as full agonists (Sokolova et al., 2004, 2006; Pratt et al., 2005; Petrenko et al., 2011, for reviews see North, 2002; Coddou et al., 2011; Fabbretti and Nistri, 2012). Despite their different structures, all these compounds applied in high concentration can generate full size responses that, when recorded as membrane currents in voltage clamp conditions, show very similar fast decay (Sokolova et al., 2006).

The scheme of **Figure 1** shows that the agonist-bound open receptor can effect a dynamic transition to the closed, desensitized state persisting even when the agonist has come off the receptor. Thus, on P2X3 receptors, as expected from the classical model of desensitization by Katz and Thesleff (1957), the desensitization onset is accelerated by increasing the agonist dose (Sokolova et al., 2006) because the rate of this transition is strongly dependent on the agonist concentration and it, therefore, ensures that a large fraction of the available receptor population dwells in this functionally inactive state. Nevertheless, unlike the phenomenon observed with nicotinic receptors, the onset of P2X3 receptors desensitization is remarkably insensitive to temperature changes (Khmyz et al., 2008). There are only a few experimental manipulations which can specifically interfere with P2X3 receptor desensitization onset. One drug is the anti-inflammatory analgesic naproxen, which is widely used as an anti-migraine agent and which can speed up the desensitization onset of recombinant P2X3 receptors expressed in HEK cells (Hautaniemi et al., 2012, see **Figure 1**). Conversely, acid pH slows down the development of desensitization, although this effect might be indirectly due to reduction of P2X3 receptor potency (Gerevich et al., 2007a).

SLOW RECOVERY FROM DESENSITIZATION

Restoration of the response after removal of agonist, indicating P2X3 receptor recovery from ATP-induced desensitization, is very slow and can take up to 20 min at room temperature (Cook et al., 1998). This outstanding property makes P2X3 (and P2X1) receptors very different from other ligand-gated receptors (Cook et al., 1998). Even though the recovery occurs very slowly, it proceeds to full return of P2X3 receptor response: this reversibility is a useful criterion to distinguish desensitization from the irreversible run-down which is a common feature of many ionotropic receptors including the P2X1 subtype (Lewis and Evans, 2000).

We have previously shown that recovery of P2X3 receptors from desensitization is an agonist-specific process (Sokolova et al., 2004). It can be very fast for agonists like β,γ-meATP, but unusually slow for 2MeS-ATP (Sokolova et al., 2006). Furthermore, recovery displays distinctive properties from desensitization onset because, in addition to its time-course, it is modulated by factors such as temperature (Khmyz et al., 2008), cibacron blue (Alexander et al., 1999), extracellular Ca^{2+} (Cook et al., 1998) and others listed in **Figure 1**. The independent modulation of desensitization onset and recovery suggests that they are likely to have different determinants (see details in the "Structural Determinants of Desensitization").

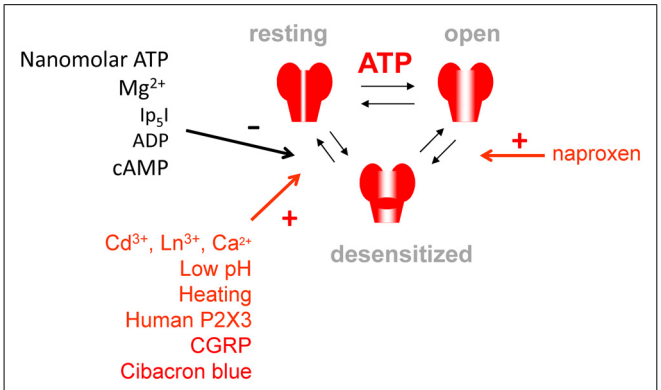

FIGURE 1 | A simplified kinetic scheme for P2X3 receptor operation indicating resting, open and desensitized receptor states. Note multiple factors accelerating (+, red arrow) or retarding (–, black arrow) recovery from desensitization, while naproxen promotes desensitization onset. Factors accelerating recovery are expected to facilitate ATP signaling via P2X3 receptor activity, whereas factors retarding recovery (or promoting desensitization onset) could provide the anti-nociceptive effect.

HIGH AFFINITY DESENSITIZATION

Apart from the "classical" desensitization arising from the open receptor state, there is an additional process of slow onset desensitization called "HAD" – that is, a low nanomolar concentration of the agonist inhibits P2X3 receptor responses without evoking a macroscopic current (McDonald et al., 2002; Sokolova et al., 2004, 2006; Pratt et al., 2005). We have shown that this phenomenon is also agonist-specific, being particularly strong for the natural agonist ATP (Sokolova et al., 2004, 2006). Thus, ATP is about a 10-fold more potent inducer of HAD than α,β-meATP (IC_{50} 2 and 20 nM, respectively), even though both agonists have almost the same EC_{50} values (Sokolova et al., 2004).

Given that the ambient level of extracellular ATP in various tissues is in the nanomolar range (Kuzmin et al., 1998; Lazarowski et al., 2003), one might suggest that a fraction of P2X3 receptors is continuously inactivated *in vivo*. Such inactivation could largely reduce the ability of P2X3 receptor expressing neurons to detect and generate nociceptive signal in response to acute ATP release. Furthermore, diadenosine polyphosphates, that are endogenously produced by bridging two adenosine moieties with a chain of two or more phosphates, can act as powerful HAD inducers (McDonald et al., 2002). This observation widens the range of substances that can modulate desensitization of P2X3 receptors and emphasizes the functional role of HAD as discussed in detail in the present review.

P2X3 ACTIVITY IS FACILITATED BY EXTRACELLULAR CALCIUM AND INHIBITED BY MAGNESIUM

One outstanding feature of the P2X3 receptor is its unusual sensitivity to extracellular Ca^{2+} which is specifically targeting receptor desensitization. The exciting finding of the facilitatory action of extracellular Ca^{2+} on P2X3 receptor was first described by McClesky's group (Cook et al., 1998). Interestingly, Ca^{2+} selectively and positively controls the recovery stage of receptor desensitization without changes in desensitization onset (Giniatullin et al., 2003). The most intriguing finding is that there is a sort of "memory" of the receptor system which remains in the facilitated state for several mins after the initial *transient* contact with the divalent cation (Cook et al., 1998). Such resensitization by Ca^{2+} on P2X3 receptor is functionally opposite to the well documented inhibitory action of Ca^{2+} on other P2X receptor subtypes (Virginio et al., 1998; Ding and Sachs, 2000). In fact, in the case of P2X2 receptors, extracellular Ca^{2+} rather promotes desensitization (Ding and Sachs, 2000).

We have previously observed that, on native P2X3 receptors, extracellular Mg^{2+} can produce an opposite effect to Ca^{2+} (Giniatullin et al., 2003). Thus, Mg^{2+} delays receptor recovery from desensitization, while the onset of desensitization remains unchanged. Ca^{2+}-free solution has the similar inhibitory action on receptor resensitization. As the modulatory effects were observed with physiological concentrations of Ca^{2+}, these finding outline a potential role of this mechanism even *in vivo*.

Our recent study has demonstrated that a single aminoacid (S275) within the left flipper region of the P2X3 receptor ectodomain is a likely determinant for the facilitatory action of extracellular Ca^{2+} (Petrenko et al., 2011). Thus, in the A275 mutant, extracellular Ca^{2+} induces an inhibitory effect on P2X3 receptor mediated responses instead of the facilitation normally seen with wildtype (WT) receptors.

UNUSUALLY HIGH TEMPERATURE SENSITIVITY

Another unexpected desensitization property of P2X3 receptors was described in detail by Krishtal's group. They showed that, while the desensitization onset is almost temperature insensitive, the recovery process is very temperature sensitive with a Q_{10} coefficient of about 10 (Khmyz et al., 2008). The temperature sensitivity of HAD is also high but clearly smaller than the one of the recovery process (Khmyz et al., 2008). These findings suggest that, unlike most *in vitro* experiments performed at room temperature, the recovery process at normal body temperature is much faster and that, in physiological conditions, the probability of repeated receptor activation is strongly enhanced.

Interestingly, the recycling of P2X3 receptors in the perimembrane region measured by the TIRF/FRAP technique also shows very high sensitivity to temperature (Pryazhnikov et al., 2011).

KINETIC MODELING OF P2X3 RECEPTOR DESENSITIZATION

A formal description of P2X3 receptor behavior using kinetic modeling allows the exploration of silent receptor conformations, prediction of new receptor desensitization properties, and provides a mechanistic explanation for experimentally observed phenomena. Modeling of P2X3 receptor kinetics clearly indicates that a cyclic scheme of receptor reversible transitions modified from the original proposal by Katz and Thesleff (1957) is the most suitable to account for the experimental data which cannot be adequately explained with linear or bifurcation models (Sokolova et al., 2006). This model integrates all the main steps of P2X3 function such as binding, gating and desensitization, which are presented in an over-simplified form in **Figure 1**.

In the complete receptor model with full activation caused by three agonist molecules bound to it, the receptor transition into the desensitized state occurs mainly from the open state. Furthermore, this model can simulate HAD, implying the existence of a single molecule bound desensitized state with high agonist affinity (Sokolova et al., 2006). As a result, the model fully reproduces all the main properties of the P2X3 receptor, including fast desensitization, slow recovery and HAD.

The rate limiting role of agonist dissociation from the desensitized state for resensitization obtained with this theoretical approach accords with agonist unbinding from P2X3 receptors measured using radiolabeled ATP (Pratt et al., 2005). Most importantly, the kinetic model predicts that desensitization, being the next step after binding of ATP and channel opening, should depend not only on "intrinsic" desensitizing properties of the P2X3 receptor, but also on agonist binding and channel gating. This view is essential when trying to find out and analyze the numerous data on structural determinants of desensitization.

To explain P2X3 receptor operation and in particular HAD, Karoly et al. (2008) have subsequently proposed an allosteric model that retains the cyclic scheme of Sokolova et al. (2006), but it adds to it an additional transition, namely a receptor open state occurring when two rather than three ATP molecules are bound. Thus, Karoly et al. (2008) argue that their revised model is the simplest way to explain increased affinity of non-occupied binding sites when receptors are partially occupied. Future biophysical studies using microscopic recording of single channel currents may be necessary to clarify this proposal.

STRUCTURAL DETERMINANTS OF DESENSITIZATION

At membrane level functional homomeric P2X3 receptors are assembled as trimers, whereby each subunit is composed of a large extracellular loop containing the ATP binding domain, two transmembrane domains and intracellular N- and C- termini (for reviews see Khakh, 2001; Khakh and North, 2006; Kawate et al., 2009, Fabbretti and Nistri, 2012). This complex structure has been extensively probed to find out the molecular determinants of desensitization.

One pioneer study showed that, using recombinant receptors expressed in Xenopus oocytes, chimeric P2X2 receptors containing P2X1 or P2X3 domains acquire strong desensitization, while chimeric P2X1 or P2X3 receptors with P2X2 domains show very weak desensitization (Werner et al., 1996). The domains necessary to alter the desensitization phenotype include the most hydrophobic segments of the molecules, which are thought to be membrane-spanning segments (Werner et al., 1996). These findings led Werner et al. (1996) to propose that desensitization requires the interaction of these receptor long segments (comprising 34 aminoacids).

Many subsequent studies were focused on finding key residues determining the desensitization process of P2X receptors. Thus, it has been shown that certain C-terminus residues are important to express the slow desensitization of P2X2 receptors (Koshimizu et al., 1998; Smith et al., 1999). Likewise, the N-terminus half of the P2X3 receptor ectodomain plays a role in the slow recovery from desensitization (Zemkova et al., 2004).

Our previous study has identified several residues in the ectodomain which determine the desensitization properties including Glu111, Asp220, and Asp266 (Fabbretti et al., 2004). By site-directed mutagenesis to alanine, it was possible to observe a pleiotropy of receptor responses like fast onset combined with either faster or slower recovery (Glu111Ala and Asp220Ala, respectively), or very slow onset (resembling the non-desensitizing P2X2 receptor) combined with fast recovery (Asp266Ala). These various combinations of kinetic properties provide additional evidence for the existence of independent determinants of desensitization onset and recovery.

Our *in silico* exploration of the P2X3 receptor molecular model predicted that S275 within the ectodomain region termed *left flipper* as a contributor to ATP binding and suggested that its manipulation would result in changes in the desensitization properties (Petrenko et al., 2011). Indeed, substituting S275 with alanine produces a mutant with slow desensitization onset, fast resensitization and lack of HAD (Petrenko et al., 2011). In this experiment, HAD remained minimal even with higher agonist concentrations (to compensate for reduced potency), suggesting that this residue is indeed important to express the inhibitory action of low nanomolar concentrations of ATP. Substituting S275 with more hydrophobic aminoacids further slows down desensitization onset and accelerates recovery, indicating these two properties to be reciprocally interrelated (Petrenko et al., 2011).

Manipulations of the transmembrane P2X3 domains have indicated additional contributors to shape desensitization. Thus, substitution with alanine (or phenylalanine) of the highly conserved Y37 yields initial fast desensitization with a large residual current, remarkably resistant to further desensitization (Jindrichova et al., 2011; see **Figure 2A**). Unlike the WT, this long lasting persistent current (highlighted by red arrow in **Figure 2B**) is systematically observed in the Y37A mutant in the presence of four different agonists (**Figure 2B**) applied at saturating concentrations that induce analogous amplitude of membrane current (**Figures 2C,D**). The latter indicated that this unusual plateau-like current is independent from the nature of the agonist. In sharp contrast, at the end of the agonist application, a highly variable (agonist-specific) rate of deactivation is observed (**Figure 2B**). This variable deactivation is related to the EC_{50} values for each agonist (**Figure 2E**). Furthermore, the rate of deactivation correlates also with the rate of agonist dissociation from the desensitized receptor state (fast deactivation, fast dissociation, see Sokolova et al., 2006). Kinetic modeling may help to explain these unusual phenomena by assuming facilitated re-opening of the P2X3 channels from the desensitized to the conducting states (Jindrichova et al., 2011).

In conclusion, current evidence shows multiple determinants of desensitization throughout the whole receptor structure (ectodomain, transmembrane and intracellular termini).

Useful clues to the biophysical nature of desensitization may also come by comparing P2X3 receptors with sister receptors containing other P2X subunits. Thus, using voltage clamp fluorometry applied to the P2X1 subtype, which is also prone to fast desensitization, Lörinczi et al. (2012) have proposed that the cys-rich *head* domain of this receptor is involved in both channel activation and desensitization. In the P2X1 subtype, high sensitivity to low doses of ATP is also reported, although desensitization in this case develops after detectable membrane currents (Rettinger and Schmalzing, 2003). Despite the fact that P2X7 receptors have low affinity for their natural ligand ATP (active at mM concentrations), ongoing desensitization has also been proposed to exist in this subtype, yet masked by the overlapping process of ion pore widening (Khadra et al., 2013).

FUNCTIONAL ROLE OF DESENSITIZATION OF P2X3 RECEPTORS

Nowadays there is strong evidence that P2X3 receptors participate in chronic pain, a most distressing clinical state often resistant to treatment (Burnstock, 2001; North, 2004). In fact, P2X3 channels are almost exclusively expressed by nociceptive neurons (Chen et al., 1995; Lewis et al., 1995), and impaired pain-evoked responses (especially of inflammatory type) are observed in P2X3 knockout mice (Cockayne et al., 2000; Souslova et al., 2000). These data accord with earlier reports that injection of α,β-meATP into the rat paw evokes strong nociceptive behavior which is paradoxically prevented by a previous application of the same agonist (Bland-Ward and Humphrey, 1997), pointing to a functional role of P2X3 receptor desensitization in shaping pain responses *in vivo*. However, in view of the fast desensitization of P2X3 receptors and the high likelihood of their HAD because of ambient ATP (and its metabolites), one may wonder how to reconcile such characteristics with a functional role of these receptors in sustained pain signaling (North, 2004).

One possible explanation is that, in healthy subjects, the main role of HAD in ATP-gated P2X3 receptors is to prevent inappropriate excitation of nociceptive pathways (and associated pain sensitivity) when there is a high probability of ATP release from surrounding tissues. In other words, HAD might be viewed as an intrinsic mechanism of "anti-nociception" in normal states, working in cooperation with fast desensitization to restrict ATP-mediated signaling. This protective process might be important in conditions like physical exercise when there is a continuous release of ATP in muscles to the level comparable with EC_{50} of P2X3 receptors (Hellsten et al., 1998; Mortensen et al., 2011).

Assuming a constitutive inhibition of P2X3 receptor activity by ambient purines, only strong, burst-like ATP release can perhaps represent a stimulus large enough to overcome any intrinsic anti-nociceptive effect (Giniatullin et al., 2008). In contrast, in chronic pain in man, particularly of inflammatory origin, P2X3 receptors might play a direct pro-nociceptive role (Burnstock, 2001) because the desensitization properties of P2X3 receptors could be modified by several cellular mechanisms that collectively or individually promote nociceptive sensitization (**Figure 1**):

(i) removal of ambient ATP via extracellular NTDases;
(ii) decreased HAD;

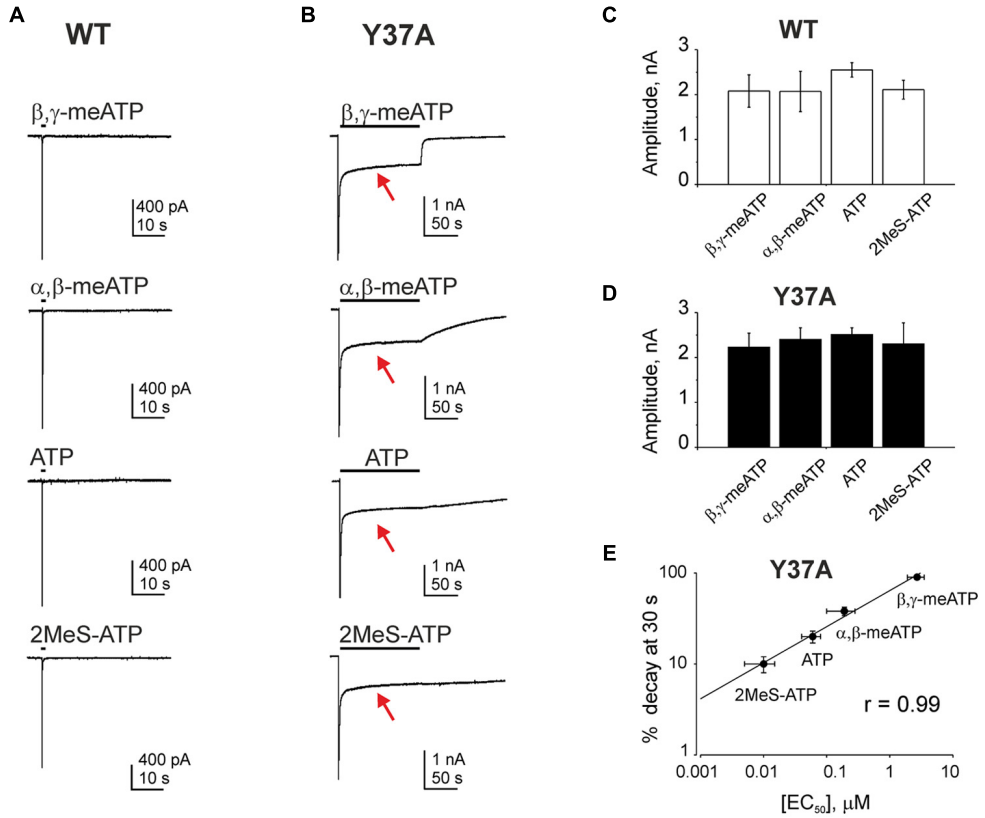

FIGURE 2 | The action of different agonists on the WT and Y37A receptor. (A) Currents activated by 2 s application of β,γ-meATP, α,β-meATP, ATP, or 2MeS-ATP (all 100 μM) on WT receptors. **(B)** Currents activated by 2 min application of β,γ-meATP, α,β-meATP, ATP, or 2MeS-ATP (in keeping with higher potency, all at 10 μM) on Y37A mutant receptors. Note similar peak amplitudes and plateau-like components (red arrows) but different current decays during the washout phase. **(C,D)** Histograms showing the mean amplitudes of peak currents induced by different agonists on WT **(C)** or Y37A receptor **(D)**. **(E)** Graph showing a correlation between differential agonist-specific current decays (measured after 30 s of washout) and corresponding EC_{50} values for each agonist. Datapoints from 3 to 12 cells. Reproduced with modifications from Jindrichova et al. (2011) with permissions from John Wiley and Sons.

(iii) accelerated recovery from desensitization;
(iv) heteromerization with non-desensitizing P2X2 receptors.

Although the *first mechanism* of sensitization has not been studied in relation to pain, its feasibility is indirectly supported by experiments on taste buds (Vandenbeuch et al., 2013). In fact, recent studies have shown expression of P2X3 receptors (along with P2X2 ones) in nerves supplying the taste buds (Finger et al., 2005). Using the NTDase-2 knockout mouse, it has been observed that excessive levels of extracellular ATP generated in the taste bud could inactivate nerve terminal P2X receptors and block the taste response by promoting desensitization via a phenomenon resembling HAD (Vandenbeuch et al., 2013). Thus, the extent of breakdown and clearance of extracellular ATP may be important to regulate nociceptive purinergic signaling.

As for the *second mechanism* of sensitization, evidence clearly shows that resensitization of P2X3 receptors *in vivo* is much faster than *in vitro* (Khmyz et al., 2008), and may be expected to occur even faster if local inflammatory reaction raises tissue temperature. Furthermore, inflammatory cells like macrophages releasing TNFα can further upregulate P2X3 receptor activity (Franceschini et al., 2012, 2013). It is also noteworthy that human P2X3 receptors recover about twice faster than analogous rodent receptors (Pratt et al., 2005). Finally, the P2X3 receptor recovery may be accelerated, via release of neuropeptides and neurotrophins (Fabbretti et al., 2006; D'Arco et al., 2007) and by extracellular acidification (Gerevich et al., 2007a) which occurs in inflammation and cancer (Tannock and Rotin, 1989). Thus, the migraine mediator CGRP not only largely increases the amplitude of P2X3 currents generated in nociceptive trigeminal neurons, but it also significantly speeds up recovery from desensitization via an intracellular kinase-dependent process of P2X3 receptor phosphorylation (Fabbretti et al., 2006; Giniatullin et al., 2008).

The possibility of the *third mechanism* of sensitization via reduced HAD is supported by decreased inhibitory action of nanomolar ATP at higher temperature (Khmyz et al., 2008).

Finally, enhanced expression of heteromeric P2X2/3 receptors producing typically non-desensitizing plateau currents might be the *fourth mechanism* of sensitization contributing to nociceptive signaling (North, 2004). However, recent study revealed the prevailing expression of P2X3 subunits in dorsal root ganglion neurons in primates, including human sensory neurons

(Serrano et al., 2012). Thus, the contribution of P2X2/3 receptors to chronic pain is probably more important at the level of nociceptive pathways within the spinal dorsal horn as indicated by the inhibition of nociceptive neuron firing by A-317491, a potent P2X2/3 antagonist (Xu et al., 2012).

A proposal for the physiological role of desensitization should also include mechanisms which augment this inhibitory process. Indeed, recovery from desensitization can be delayed via GPCR signaling (**Figure 1**) involving P2Y receptors (Gerevich et al., 2007b), $GABA_B$ receptors (Sokolova et al., 2003) and unidentified membrane receptors activated by extracellular cAMP (Mamenko et al., 2010). These mechanisms may be additional to the intrinsic inhibition of P2X3 receptor activity via C-terminal Src inhibitory kinase (Csk)-mediated tyrosine phosphorylation of the receptor (D'Arco et al., 2009). Future studies should address whether in chronic pain models there is a disruption in the intricate molecular mechanisms regulating P2X3 expression and function.

ANALGESIC DRUGS PROMOTING DESENSITIZATION OF P2X3 RECEPTORS

INFLAMMATORY AND NEUROPATHIC PAIN

The anti-nociceptive action of the selective P2X3 antagonist A-317491 in inflammatory and neuropathic pain was first reported in a study aimed at counteracting chronic pain in animal models (Jarvis et al., 2002). The traditional pharmacological approach to anti-nociception is usually based on the development of receptor antagonists with good bioavailability, high specificity, and minimal side-effects. One such example is a novel selective P2X3/P2X2/3 receptor antagonist AF-353 which is orally bioavailable (Gever et al., 2010). Other potential analgesic agents with different structure based on P2X3 receptor antagonism are reviewed by Gum et al. (2012) and by A. P. Ford in this series. Some of these compounds are already investigated in clinical trials, although none of them has yet received statutory approval by regulatory agencies for human use (Ford, 2012). A new line in drug development for the prevention of P2X3 mediated pain should target facilitation of desensitization. In fact, Bland-Ward and Humphrey (1997) have previously shown that an analgesic effect could be induced by injection of the full P2X3 agonist, α,β-meATP after an initial pro-nociceptive effect. More promising clues for pain suppression treatment may, however, come from the use of promoters of desensitization which show little or no agonist activity. Thus, purotoxin-1, a peptide recently isolated from the Asian wolf spider Geolycosa, powerfully inhibits P2X3 receptors by strongly promoting their desensitized state and provides effective anti-nociception in various pain models *in vivo* (Grishin et al., 2010) while the antagonist P1, P5-di[inosine-5′] pentaphosphate binds to the desensitized state of the P2X3 receptor in DRG neurons (Ford et al., 2005).

MIGRAINE AND TRIGEMINAL PAIN

Mg^{2+} can hardly be considered as a selective anti-migraine agent, and is typically known to be the endogenous blocker of NMDA channels (Nowak et al., 1984). There are, however, repeated suggestions to include this agent in the complex therapy of trigeminal migraine pain (this subject has been extensively discussed by Pardutz and Vecsei, 2012; and Mauskop and Varughese, 2012). As Mg^{2+} inhibits desensitizing ATP-evoked currents in rat cultured sensory neurons (Giniatullin et al., 2003), this effect might be considered to contribute to its analgesic action *in vivo* (Crosby et al., 2000). Given that Mg^{2+} specifically interferes with desensitized receptor states, one could expect the use-dependent anti-nociceptive action of this divalent cation in pain mediated by high concentrations of ATP acting on the P2X3 receptor. Of course, modulation by Mg^{2+} of P2X3 receptor activity may be complementary to the divalent cation role in NMDA receptor function that is thought to be dysregulated in trigeminal ganglia and underlying pain sensitization (Laursen et al., 2013).

The high expression of P2X3 receptors by the vast majority of trigeminal sensory neurons (Simonetti et al., 2006) provides the molecular substrate for P2X3 mediated trigeminal pain including migraine (Giniatullin et al., 2008). In experimental model of trigeminal pain *in vitro*, expression of P2X3 membrane receptors by trigeminal neurons is amplified by the migraine mediator CGRP that operates via intracellular kinases and enhanced P2X3 receptor trafficking from intracellular compartments (Fabbretti et al., 2006). Likewise, in transgenic mice expressing human mutated P/Q calcium channels as observed in Familial Hemiplegic Migraine type 1, the activity of P2X3 channels is constitutively enhanced (Nair et al., 2010) probably because of the higher background release of CGRP as shown by the phenotype reversal evoked by a CGRP receptor antagonist (Hullugundi et al., 2013). To further support the role of PX3 receptors in migraine pain, there are data obtained with well-established anti-migraine drugs. Thus, naproxen, a popular anti-headache analgesic, directly inhibits P2X3 receptors by facilitating receptor desensitization (**Figure 1**), an inhibitory effect that is potentiated in the presence of the algogen nerve growth factor (Hautaniemi et al., 2012), the level of which is elevated in patients with chronic migraine (Jang et al., 2011).

CANCER PAIN

Accumulating evidence suggests the involvement of P2X3 receptors in bone tissue cancer pain. The bone has a very specific environment including diverse cell populations and a specialized hormonal control by parathyroid hormone and parathyroid hormone-related protein which regulate Ca^{2+} release from osteoclasts (Soki et al., 2012). Thus, in bone cancer there is a massive release of Ca^{2+} leading eventually to hypercalcaemia which as mentioned above is a potent and unique mechanism to facilitate, via resensitization, the function of P2X3 receptors. In some pathological conditions, for instance, during parathyroid hormone-related protein-mediated hypercalcemic crisis, the serum Ca^{2+} can raise from normal level of ∼1.5 mM up to 4.8 mM (Rahil and Khan, 2012). There can be also malignancy-associated hypercalcemia, often associated with headache when the level of Ca^{2+} released from osteoclasts is rising locally within the bone (Basso et al., 2011). When the intense ATP release coincides with local enhancement of extracellular Ca^{2+} this could result in a strong nociceptive firing through increased activity of P2X3 receptors. The antagonism of Ca^{2+} effect on P2X3 receptor by Mg^{2+} (Giniatullin et al., 2003) can provide a rationale for the high analgesic efficiency of the latter in cancer pain (Crosby et al., 2000). Thus, the selective P2X3, P2X2/3 receptor antagonist A-317491

transiently attenuates cancer-induced bone pain in mice, but has no effect at the late stage of bone cancer (Hansen et al., 2012). Bone cancer pain in rats is reduced by the blockade of P2X3 and P2X2/3 receptors with AF-353 (Kaan et al., 2010). Furthermore, there is an increased expression of P2X3 receptors in CGRP immunoreactive nerves during tumor growth suggesting their role in cancer-related pain (Gilchrist et al., 2005). Likewise, P2X3 receptors are functionally up-regulated in dorsal root ganglion neurons of a rat model of bone cancer (Wu et al., 2012).

CONCLUSION

Desensitization of nicotinic ACh receptors is now considered an important process for neuronal signaling in health and disease (Giniatullin et al., 2005). In fact, there are ongoing efforts to develop drugs ("silent desensitizers"; Buccafusco et al., 2009) to modulate cholinergic function via receptor desensitization. The path is, therefore, open to look for chemical agents to desensitize P2X3 receptors selectively and to discover their impact on physiological or pathological conditions.

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AUTHOR CONTRIBUTIONS

Rashid Giniatullin and Andrea Nistri contributed to the writing of this review.

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P2X receptors as targets for the treatment of status epilepticus

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Prolonged seizures are amongst the most common neurological emergencies. Status epilepticus is a state of continuous seizures that is life-threatening and prompt termination of status epilepticus is critical to protect the brain from permanent damage. Frontline treatment comprises parenteral administration of anticonvulsants such as lorazepam that facilitate γ-amino butyric acid (GABA) transmission. Because status epilepticus can become refractory to anticonvulsants in a significant proportion of patients, drugs which act on different neurotransmitter systems may represent potential adjunctive treatments. P2X receptors are a class of ligand-gated ion channel activated by ATP that contributes to neuro- and glio-transmission. P2X receptors are expressed by both neurons and glia in various brain regions, including the hippocampus. Electrophysiology, pharmacology and genetic studies suggest certain P2X receptors are activated during pathologic brain activity. Expression of several members of the family including $P2X_2$, $P2X_4$, and $P2X_7$ receptors has been reported to be altered in the hippocampus following status epilepticus. Recent studies have shown that ligands of the $P2X_7$ receptor can have potent effects on seizure severity during status epilepticus and mice lacking this receptor display altered seizures in response to chemoconvulsants. Antagonists of the $P2X_7$ receptor also modulate neuronal death, microglial responses and neuroinflammatory signaling. Recent work also found altered neuronal injury and inflammation after status epilepticus in mice lacking the $P2X_4$ receptor. In summary, members of the P2X receptor family may serve important roles in the pathophysiology of status epilepticus and represent novel targets for seizure control and neuroprotection.

Keywords: anticonvulsant, ATP, epilepsy, hippocampus, interleukin-1β, microglia, neuroprotection

INTRODUCTION

Status epilepticus is a potentially devastating neurological condition of continuous seizures. Current treatments are often unsuccessful in achieving complete seizure suppression, particularly when delivered late, so novel targets must be identified. ATP-gated ion channels—P2X receptors—are an interesting new focus of status epilepticus research. The pleiotropic effects of P2X receptor activation, including neuromodulation under conditions of excessive neuronal firing and indirect effects on excitability via control of neuroinflammation and gliosis offer a "multi-targeting" mode of action that may be particularly well suited to suppressing both the immediate pathologic brain activity and its downstream consequences. This review summarizes recent work on P2X receptors in status epilepticus, with particular emphasis on the $P2X_7$ receptor ($P2X_7R$), and speculates on the potential of these receptors as future drug targets for seizure control.

STATUS EPILEPTICUS AND LIMITATIONS OF CURRENT TREATMENT

Status epilepticus is a state of continuous seizures, with an annual incidence ranging from 10 to 86 per 100,000 individuals (Chen and Wasterlain, 2006). Status epilepticus is traditionally defined as seizures lasting 30 min or more, but the current operational definition is clinical or electrographic seizures lasting beyond 5 min (Brophy et al., 2012). Status epilepticus represents a neurological emergency that is associated with profound morbidity and mortality. In humans and animal models, status epilepticus results in selective neuronal loss and gliosis, particularly within the hippocampus, as well as cognitive deficits and lasting hyper-excitability (Lowenstein, 2005; Chen and Wasterlain, 2006). Status epilepticus may result from metabolic disturbances, infection, drug toxicity or withdrawal, and non-compliance with the taking of anti-epileptic drugs (AEDs) (Brophy et al., 2012). Identifying the underlying cause of status epilepticus and treating it appropriately is paramount to alleviating the condition (Shorvon, 2011).

Pharmacological treatment of status epilepticus has been reviewed elsewhere (Lowenstein, 2005; Chen and Wasterlain, 2006) and new guidelines were recently published (Brophy et al., 2012). Initial therapy is to provide parenteral benzodiazepines such as lorazepam. Where benzodiazepines fail to control seizures, second-line therapy is usually with certain AEDs,

including phenytoin. If both groups of drug fail and status epilepticus has become refractory, treatment options include ongoing intravenous combinations of the above or alternative treatments including hypothermia (Brophy et al., 2012). Recent work supports the use of the *N*-methyl-D-aspartate (NMDA) receptor antagonist ketamine for refractory status epilepticus (Synowiec et al., 2013).

ANIMAL MODELS OF STATUS EPILEPTICUS

Animal models of status epilepticus have been critical for understanding the pathophysiology and treatment of status epilepticus. During status epilepticus there is a failure of the normal mechanisms for seizure termination, such as build-up of the anticonvulsant adenosine, acidosis or ion channel block. Other changes also accompany status epilepticus, including internalization of receptors for the inhibitory neurotransmitter γ-amino butyric acid (GABA) and externalization of receptors for the excitatory neurotransmitter glutamate (Wasterlain and Chen, 2006; Loscher, 2009). This is thought to underlie the development of benzodiazepine resistance which is common in status epilepticus. Optimal therapy is still lacking and there remains a need to identify other targets.

The most common animal models of status epilepticus use a chemoconvulsant or neurotoxin which is systemically administered or injected directly into the brain. Status epilepticus can also be triggered via electrical stimulation of the brain (e.g., perforant pathway, amygdala). Each has advantages and disadvantages, which have been reviewed elsewhere (Sperk, 1994; Loscher, 2002; Curia et al., 2008). To date, only the pilocarpine and kainic acid models have been used to investigate P2X modulation *in vivo*. Pilocarpine is a cholinergic agonist which produces status epilepticus and a pattern of hippocampal damage similar to that observed in epilepsy patients with mesial temporal sclerosis. However, induction of status epilepticus by pilocarpine appears to be secondary to peripheral immune responses and opening of the blood-brain barrier and the model is associated with high mortality, inter-animal variability in hippocampal pathology, and neuronal injury caused by ischemic as well as excitotoxic mechanisms (Fabene et al., 2007; Marchi et al., 2009). While the use of systemic kainic acid is also associated with variable hippocampal pathology, triggering status epilepticus using an intracerebral (e.g., intra-amygdala) injection of kainic acid produces a highly consistent focal and unilateral hippocampal lesion, with minimal mortality and reliable onset of spontaneous seizures (Li et al., 2008; Mouri et al., 2008; Liu et al., 2013). Such differences are important in critical evaluation of some of the conflicting findings with P2X ligands and genetically-modified mice.

ATP-GATED RECEPTORS; POTENTIAL TARGETS FOR SEIZURE CONTROL?

ATP acts as a neurotransmitter/co-transmitter in the CNS where it has important neuromodulatory and trophic effects (Burnstock, 2008). ATP is released from neurons and glia in response to neuronal activity via exocytosis as well as through alternative routes, including hemichannels and other mechanisms (Lazarowski et al., 2003; Dale and Frenguelli, 2009). ATP can also accumulate because of release from damaged cells. Convulsive activity produces an overall reduction in brain ATP levels but intense activation of neuronal pathways also triggers ATP release (Dale and Frenguelli, 2009). Once released, ATP acts on ionotropic P2X and metabotropic P2Y receptors, and produces a mixture of excitatory and inhibitory effects [for review, see Burnstock (2007); Abbracchio et al. (2009)]. The other major class of purinoceptor, P1 receptors, is activated by adenosine. Adenosine is a potent anticonvulsant, and its important contribution to seizure control has recently been reviewed (Boison, 2013a,b).

P2X receptors are all ATP-gated ion channels, usually composed of heterotrimers of individual subunits encoded by seven different genes (*P2rx1-7*) that gate fast depolarizing sodium (Na^+) and calcium (Ca^{2+}) entry. A number of additional properties are attributed to P2X receptors. Extended activation of certain P2X receptors leads to the formation of a large pore with permeability to small molecules. This is best understood for the $P2X_7R$, in which the response has been linked to a direct cytolytic effect, but other members of the P2X family may also form such channels (Surprenant et al., 1996; Di Virgilio et al., 1998; Virginio et al., 1999). The pore is not necessarily cytolytic, however, and there is controversy over whether the pore is instead formed by adjacent pannexin-1 channels (Duan et al., 2003; Pelegrin and Surprenant, 2006; Iglesias et al., 2008). The $P2X_7R$ has a number of other distinct characteristics. There is a large intracellular domain that enables it to directly interact with downstream pathways, including structural proteins (Kim et al., 2001). The receptor generally does not form heterotrimers and is found as a homotrimer, although recent work suggests it can interact with $P2X_4R$ in some cells (Craigie et al., 2013). The $P2X_7R$ also has low affinity for ATP, requiring mM levels for activation (Gever et al., 2006; Skaper et al., 2010). The implication is that the $P2X_7R$ is not activated under physiological conditions. The necessary conditions to generate sufficient extracellular ATP to activate $P2X_7R$ activation might include after cell lysis (e.g., neuronal necrosis due to excitotoxicity) or pathologic brain activity such as during prolonged or repeated brief seizures. Another feature of the $P2X_7R$ is that repeated agonist application under certain conditions results in sensitization and increased inward currents (Chessell et al., 1998; North and Surprenant, 2000; Armstrong et al., 2002).

Distribution of the P2X receptors has been previously reviewed, although characterization of their exact subunit composition in different tissues and cells is not yet complete (Norenberg and Illes, 2000; North and Surprenant, 2000; Gever et al., 2006). P2X receptors are found on neurons, where they may localize to both pre- and post-synaptic sites, and on non-neuronal cell types. The main subtypes expressed in the brain, including neurons in the hippocampus, are P2X2, 4 and 6 although P2X1, 3 and 5 receptor transcript and/or immunoreactivity has also been reported in the hippocampus (Papp et al., 2004; Dona et al., 2009; Engel et al., 2012a; Ulmann et al., 2013). The $P2X_7R$ was originally cloned from rat brain but considerable controversy has surrounded the exact localization in the brain. Early studies reported only microglial expression of the $P2X_7R$ in the adult brain (Collo et al., 1997). A number of groups have since reported $P2X_7R$ expression in neurons, including in the hippocampus (Deuchars et al., 2001; Armstrong et al., 2002; Dona

et al., 2009; Engel et al., 2012a). However, the specificity of the antibodies used in those studies was questioned by the finding of widespread immunostaining with different antibodies in $P2X_7R$ knockout mice (Sim et al., 2004). Although that study concluded that the $P2X_7R$ was not expressed at appreciable levels in neurons of the hippocampus, other studies have identified the mRNA for $P2X_7R$ in neurons in the rodent hippocampus (Yu et al., 2008). New evidence for constitutive expression of the $P2X_7R$ in hippocampal neurons has come from studies of mice expressing enhanced green fluorescent protein (EGFP) under the control of the $P2X_7R$ promoter (Engel et al., 2012a,b; Jimenez-Pacheco et al., 2013). In the normal mouse brain, EGFP is seen in few dentate granule neurons and also in certain populations of neurons in the neocortex (Engel et al., 2012a,b; Jimenez-Pacheco et al., 2013).

The other major cell type expressing $P2X_7R$ in the brain is microglia (Collo et al., 1997; Rappold et al., 2006; Dona et al., 2009). Oligodendrocytes also express $P2X_7R$ (Matute et al., 2007; Yu et al., 2008). Although expression of $P2X_7R$ has been reported in cultured astrocytes (Duan et al., 2003), there is limited evidence for such expression *in vivo* (Yu et al., 2008; Engel et al., 2012a; Jimenez-Pacheco et al., 2013). Thus, multiple members of the P2X receptor family are expressed in brain where they may exert important modulatory effects on neuro- and glio-transmission (Khakh, 2001; Abbracchio et al., 2009).

EXPRESSIONAL RESPONSE OF P2X RECEPTORS FOLLOWING STATUS EPILEPTICUS

Injury to the brain produces widespread changes to the expression of P2X receptors (Burnstock, 2008). Following status epilepticus, there is a prominent increase in $P2X_7R$ immunoreactivity and functional responses in microglia (Rappold et al., 2006; Avignone et al., 2008). Protein levels of the $P2X_7R$ measured by immunoblotting also increase after status epilepticus in the hippocampus and neocortex, including in the synaptodendritic compartment (Dona et al., 2009; Engel et al., 2012a; Jimenez-Pacheco et al., 2013). Transcript levels of $P2X_7R$ are increased in hippocampal neurons, particularly granule neurons, and microglia after status epilepticus (Avignone et al., 2008; Engel et al., 2012a). There has not been convincing *in vivo* evidence of changes to $P2X_7R$ expression in astrocytes or oligodendrocytes after status epilepticus (Rappold et al., 2006; Engel et al., 2012a; Jimenez-Pacheco et al., 2013).

There is less data on expressional responses of other P2X receptors after status epilepticus. Down-regulation of $P2X_2R$ has been reported after status epilepticus (Engel et al., 2012a) and $P2X_2R$ expression is also decreased in seizure-sensitive gerbils (Kang et al., 2003). For the $P2X_4R$, studies have reported both up- and down-regulation in the hippocampus after status epilepticus (Avignone et al., 2008; Dona et al., 2009). The $P2X_4R$ was recently reported to be up-regulated on hippocampal microglia after status epilepticus in rats (Ulmann et al., 2013) but is expressed at lower levels in the seizure-sensitive gerbil (Kang et al., 2003). Hippocampal protein levels of P2X1, 3, and 5 receptors, as measured by immunoblotting, were all unchanged after status epilepticus (Engel et al., 2012a). Thus, status epilepticus produces select changes to levels of P2X receptors which are likely to result in altered responsiveness of glia and neurons to ATP signaling in the brain. A summary of status epilepticus-induced changes to P2X receptor expression is provided in **Table 1**.

ROLE OF P2X RECEPTORS IN BRAIN EXCITABILITY

Under physiological circumstances, P2X gated currents at synapses are thought to be small and not uniformly detected (North, 2002; Khakh and North, 2006). Intracellular recordings have estimated the ATP-dependent fast excitatory component to comprise 5–20% of the total synaptic current in CA1 pyramidal cells (Pankratov et al., 2002). The real significance of P2X receptor-mediated current may be to facilitate Ca^{2+} entry into cells. Together with the presynaptic location of certain P2X receptors, this implicates them in control of neurotransmitter release (Sperlagh et al., 2007). The distribution of P2X receptors at synapses—particularly at the periphery of the post-synaptic density—suggests their contribution becomes more important under conditions of intense neuronal activity (Khakh and North, 2006).

A number of studies have investigated the effects of P2X receptor activation or blockade on hippocampal excitability and there is evidence for both pro- and anti-excitatory consequences. An excitatory effect of the P2X agonist α, β-meATP was found in rat hippocampal slices (Ross et al., 1998) and hippocampal slices from seizure-prone mice release more extracellular ATP when stimulated (Wieraszko and Seyfried, 1989). In contrast, blockade of post-synaptic P2X receptors was observed to facilitate long-term potentiation, suggesting some P2X receptor functions restrict certain aspects of synaptic plasticity (Pankratov et al., 2002). $P2X_2R$ are present on the presynaptic terminals of CA3 axons (Schaffer collaterals) that terminate on inhibitory interneurons in the CA1 subfield and are thought to function as a physiological brake on excessive neuronal activity (Khakh and North, 2006). Activation of $P2X_2R$ enhances release of excitatory neurotransmitter onto CA1 interneurons, which in turn increases release of inhibitory neurotransmitter to reduce excitatory drive onto CA1 pyramidal neurons (Khakh et al., 2003). Notably, down-regulation of the $P2X_2R$ has been reported in models of status epilepticus, suggesting loss of this receptor might represent a novel "channelopathy" (Engel et al., 2012a).

$P2X_7R$ also mediate effects on hippocampal excitability. Stimulation of $P2X_7R$ present on the presynaptic terminals of mossy fibers reduced excitatory field potentials recorded in the CA3 subfield (Armstrong et al., 2002). These data are consistent with a model whereby pre-synaptic $P2X_7R$ are activated during high-level neuronal excitability and function to reduce further release of glutamate from mossy fiber terminals (Armstrong et al., 2002). The $P2X_7R$ effect to decrease transmitter release probability at mossy fiber synapses is therefore the opposite of what was found for pre-synaptically-located $P2X_2R$, which enhanced transmitter release probability, but functionally these actions are synergistic, to limit over-excitation within the hippocampus. These results are also consistent with the main effects of ATP being presynaptic, not post-synaptic in the hippocampus (Khakh et al., 2003). Other recent work in a model of recurrent epileptiform activity found a small effect of $P2X_7R$ antagonists against slow

Table 1 | P2X receptors in status epilepticus.

	Expression in seizure-relevant brain regions	Expressional response to status epilepticus	Effect of agonists/antagonists or knockout on status epilepticus, seizure-induced cell death and inflammation
$P2X_1$	Hippocampus[a] Cortex[b]	*Hippocampus* No change (W)[c] Up-regulated (qPCR)[d] *Cortex* not studied	Not studied
$P2X_2$	Hippocampus[e,f] Cortex[e]	*Hippocampus* Decreased (W)[c] No change (W)[g] *Cortex* not studied	Not studied
$P2X_3$	Hippocampus[h] Cortex[h]	*Hippocampus* No change (W)[3] *Cortex* not studied	Not studied
$P2X_4$	Hippocampus[f,i,j] Cortex[i,j]	*Hippocampus* Increased (W, IH)[k] No change (W)[c,g] Up-regulated (qPCR)[d] *Cortex* not studied	*$P2X_4$ knock-out mice:* Decreased seizure-induced cell death (i.p. KA)[k] No effect on seizures (i.p. KA)[k] Decreased inflammation and microglia density (i.p. KA)[k] No change in IL-1β levels (i.p. KA)[k]
$P2X_5$	Hippocampus[l] Cortex[l]	*Hippocampus:* No change (W)[c] *Cortex* not studied	Not studied
$P2X_6$	Hippocampus[f,i] Cortex [i]	Not studied	Not studied
$P2X_7$	Hippocampus[c,m,n] Cortex[n,o]	*Hippocampus:* Increased (W, GFP)[c,g] Up-regulated (qPCR)[d] Increased (IH)[p] *Cortex:* Increased (W, GFP)[o]	*Agonists (BzATP):* Increased seizures (i.a. KA)[c] No effect on seizures (Pilo)[q] Increased microglia activation (Pilo)[r] Increase in astrocyte loss (Pilo)[s] Increased TNF-α immunoreactivity (Pilo)[t] Decreased seizure-induced cell death (Pilo)[t] *$P2X_7R$ knock-out mouse:* Decreased seizures (i.a. KA)[c] Increased seizures (Pilo)[q] No effect on seizures (i.p. KA and i.p. Pic)[q] *Antagonists (A-43, A-74, BBG, OxATP, IgG-$P2X_7$):* Decreased seizures (i.a. KA)[c,o] Increased seizures (Pilo)[q] Decreased seizure-induced cell death (i.a. KA)[c] Increased seizure-induced cell death (Pilo)[t] Decreased astrocyte loss (Pilo)[s] Decreased microglia activation (i.a. KA and Pilo)[c,r] Decreased Il-1β levels (i.a. KA)[c]

A-43, A-438079; A-74, A-740003; BzATP, 2′,3′-O-(4-benzoylbenzoyl)-adenosine 5′-triphosphate; OxATP, oxidised ATP GFP, $P2X_7R$-GFP-reporter mouse; i.a. KA., intra-amygdala KA; i.p. KA, intra-peritoneal KA; Pilo, Pilocarpine; i.p. Pic, intra-peritoneal Picrotoxin; IgG-$P2X_7$, anti-$P2X_7$ antibodies; Il-1β, interleukin-1beta; qPCR, quantitative polymerase chain reaction; IH, Immunohistochemistry; TNF-α, Tumor necrosis factor alpha; W, Western blot.

[a] *Cavaliere et al., 2007*; [b] *Lalo et al., 2008*; [c] *Engel et al., 2012a*; [d] *Avignone et al., 2008*; [e] *Kanjhan et al., 1999*; [f] *Rubio and Soto, 2001*; [g] *Dona et al., 2009*; [h] *Seguela et al., 1996*; [i] *Collo et al., 1996*; [j] *Le et al., 1998*; [k] *Ulmann et al., 2013*; [l] *Guo et al., 2008*; [m] *Armstrong et al., 2002*; [n] *Yu et al., 2008*; [o] *Jimenez-Pacheco et al., 2013*; [p] *Rappold et al., 2006*; [q] *Kim and Kang, 2011*; [r] *Choi et al., 2012*; [s] *Kim et al., 2011a*; [t] *Kim et al., 2011b*.

field potentials induced by potassium-bicuculline treatment of rat cortical slices (Klaft et al., 2012).

Overall, the properties of the P2X system—activation under high levels of neuronal activity—are particularly relevant to status epilepticus and raise the prospect of a class of receptor that when targeted may influence pathologic brain activity while leaving normal neurotransmission largely unaffected. Nevertheless, until very recently, no study had directly investigated the effect of ligands acting at P2X receptors on status epilepticus.

In vivo STUDIES WITH $P2X_7R$ LIGANDS IN STATUS EPILEPTICUS

There has been significant interest in $P2X_7R$ ligands as therapeutics for neurological conditions (Skaper et al., 2010). The leading clinical application of $P2X_7R$ receptor antagonists is for treatment of neuropathic pain but there are indications in acute neurologic injuries. For example, $P2X_7R$ antagonists have been reported to reduce injury or inflammation following intracerebral hemorrhage (Chen et al., 2013) and global ischemia (Yu et al., 2013). In focal cerebral ischemia, protective effects were reported in some studies (Melani et al., 2006; Arbeloa et al., 2012) but not others (Le Feuvre et al., 2003). Also of interest, $P2X_7R$ agonists have been shown to trigger a protective state, a form of "chemical preconditioning," that rendered cardiac tissue resistant to subsequent ischemia (Vessey et al., 2011). Protective effects have also been reported for $P2X_7R$ antagonists in models of neurodegeneration, including Huntington's disease (Diaz-Hernandez et al., 2009), Parkinson's disease (Marcellino et al., 2010), amyotrophic lateral sclerosis (Cervetto et al., 2013) and Alzheimer's disease (Diaz-Hernandez et al., 2012; Murphy et al., 2012).

$P2X_7R$ antagonists have been reported to produce potent anticonvulsant effects in some, but not all models of status epilepticus (see **Table 1**). Studies by the authors demonstrated that a central (intracerebroventricular) injection the $P2X_7R$ antagonists BBG or A-438079 resulted in as much as a 50% reduction in electrographic seizures during status epilepticus triggered by intra-amygdala microinjection of kainic acid in mice (Engel et al., 2012a; Jimenez-Pacheco et al., 2013). Behavioral convulsions were also reported to be reduced in mice treated with A-438079 prior to status epilepticus (Jimenez-Pacheco et al., 2013). Experiments using the "Pfizer" $P2X_7R$ knockout mice (Solle et al., 2001) supported these pharmacological studies, with seizure severity reduced compared to wild-type animals. Further complementing these findings, intracerebroventricular injection of a $P2X_7R$ blocking antibody suppressed seizures while BzATP, a $P2X_7R$ agonist, exacerbated seizures in the model (Engel et al., 2012a). Analysis of the hippocampus and neocortex of mice pre-treated with $P2X_7R$ antagonists found reductions in neuronal death, microgliosis and interleukin-1β (Engel et al., 2012a; Jimenez-Pacheco et al., 2013). Treatment of mice with $P2X_7R$ antagonists 20 min after triggering status epilepticus - a more clinically-relevant scenario—also reduced seizure severity and protected the hippocampus (Engel et al., 2012a). Finally, injection of A-438079 1 h after status epilepticus began, at a time when sensitivity to lorazepam was reduced, also had modest seizure-suppressive effects (Engel et al., 2012a). This finding is important since it supports the possible use of P2X ligands as adjunctive treatments for status epilepticus alongside frontline drugs such as lorazepam.

These findings add complexity to the pathophysiological functions assigned to the $P2X_7R$ in the brain. *In vitro* data had supported the $P2X_7R$ as a "physiological brake" on overexcitation (Armstrong et al., 2002) but these *in vivo* data indicate blocking the $P2X_7R$ reduces hyper-excitation. It will be important to establish mechanisms that account for the observed *in vivo* effects of $P2X_7R$ antagonists against status epilepticus. Direct effects of $P2X_7R$ on neuronal activity may behave differently *in vivo* during status epilepticus. For example, the pre-synaptic $P2X_7R$ thought present on mossy fibers facilitating rather than opposing glutamate release, as demonstrated for $P2X_2R$ (Khakh et al., 2003). Also, perhaps blocking $P2X_7R$ on glia (e.g., microglia) confers anti-excitatory effects that functionally supersede pre-synaptic effects limiting transmitter release. As always, findings based mainly on pharmacology require careful consideration of the specificity of the ligands involved (Anderson and Nedergaard, 2006).

OTHER P2X RECEPTOR LIGANDS IN STATUS EPILEPTICUS

The only other member of the P2X family for which *in vivo* data exist in a model of status epilepticus is the $P2X_4R$. Mice lacking the $P2X_4R$ display a reduction in neuronal death after status epilepticus, although seizures themselves were not altered in these mice (Ulmann et al., 2013). Notably, while some inflammatory signaling was also reduced, the induction of interleukin-1β was not found to be different, supporting other work linking modulation of this pathway to the $P2X_7R$ (see below).

We can speculate that targeting other members of the P2X family would have seizure-modulating effects *in vivo*, although there are fewer ligands selective for the other P2X receptors. An obvious candidate would be an agonist of the $P2X_2R$. Activation of this receptor promotes inhibitory transmission within the hippocampus (Khakh and North, 2006). Delivery of such a ligand might enhance endogenous mechanisms of seizure suppression.

GLIA-RELATED FUNCTIONS OF THE $P2X_7R$ IN STATUS EPILEPTICUS

The immediacy of the seizure-suppressive effects of $P2X_7R$ antagonists implies a direct action on neurons, for which there is supporting evidence (Armstrong et al., 2002; Engel et al., 2012a,b). However, expression and activation of the $P2X_7R$ on glia may have important effects on excitability that influence the pathophysiology and outcome of status epilepticus. First, $P2X_7R$ activation has been associated with production of cytokines from microglia (Ferrari et al., 1996; Chakfe et al., 2002). In particular, activation of the $P2X_7R$ leads to processing and release of interleukin-1β, which is a potent pro-convulsive molecule implicated as a target for seizure control (Vezzani et al., 2010). Activated microglia also exacerbated excitotoxicity in hippocampal cultures, an effect shown to be $P2X_7R$-dependent (Bernardino et al., 2008). Thus, targeting $P2X_7R$ effects on microglia may reduce post-status epilepticus inflammation and susceptibility to excitotoxicity.

$P2X_7R$ have been implicated in certain trophic functions, including the activation and proliferation of microglia. This

serves both restorative and pathologic functions after status epilepticus, contributing to tissue repair but also releasing pro-inflammatory mediators which may contribute to hyperexcitability (Devinsky et al., 2013). Increased $P2X_7R$ expression and receptor activation was found to transform resting microglia to an activated state (Monif et al., 2009). Activation of the pore-forming function of the $P2X_7R$ was also found to be required for microglial proliferation (Monif et al., 2009). Consistent with this model, blockade of $P2X_7R$ reduces microglia activation after status epilepticus (Kim et al., 2009; Choi et al., 2012; Engel et al., 2012a). Targeting the pore-forming functions of the $P2X_7R$ may therefore be a novel approach to limit microglia responses following status epilepticus.

Astrocytes represent another non-excitable cell involved in mediating the effects of ATP. There is *in vitro* evidence that $P2X_7R$ activation on astrocytes triggers glutamate release, perhaps directly through the channel/pore (Duan et al., 2003). Such $P2X_7R$-mediated glutamate release from astrocytes may contribute to astrocyte signaling, promote excitability or even excitotoxicity. $P2X_7R$ activation on astrocytes may also serve a cell-killing function. Injection of rats with the $P2X_7R$ agonist BzATP was found to reduce astrocyte numbers after status epilepticus and $P2X_7R$ antagonists prevented astrocyte death (Kim et al., 2009, 2011a). Thus, secondary effects of P2X7R ligands will need to be considered as modulation of astrocyte number or activation has profound effects on excitability in the brain (Boison, 2008). Oligodendrocytes are also sensitive to the toxic effects of ATP acting on $P2X_7R$ (Matute et al., 2007).

Finally, it has emerged that the $P2X_7R$ may promote axonal growth and branching in the hippocampus (Diaz-Hernandez et al., 2008). Studies have also reported that $P2X_7R$ antagonists improved recovery after spinal cord injury (Peng et al., 2009). Synaptic reorganization is long-recognized following status epilepticus and has been implicated in establishing recurrent excitatory circuits (e.g., mossy fiber sprouting) that may contribute to epileptogenesis or chronic epilepsy (Houser et al., 2012). The benefits from targeting the $P2X_7R$ could therefore extend well beyond the initial period of seizure activity. As with effects on astrocytes and microglia, these data suggest targeting the $P2X_7R$ may have pleiotropic effects and the timing of manipulations or site of targeting may be critical to obtain optimal therapeutic benefit.

Figure 1 summarizes the mechanisms of ATP release during seizures, the receptors upon which ATP may act, expressional changes, and some of the downstream effects of P2X receptor modulation relevant to the pathophysiology of status epilepticus.

LIMITATIONS OF TARGETING $P2X_7$ RECEPTORS

While there is significant support for a role for $P2X_7R$ in neuronal injury and/or glial activation, there are also conflicting findings. Excitotoxic injury has been reported to be unchanged in mice lacking $P2X_7R$ or in response to $P2X_7R$ antagonists (Le Feuvre et al., 2003). Recent work by Frenguelli's group found a limited role for P2X receptors in electrically-evoked seizure-like activity in rat hippocampal slices, and no effect of $P2X_7R$ antagonists on these events (Lopatar et al., 2011). Similarly, no effects of $P2X_7R$ antagonists on *in vitro* epileptiform activity were detected in acute cortical slices from epileptic rats (Klaft et al., 2012). Inter-species differences could be to blame and have been reported for the $P2X_7R$ (Chessell et al., 1998). Last, Kang's group reported that pilocarpine-induced seizures were in fact exacerbated in mice lacking $P2X_7R$ and in wild-type animals treated with $P2X_7R$ antagonists (Kim and Kang, 2011). These data are in sharp contrast to the findings with intra-amygdala kainic acid-induced

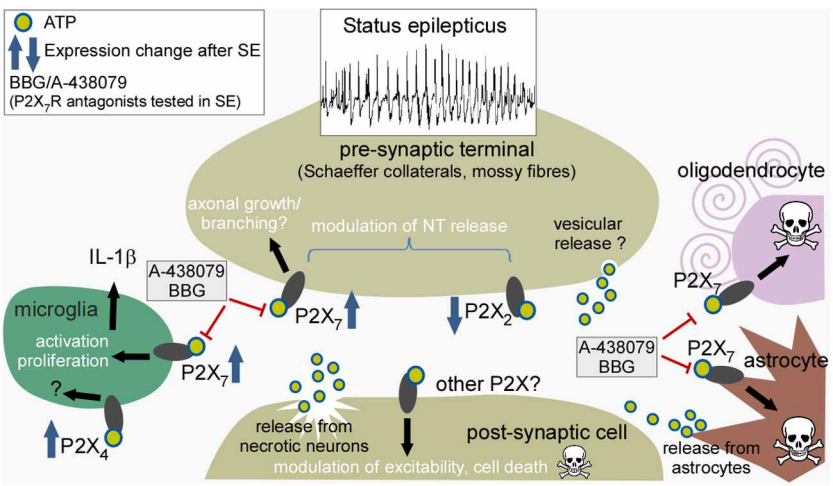

FIGURE 1 | Potential sites of action of ATP released during status epilepticus, expressional responses of individual P2X receptors, and consequences of receptor activation. Cartoon depicts the various different cell types reported to express P2X receptors and their presumed cellular locations. ATP is released during sustained neuronal activity and from damaged neurons to act pre- and post-synaptically on neurons, particularly targeting pre-synaptic receptors to modulate neurotransmitter release. ATP may also act on receptors of microglia to promote activation and release of interleukin-1β, and act on astrocytes and oligodendrocytes to trigger cell death. Drugs such as A-438079 and BBG have been reported to reduce seizures and gliosis after status epilepticus. SE, status epilepticus; IL-1β, interleukin-1β.

status epilepticus (Engel et al., 2012a; Jimenez-Pacheco et al., 2013). Although the reasons underlying these contradictory findings are uncertain they may relate to the specificity and dose of the ligands used, models or genetic tools. Indeed, the findings may be peculiar to the pilocarpine model because seizures induced by kainate or picrotoxin were not exacerbated in mice lacking $P2X_7R$ in that study (Kim and Kang, 2011). Another factor may be the variable influence of tissue responses affecting the pharmacological properties of the receptor. The $P2X_7R$ is inhibited by acidosis (North, 2002), which develops during status epilepticus (Ziemann et al., 2008). Does $P2X_7R$ blockade develop regardless of pharmacological antagonism in some models of status epilepticus? Further studies will be needed to resolve these complicated issues.

OTHER CONSIDERATIONS IN THE DEVELOPMENT OF P2X LIGANDS FOR STATUS EPILEPTICUS

New P2X receptor ligands have recently emerged while several of the known P2X antagonists show potentially suitable drug profiles. This includes the $P2X_7R$ antagonist A-438079, a relatively small molecule that crosses the blood-brain barrier following systemic delivery (McGaraughty et al., 2007). A recently developed $P2X_7R$ antagonist is also able to pass the blood brain barrier (Bhattacharya et al., 2013). More complete reviews on the potential of P2X receptor ligands as drugs can be found elsewhere (Burnstock, 2008; North and Jarvis, 2013).

Despite the need for new therapies, there has been disengagement of several of the major pharmaceutical companies from developing new anticonvulsants and treatments for epilepsy. It has been suggested that the identification of additional clinical applications (i.e., non-epileptic conditions) would make a potential anticonvulsant drug candidate significantly more attractive for development (Bialer and White, 2010). One example is with the effectiveness of certain AEDs for the treatment of pain. Notably, a key area of deployment of $P2X_7R$ antagonists is for the treatment of pain conditions (Trang et al., 2012; Alves et al., 2013).

REMAINING CHALLENGES

There are opportunities for the use of P2X receptor ligands in the control or prevention of seizures but significant challenges remain. Foremost, $P2X_7R$ antagonists need to be evaluated in other models because of the conflicting reports between kainic acid and pilocarpine models. These issues are not unique to status epilepticus; conflicting data on the $P2X_7R$ have emerged in the stroke field (Le Feuvre et al., 2003; Melani et al., 2006; Yanagisawa et al., 2008; Arbeloa et al., 2012). A genetic approach will be needed to confirm specific drug effects are lost in animals lacking the $P2X_7R$, although such studies are not without problems (Nicke et al., 2009; Masin et al., 2012).

Assuming $P2X_7R$ antagonists display consistent anticonvulsive profiles, work will be needed to explore dosing and route of delivery. Do these drugs suppress seizures when given systemically and are there off-target effects? Increased attention is needed on assessing the pleiotropic actions of these drugs, and consideration given to looking for effects on glia and inflammation, including long after the initial period of status epilepticus. Are anti-inflammatory effects of $P2X_7R$ antagonists countered by trophic effects that promote gliosis? P2X ligands may have effects against spontaneous seizures, which would raise their potential as future AEDs. P2X receptors serve important roles in neurodevelopment and ligands may also have potential to treat seizures in the developing brain, a condition currently poorly served by available treatments (Slaughter et al., 2013). It will also be important to determine the mechanism(s) controlling P2X receptor expression after status epilepticus. Indeed, efforts to "rescue" the post-status epilepticus decline in $P2X_2R$ levels could promote inhibitory transmission. Last, we lack relevant human data. Although there is evidence for altered expression of P2X receptors in human epilepsy (Jimenez-Pacheco et al., 2013), their expression and function in status epilepticus is unknown.

In summary, there is increasing evidence for a role for ATP and P2X receptors in seizure states such as status epilepticus and epilepsy. Certain P2X receptors may represent novel drug targets for seizure control. Targeting these receptors could provide frontline or adjunctive seizure suppression during status epilepticus, as well as influencing post-injury glial function that may help mitigate outcomes including epileptogenesis.

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A taste for ATP: neurotransmission in taste buds

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Not only is ATP a ubiquitous source of energy but it is also used widely as an intercellular signal. For example, keratinocytes release ATP in response to numerous external stimuli including pressure, heat, and chemical insult. The released ATP activates purinergic receptors on nerve fibers to generate nociceptive signals. The importance of an ATP signal in epithelial-to-neuronal signaling is nowhere more evident than in the taste system. The receptor cells of taste buds release ATP in response to appropriate stimulation by tastants and the released ATP then activates P2X2 and P2X3 receptors on the taste nerves. Genetic ablation of the relevant P2X receptors leaves an animal without the ability to taste any primary taste quality. Of interest is that release of ATP by taste receptor cells occurs in a non-vesicular fashion, apparently via gated membrane channels. Further, in keeping with the crucial role of ATP as a neurotransmitter in this system, a subset of taste cells expresses a specific ectoATPase, NTPDase2, necessary to clear extracellular ATP which otherwise will desensitize the P2X receptors on the taste nerves. The unique utilization of ATP as a key neurotransmitter in the taste system may reflect the epithelial rather than neuronal origins of the receptor cells.

Keywords: purinergic, P2Y, adenosine, glossopharyngeal, chorda tympani, hemichannel, pannexin, connexin, CALHM1

INTRODUCTION

Epithelia are faced with seemingly conflicting tasks – first, they serve as a barrier between the external world and the innards of an organism, and second, they are extended sensory organs responding to varied stimuli in the external world including temperature, pressure, and even illumination. The epithelial barrier is formed by junctional complexes between epithelial cells obstructing the free diffusion of materials from outside to in. The sensory functions are accomplished either by direct responses by keratinocytes, or by activation of appropriate sensors on intraepithelial nerve fibers. The sensory responses by keratinocytes are relayed to the sensory nerve fibers by release of appropriate mediators or transmitters including ATP, which activates neural purinergic receptors (e.g., Mandadi et al., 2009; Barr et al., 2013). Thus purinergic signaling is a common means by which epithelial keratinocytes communicate with sensory nerve fibers. In contrast, typical epithelial sensory endorgans, e.g., photoreceptors, auditory hair cells, and olfactory receptor cells, utilize conventional neurotransmitters such as glutamate, for neurotransmission.

Taste buds, the sensory endorgans of gustation consist of a collection of 50–100 specialized, columnar taste cells embedded in the relatively non-specialized, lightly keratinized stratified squamous lingual epithelium. The keratinocytes of the non-specialized lingual epithelium are similar to epithelial cells elsewhere in the body in that they respond to a variety of external stimuli, e.g., pressure, or chemicals, by releasing ATP along with other intercellular signaling molecules (Mandadi et al., 2009; Lazarowski et al., 2011; Barr et al., 2013). Taste cells, like other epithelial cells, but unlike other epithelial sensory endorgans, rely on ATP to activate the sensory nerves innervating the taste buds (Bo et al., 1999; Finger et al., 2005). However, although taste cells have an epithelial origin, they do have neuron-like voltage-gated ion channels and generate action potentials to most taste stimuli (Damak et al., 2006; Yoshida et al., 2009). While depolarization is required for ATP release and the amount of ATP released is proportional to the frequency of action potentials (Murata et al., 2010), the requirement for action potentials in ATP release is controversial (Huang and Roper, 2010).

ATP is utilized for intercellular communication in a wide variety of biological contexts including neural signaling. At many synapses, ATP is co-released with a conventional neurotransmitter and serves in a trophic or modulatory role modifying the responsivity of the sensory cells or modifying actions of a conventional neurotransmitter. For example, in the auditory system, ATP serves a protective function helping maintain epithelial integrity in the face of extreme stimulation (Thorne et al., 2004). In the olfactory system, ATP modulates neural sensitivity, induces production and release of growth factors, and modulates cell division of proliferative basal cells (Jia et al., 2011). In these systems, interruption of purinergic signaling leads to relatively minor disruption of function or cell turnover. Conversely, in the taste system, ATP is necessary for transmission of information from the sensory cells to the afferent nerve fibers. Genetic elimination of the P2X receptors on the sensory nerve fibers (P2X2 and P2X3) totally eliminates transmission of the signal from taste receptor cells to nerve fibers (Finger et al., 2005). In this review, we describe the evidence that ATP serves a unique, crucial role in transmission of taste information from the taste buds to the taste nerves.

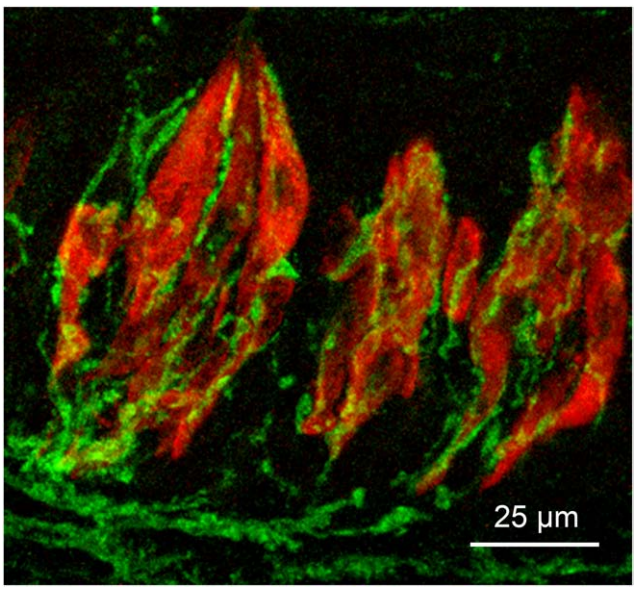

FIGURE 1 | Micrograph of longitudinal section through three taste buds in the circumvallate papilla of a mouse. Type II taste cells are stained red with antiserum to PLCβ2; gustatory afferent fibers are stained green with an antiserum to P2X3. The surface of the epithelium is at the top of the micrograph.

TASTE BUDS

Taste cells, the sensory cells of taste buds, arise during late embryogenesis, not from neural progenitors such as neural crest or neurogenic placodes, but from the lingual epithelium itself (Stone and Finger, 1994; Barlow and Northcutt, 1995). Thus, unlike other receptor cells, e.g., olfactory receptor cells and hair cells, taste cells are a specialized component of the epithelium rather than being derived from neurogenic progenitors. Molecular differentiation of the undifferentiated lingual epithelium begins about day E12 in mice, and can first be recognized by small clusters of cells expressing a variety of developmental signaling molecules such as sonic hedgehog, wnts, and BMP (Hall et al., 1999; Jung et al., 1999; Mistretta et al., 2003; Iwatsuki et al., 2007; Liu et al., 2007; Kapsimali and Barlow, 2013). Fully differentiated taste cells first appear shortly before birth although full elaboration of the peripheral taste system does not occur until several days postnatally.

A mature taste bud contains multiple types of taste cells distinguished morphologically, physiologically, and molecularly (**Figure 1**). Conventionally, taste cells are divided into three types, which although based originally on staining characteristics, correlates well with molecular and functional expression profiles (Yee et al., 2001; Finger, 2005; Finger et al., 2005; Huang et al., 2007; Romanov et al., 2007; Roper, 2013). *Type I cells*, which constitute the majority of cells within each bud, are glial-like in that they exhibit several features common to astrocytes. They enwrap other cells with flattened processes, express proteins associated with neurotransmitter reuptake or catabolism (including NTPDase2; Bartel et al., 2006), and form no apparent specialized contacts with the sensory nerve fibers. *Type II cells* are the receptor cells for the taste qualities of sweet, bitter, and umami (savory) mediated by the taste receptor (TR) family of G-protein coupled taste receptors and the related phospholipase C (PLC)-mediated downstream cascade (Yarmolinsky et al., 2009; Roper, 2013). The points of contact between the type II cells and nerve fibers often exhibit a non-conventional specialization involving subsurface cisternae and atypical mitochondria (Royer and Kinnamon, 1988; Clapp et al., 2004). No conventional synapses, complete with presynaptic vesicles and postsynaptic membrane thickening are evident at contacts between type II cells and nerve fibers. *Type III cells* do not express the TR family taste receptor proteins or downstream cascade, but do form conventional synapses with the afferent nerves (Royer and Kinnamon, 1988). Type III cells are required for sour taste transduction but are not required for transmission of taste information from type II cells to the afferent nerves since genetic deletion of these cells does not disrupt sweet, bitter, and umami detection (Huang et al., 2006).

The cellular basis of salt taste is poorly understood but likely involves multiple cell types and mechanisms (Chandrashekar et al., 2010; Oka et al., 2013). High concentrations of salt stimulate both type III cells and type II cells via an amiloride-insensitive mechanism (Oka et al., 2013). In contrast, low concentrations of salt primarily utilize the amiloride-sensitive epithelial sodium channel, ENaC, in a cell type (Chandrashekar et al., 2010), lacking voltage-gated ion channels (Vandenbeuch et al., 2008), i.e., not a typical type II or type III cell. If these ENaC-expressing taste cells are type I cells, as suggested by Vandenbeuch et al. (2008), how these glial-like taste cells might communicate with the afferent nerve fibers is unclear. One possibility is that ENaC-expressing taste cells communicate via a paracellular mechanism to electrically excitable cells in the taste bud which relay the signal to the nerve fibers.

Since type III cells are the only cells that possess conventional synapses, then transmission of taste information from type II cells to nerve fibers must utilize a non-conventional functional contact, perhaps the contacts with subsurface cisternae and specialized mitochondria.

NEUROTRANSMITTERS IN TASTE CELLS

Several potential neurotransmitters have been identified in taste buds (for detailed review, Roper, 2013). These include serotonin (Kaya et al., 2004; Huang et al., 2005, 2009), GABA (Cao et al., 2009; Starostik et al., 2010; Dvoryanchikov et al., 2011; Huang et al., 2011a), and noradrenalin (Huang et al., 2008; Zhang et al., 2010), which are released from type III cells, and acetylcholine, released from type II cells (Dando and Roper, 2012). In addition, several peptide transmitters have been identified in taste buds (for recent review, Dotson et al., 2013). These include CCK, VIP, NPY, and PYY, and glucagon in type II cells, and GLP-1 and galanin in both type II and type III cells. Ghrelin appears to be expressed non-specifically in all taste cells. Rather than primarily activating afferent nerve fibers, these transmitters and peptides appear to exert their effects largely by binding to cognate receptors on adjacent taste cells, modulating the output of the taste bud. The possible exceptions to this are serotonin, which may activate 5-HT3 receptors on afferent nerve fibers (Kaya et al., 2004) and GLP-1, which may activate GLP-1 receptors on both nerve fibers and other taste cells to modulate sweet taste (Shin et al., 2008).

However, knockout (KO) of either 5-HT3 (Finger et al., 2005) or GLP-1R (Shin et al., 2008) fails to block taste behaviors, suggesting that while these transmitters may play a role in activating nerve fibers, they are not required.

Glutamate also has been suggested to serve as a taste transmitter (Vandenbeuch et al., 2010a), primarily because the glutamate transporter GLAST is co-expressed in type I taste cells (Lawton et al., 2000). However, the expression of vesicular glutamate transporters VGLUT1 and 2 is restricted to afferent nerve fibers (Vandenbeuch et al., 2010a), suggesting glutamate may be released from afferent nerve fibers via an axon reflex, thereby modulating taste bud function by activating ionotropic glutamate receptors on the type III taste cells (Caicedo et al., 2000; Vandenbeuch et al., 2010a; Niki et al., 2011; Huang et al., 2012). Although all of these potential transmitters may play roles in modulating taste, none has been shown to meet *all* of the criteria for a substance to be accepted as an afferent transmitter: presence in the presynaptic cell, release upon stimulation, activation of postsynaptic receptors on afferent nerve fibers, and a mechanism for degradation or removal of the transmitter from the extracellular space. Only ATP meets all four of these criteria.

ATP RELEASE

ATP is present at mM concentrations in the cytoplasm of all cells, so the question is, whether ATP is released by taste stimulation, and if so, by what mechanisms? ATP release with taste stimulation was first described at a tissue level by a luciferin–luciferase assay (Finger et al., 2005) and subsequently characterized at a cellular level by a variety of techniques, including biosensor cells containing purinergic receptors (Huang et al., 2007; Romanov et al., 2007), and luciferin–luciferase assays from patch pipets contacting identified taste cells (Murata et al., 2010). All of these studies suggested that release occurred by an unconventional, non-vesicular mechanism likely involving depolarization-activated ATP release channels. Curiously, release was only detected from type II taste cells, i.e., those that lack conventional synapses with the afferent nerve fibers. The identity of the ATP release channel is still in question, since several putative release channels are expressed in taste buds: connexins 43 and 30 in both taste cells and non-gustatory epithelial cells (Huang et al., 2007; Romanov et al., 2007), pannexin-1, expressed primarily in type II cells (Huang et al., 2007; Romanov et al., 2007), and a recently discovered ATP release channel, CALHM1, expressed in most type II taste cells (Taruno et al., 2013). The only channel knockout that has been examined at the systems level in taste buds is CALHM1, which shows severely diminished responses to bitter, sweet, and umami taste stimuli (all type II cell qualities), with little effect on other qualities. These data suggest that CALHM1 plays a role in the release process. However, the pharmacology of taste-evoked release suggests the ATP release channels are likely composed of pannexin-1, since release is blocked by low concentrations of carbenoxolone (Huang et al., 2007, 2011b; Dando and Roper, 2009; Murata et al., 2010). Taste buds of pannexin-1 knockouts are capable of ATP release (Romanov et al., 2012), but these knockouts have not been examined with either taste nerve recording or behavior, so the mechanism of release remains in question.

PURINERGIC RECEPTORS IN AFFERENT FIBERS AND TASTE BUDS

The presence of purinergic receptors on the afferent nerve fibers was first discovered by Bo et al. (1999), who found both P2X2 and P2X3 on nerve fibers innervating taste buds. This has been examined more recently by Ishida et al. (2009), who showed that all geniculate ganglion neurons in rodents express P2X3, with approximately 70% also expressing P2X2. If P2X2 and P2X3 are required for transmitting taste information to the nervous system, then the double knockout of P2X2 and P2X3 should abolish taste-evoked behavior. Indeed, not only were responses to sweet, bitter, and umami abolished in the double knockout, but responses to other taste stimuli were abolished as well, suggesting ATP was required for transmission of all taste qualities to the nervous system (Finger et al., 2005; Ohkuri et al., 2012). Interestingly, single knockouts of either P2X2 or P2X3 had only a minor taste phenotype, suggesting that either P2X2 or P2X3 is capable of forming functional homomeric receptors in the taste afferents (Finger et al., 2005) although the typical receptor in wildtype mice is likely a P2X2/P2X3 heteromer.

As is true for any genetic deletions, global knockout of P2X2 and P2X3 may affect development or carry other unintended effects that could negatively impact taste functions. Indeed, the P2X2/P2X3 double knockout mice fail to release ATP normally in response to taste stimulation, suggesting the lack of taste responses could be due to a presynaptic rather than a postsynaptic defect (Huang et al., 2011b). Nevertheless, our recent discovery of a pharmacological recapitulation of the knockout findings is further confirmation of the importance of purinergic signaling in taste transmission. After i.p. injection of an antagonist selective for P2X3-containing receptors (Vandenbeuch et al., 2013a), responses to all taste qualities are eliminated or substantially reduced. The lack of responses to sour and salty stimuli as in the double P2X knockout is especially noteworthy since ATP release has not been detected from type III cells in response to either depolarization or sour stimuli (Huang et al., 2007; Romanov et al., 2007). However, recent studies indicate that nerve fibers contacting type III cells do express P2X2 (Yang et al., 2012) and presumably P2X3 as well (based on Ishida et al., 2009), so the morphological substrate for ATP signaling from type III cells is present.

P2X receptors also are present on taste cells themselves (**Figure 2**). P2X2 is present on the membranes of type II taste cells (Hayato et al., 2007; Huang et al., 2011b), where it offers a positive feedback loop for potentiation of ATP release. Other P2X receptors identified in taste tissue by RT-PCR include P2X4 and P2X7 (Hayato et al., 2007), although the functional significance of these receptors is not clear. Taste cells also possess metabotropic P2Y receptors, as first documented by calcium imaging studies showing ATP-induced calcium responses in taste cells that are mediated by release of calcium from intracellular stores (Baryshnikov et al., 2003). Several isoforms have been identified by molecular and pharmacological approaches, including P2Y1, P2Y2, and P2Y4 (Kataoka et al., 2004; Bystrova et al., 2006; Huang et al., 2009). P2Y1 is expressed primarily on type II cells, where it potentiates the release of ATP, while P2Y4 is expressed on type III cells, where

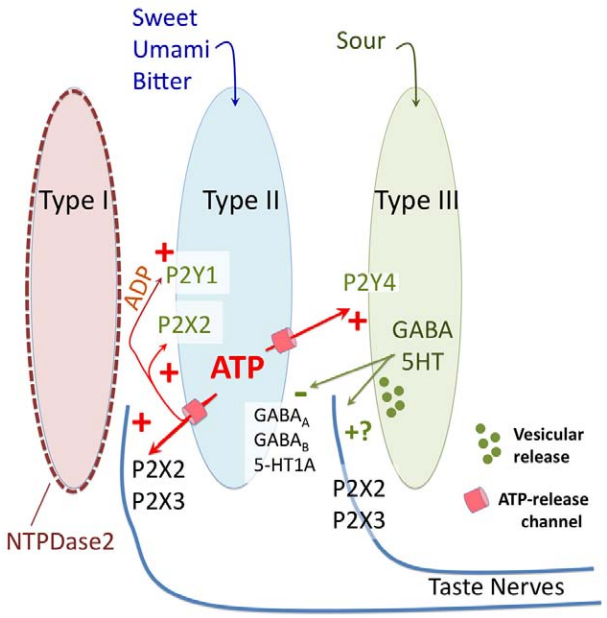

FIGURE 2 | Diagrammatic representation of purinergic signaling in taste buds. In response to bitter, sweet, and umami stimuli type II taste cells release ATP via non-vesicular release channels. The released ATP activates afferent nerve fibers by binding to ionotropic receptors containing P2X2 and P2X3 subunits. The released ATP is hydrolyzed to ADP by a specific ectoATPase, NTPDase2, expressed on the membranes of type I taste cells. In addition to activation of afferent nerves, the released ATP (and its breakdown product ADP) activate purinergic receptors (P2X2 and P2Y1) on the type II cells themselves to potentiate further ATP release. ATP also activates the sour-sensitive type III taste cells via P2Y4, causing vesicular release of 5HT and possibly GABA, which in turn inhibits further ATP release from the type II taste cells via a paracrine feedback mechanism. Unclear is whether the 5HT and GABA also activate the afferent nerve fibers, and what the source of ATP is for sour taste, since all taste qualities require ATP for activation of afferent fibers.

it stimulates the release of 5-HT in response to ATP release from type II cells (Huang et al., 2009).

CLEARANCE OF ATP RELEASED FROM TASTE BUDS

All neurotransmitter systems have mechanisms for either uptake or degradation of transmitter following release. In taste buds this is accomplished by a single ectoATPase, NTPDase2, expressed on the membranes of type I taste cells (Bartel et al., 2006). When ATP is released from taste cells, NTPDase2 degrades the ATP to ADP, which is further degraded to adenosine by other less specific ectonucleotidases including ecto-5'-nucleotidase expressed in type III cells (Dando et al., 2012). Mice globally lacking NTPDase2 have highly elevated levels of ATP in the extracellular space surrounding taste buds (Vandenbeuch et al., 2013b). The increased levels of ATP cause severely diminished taste responses to most taste stimuli, including sour stimuli that activate type III taste cells. The diminished responses are likely caused by desensitization of rapidly adapting P2X3-containing receptors on the afferent fibers. As described above, the co-expression of P2X2 and P2X3 in the majority of taste ganglion cells implies that most P2X receptors on the nerve fibers will be P2X2/P2X3 heteromers. However, we cannot rule out other possibilities for reduced taste function in NTPDase KO animals, such as inhibition of the ATP release channel by high levels of extracellular ATP (Qiu and Dahl, 2009). Nonetheless, these NTPDase2 knockout data provide further support for the requirement of ATP for all taste qualities, although the cellular source of the ATP for sour and salty stimuli remains enigmatic.

The adenosine that is ultimately produced by the degradation of ATP also modulates taste function. Of the known receptors for adenosine, only the A2B receptor is expressed in taste buds. The receptor is specifically expressed in posterior tongue, on the subset of type II taste cells that expresses sweet taste receptors (Dando et al., 2012; Kataoka et al., 2012). Adenosine enhances ATP release in response to sweet stimuli, thereby potentiating sweet responses (Dando et al., 2012). Knockout of the A2B receptor specifically diminishes sweet taste responses in the glossopharyngeal nerve, with no effect on other taste qualities (Kataoka et al., 2012).

FUTURE DIRECTIONS AND CONCLUSIONS

Taste buds are unusual sensory endorgans in that they utilize ATP as the primary neurotransmitter connecting the sensory cells to the afferent nerve supply. In other special sensory systems ATP may be co-released with a conventional neurotransmitter, but it is not the primary substance necessary for neural communication (Housley et al., 2009). In contrast, in the carotid body, an interoceptive chemosensor, ATP does play a crucial role in transmission of information from the chemoreceptor cells to the vagal afferent nerve terminals (Piskuric and Nurse, 2013). In this respect, taste buds are more similar to a visceral interoceptor than to special sensory modalities.

The carotid body and taste buds are similar in other ways as well. In both carotid body and taste buds, ATP release is effected at least in part via gated ion channels. Once released, the ATP gates P2X2 and P2X3 receptors on the afferent nerve fibers. Both carotid body and taste buds possess multiple cell types including a glial-like cell (type I cells in taste buds; type II cells in carotid body). Both endorgans also possess cells with diverse neurochemical characteristics in addition to the purinergic phenotype. But significant differences exist between these two systems. In carotid body, the glial-like cells have P2Y receptors, which amplify the initial purinergic signal by triggering additional release of ATP (Piskuric and Nurse, 2013). In taste buds, the glial-like cells express an ectoATPase whose function is to rapidly break down ATP in extracellular space.

Many features and functions of taste buds remain unexplained. Foremost is the fashion in which signal specificity is maintained. Each taste bud contains taste cells responding to each of the various taste qualities, i.e., different cells responsive to bitter, sweet, umami, sour, and salty (Tomchik et al., 2007; Yoshida et al., 2009). If all the cell types utilize the same neurotransmitter, i.e., ATP, how can the system maintain specificity? How can ATP released into the tight confines of a taste bud activate only a single class of nerve fiber? Do other neurotransmitters and neuropeptides expressed by the different types of taste cells contribute to specificity by activating only appropriately matched nerve fibers? For example do sweet-responsive taste cells release both ATP and GLP-1 so that only sweet-best taste nerve fibers, which express both P2X

receptors and GLP-1 receptors (Shin et al., 2008), become activated maximally?

A second major unanswered question relating to purinergic signaling in taste buds, is how and if ATP is released by sour and salt-responsive type III taste cells which possess conventional synapses. Type III cells do release serotonin and GABA using a vesicular release mechanism (Vandenbeuch et al., 2010b; Huang et al., 2011a), but do they co-release ATP? Blockade or abolition of P2X receptors prevents transmission of sour taste information, yet no one has yet succeeded in measuring ATP release from type III taste cells. Might type III taste cells excite type II cells to release ATP via hemichannels, similar to the way that type I carotid body cells excite type II carotid cells to release ATP via hemichannels? Alternatively ATP might be co-released via a vesicular mechanism along with serotonin or GABA, but the type III taste cells which possess classical synapses do not express vesicular nucleotide transporter (VNUT), the vesicular transporter for ATP (Iwatsuki et al., 2009). Hence it is unclear how the ATP would be packaged into the synaptic vesicles in this system.

Although taste buds contain fewer than 100 cells, they remain a complex and enigmatic endorgan. Clearly ATP and P2X receptors play a crucial role in linking taste buds to the afferent nerves. The recent decades of intense anatomical, physiological, and molecular characterization have permitted elucidation of many of the fundamental principles of taste bud organization, but much remains to be explained. These seemingly simple, small sensory endorgans remain a rich field for future studies.

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A new role for $P2X_4$ receptors as modulators of lung surfactant secretion

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In recent years, P2X receptors have attracted increasing attention as regulators of exocytosis and cellular secretion. In various cell types, P2X receptors have been found to stimulate vesicle exocytosis directly via Ca^{2+} influx and elevation of the intracellular Ca^{2+} concentration. Recently, a new role for $P2X_4$ receptors as regulators of secretion emerged. Exocytosis of lamellar bodies (LBs), large storage organelles for lung surfactant, results in a local, fusion-activated Ca^{2+} entry (FACE) in alveolar type II epithelial cells. FACE is mediated via $P2X_4$ receptors that are located on the limiting membrane of LBs and inserted into the plasma membrane upon exocytosis of LBs. The localized Ca^{2+} influx at the site of vesicle fusion promotes fusion pore expansion and facilitates surfactant release. In addition, this inward-rectifying cation current across $P2X_4$ receptors mediates fluid resorption from lung alveoli. It is hypothesized that the concomitant reduction in the alveolar lining fluid facilitates insertion of surfactant into the air–liquid interphase thereby "activating" it. These findings constitute a novel role for $P2X_4$ receptors in regulating vesicle content secretion as modulators of the secretory output during the exocytic post-fusion phase.

Keywords: $P2X_4$ receptor, lamellar body, alveolar epithelial cell, exocytosis, calcium, cellular secretion, pulmonary surfactant

INTRODUCTION

In recent years, P2X receptors have attracted increasing attention as regulators of exocytosis and cellular secretion in a wide variety of organs including the lungs (Burnstock et al., 2012). P2X receptors are membrane cation channels that are activated by extracellular adenosine triphosphate (ATP), the molecular and functional properties of which have been reviewed in detail elsewhere (Surprenant, 1996; North, 2002; Khakh and North, 2006; Burnstock and Kennedy, 2011; Kaczmarek-Hajek et al., 2012). ATP has been known to stimulate cellular secretion for several decades (Rodriguez Candela and Garcia-Fernandez, 1963; Diamant and Kruger, 1967). One of the earliest indications for involvement of P2X receptors in stimulating secretion came from the studies of Cockcroft and Gomperts (1979a,b, 1980). They found that ATP triggers degranulation and histamine release in mast cells via activation of P_{2Z} (Cockcroft and Gomperts, 1980), which later turned out to be $P2X_7$ (Surprenant et al., 1996). Since the first cloning of P2X receptor subunits in 1994 (Brake et al., 1994; Valera et al., 1994), P2X receptors have been found to stimulate and modulate various cellular secretion pathways, including fluid secretion in exocrine glands and epithelia (Novak, 2011), secretion of cytokines via release of plasma-derived microvesicles (Solini et al., 1999; MacKenzie et al., 2001) or exosomes (Qu et al., 2007; Qu and Dubyak, 2009).

Moreover, several members of the P2X family have been implicated in regulating exocytosis of secretory organelles in a variety of cell types (Gu and MacDermott, 1997; Ulmann et al., 2008; Jacques-Silva et al., 2010; Gutierrez-Martin et al., 2011; Huang et al., 2011). Substantial evidence suggests that P2X receptor activation stimulates exocytosis directly via influx of Ca^{2+} from the extracellular space and elevation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$; Kim et al., 2004; Shigetomi and Kato, 2004; Jacques-Silva et al., 2010; Hayoz et al., 2012). It is well established that a series of Ca^{2+}-dependent steps during the exocytic pre-fusion stage is essential for fusion of exocytic vesicles with the plasma membrane (Burgoyne and Morgan, 1998; Sudhof, 2004; Neher and Sakaba, 2008). Ca^{2+} can either enter through P2X receptor pores themselves or through voltage-gated Ca^{2+} channels, which are activated as a consequence of the P2X receptor-mediated membrane depolarization (Khakh and North, 2006). In line with these findings, several studies proposed a role for $P2X_4$ receptors in exocytosis that is mediated via an increase in the intracellular Ca^{2+} concentration. $P2X_4$ receptors have a relatively slow desensitization (5–10 s) and a high Ca^{2+} permeability, Ca^{2+} contributes 8% of the whole current in human $P2X_4$ (Wang et al., 1996; Garcia-Guzman et al., 1997; North, 2002; Egan and Khakh, 2004). Hence, activation of $P2X_4$ receptors can generate sufficient increases in $[Ca^{2+}]_c$ to stimulate regulated exocytosis. Indeed, insulin secretion from pancreatic islets (Ohtani et al., 2011) and exocytic response in parotid acinar cells (Bhattacharya et al., 2012) following stimulation with ATP were augmented in the presence of ivermectin, a selective potentiator of $P2X_4$ receptor currents (Khakh et al., 1999). $P2X_4$ activation was also found to modulate glutamate and gamma-aminobutyric acid (GABA) release in hypothalamic neurons (Vavra et al., 2011) and brain-derived neurotrophic factor (BDNF) in microglial cells (Trang et al., 2009).

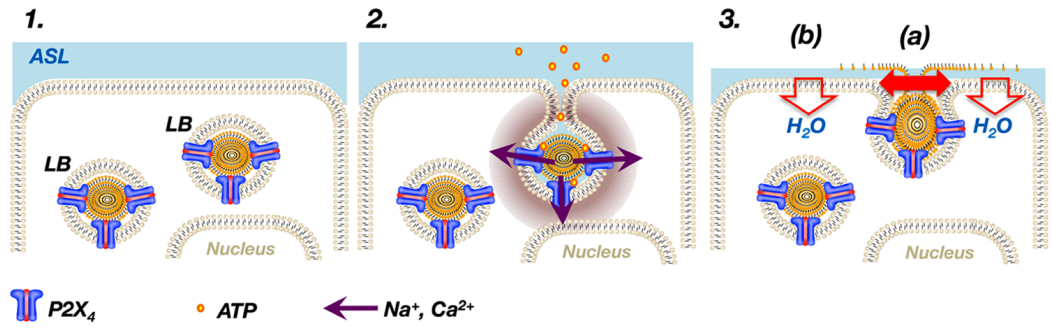

FIGURE 1 | $P2X_4$ receptors on LBs modulate surfactant secretion. $P2X_4$ receptors are expressed on LBs, large storage organelles for pulmonary surfactant in ATII cells **(1)**. Upon exocytosis of LBs, $P2X_4$ receptors readily become part of the apical membrane and activation of $P2X_4$ by extracellular ATP results in a transient, non-selective, inward-rectifying, cation current at the site of the fused vesicle causing a local increase in Ca^{2+} around the fused vesicle **(2)**. The local increase in Ca^{2+} promotes fusion pore expansion **(3a)**. In addition, the inward-rectifying cation current on the apical side results in vectorial ion transport across ATII cells, which in turn promotes fluid resorption and thereby facilitates adsorption of newly released surfactant into the air–liquid interface **(3b)**. ASL = alveolar surface liquid.

In all of these systems, activation of P2X receptors adjusts the secretory output predominantly by modulating the number of vesicles that fuse with the plasma membrane. Depending on the cell type and the shape of the Ca^{2+} signal, the rise in $[Ca^{2+}]_c$ triggers fusion of secretory vesicles with the plasma membrane, but also affects maturation and trafficking of secretory vesicles to the plasma membrane (Neher and Sakaba, 2008; Dolensek et al., 2011; Gutierrez-Martin et al., 2011).

VESICULAR $P2X_4$ RECEPTORS PROMOTE SURFACTANT SECRETION VIA FACE – "FUSION-ACTIVATED Ca^{2+}-ENTRY"

Apart from regulating secretion via adjusting the number of fusing organelles the amount and composition of the secretory output is – at least for exocytosis of large secretory granules and secretion of bulky vesicle contents – modulated following fusion of the vesicle with the plasma membrane during the so-called exocytic "post-fusion" phase. Recent evidence also suggests a role for $P2X_4$ receptors therein. It has been demonstrated that activation of $P2X_4$ receptors following vesicle–plasma membrane fusion modulates the secretion and activation of pulmonary surfactant (Miklavc et al., 2011; Dietl et al., 2012; Thompson et al., 2013).

Pulmonary surfactant is secreted via exocytosis of lamellar bodies (LBs), large lysosome-related storage organelles in alveolar type II (ATII) epithelial cells. Surfactant is stored in LBs as densely packed membranous structures that do not readily diffuse out of fused LBs following opening of the exocytic fusion pore. Rather, surfactant is so insoluble, that it may remain entrapped within the fused vesicle for many minutes and the slowly expanding fusion pore acts as a mechanical barrier for the release (Dietl et al., 2001; Haller et al., 2001; Singer et al., 2003; Dietl and Haller, 2005; Miklavc et al., 2012).

Miklavc et al. (2010) initially discovered that exocytosis of LBs results in localized Ca^{2+} influx at the site of vesicle fusion which they termed "FACE" for "fusion-activated Ca^{2+}-entry". Subsequently, they found that FACE is mediated via activation of $P2X_4$ receptors expressed on the limiting membranes of LBs (Miklavc et al., 2011). Upon exocytosis of LBs, the $P2X_4$ receptor is readily part of the apical membrane as soon as membrane fusion is completed (Miklavc et al., 2009). Activation of $P2X_4$ in the presence of extracellular ATP then results in a transient, non-selective, inward-rectifying, cation current at the site of the fused vesicle (Miklavc et al., 2011; Thompson et al., 2013) (**Figure 1**). The relatively high Ca^{2+} permeability of $P2X_4$ receptors (North, 2002) causes a local, transient rise of $[Ca^{2+}]_c$ around the fused vesicle which promotes fusion pore expansion (Miklavc et al., 2011). In ATII cells, vesicle content (i.e., surfactant) release is tightly regulated via Ca^{2+}-dependent fusion pore expansion (Haller et al., 2001) and it has been demonstrated that FACE via $P2X_4$ receptors on LBs directly facilitates surfactant release in the alveolus (Miklavc et al., 2011).

Ca^{2+} channels localized in the membranes of the secretory vesicles that respond to changes in the membrane potential or extracellular agonists upon fusion are ideally suited for generating a localized rise in Ca^{2+} and selectively affect the individual fused vesicle. Yet, so far such mechanisms have only been known in invertebrates (Smith et al., 2000; Yao et al., 2009; Miklavc and Frick, 2011) and $P2X_4$ receptors on LBs resemble the first analog mechanism in mammals. It will be interesting to see whether a similar role for P2X receptors is present in other secretory cells. Similar to LBs in ATII cells, many different cell types harbor secretory lysosomes or lysosome-related organelles to store for secretory products that are released via exocytosis of these organelles (Dell'Angelica et al., 2000; Blott and Griffiths, 2002; Luzio et al., 2007). Many of these contain rather bulky, macromolecular vesicle contents and release is modulated via the exocytic post-fusion phase (Thorn, 2009). In addition, it is well established that P2X receptors, in particular $P2X_4$, are predominantly located within lysosomal compartments and inserted into the cell surface upon exocytosis (Qureshi et al., 2007; Toyomitsu et al., 2012).

VESICULAR $P2X_4$ RECEPTORS FACILITATE "ACTIVATION" OF SURFACTANT

Following release into the alveolar hypophase surfactant maintains its compact organization, constituting lamellar body-like particles (LBPs; Haller et al., 2004). To gain its vital function of reducing the surface tension within alveoli, it needs to be inserted

into the air–liquid interface. Freshly released LBPs disintegrate when they contact an air–liquid interface, leading to instantaneous spreading and insertion of surfactant material at this interface (Dietl and Haller, 2005). Thompson et al. (2013) demonstrated that, in addition to facilitating fusion pore dilation, FACE via $P2X_4$ also drives fluid resorption from the alveolar lumen. The $P2X_4$ mediated inward-rectifying cation current on the apical side results in vectorial ion transport across ATII cells, which in turn promotes apical to basolateral fluid transport (Thompson et al., 2013) (**Figure 1**). FACE-dependent transepithelial fluid resorption is a rather transient process which requires the presence of luminal ATP or other $P2X_4$ agonists and hence it is unlikely that it is a major contributor to regulation of alveolar liquid homeostasis under physiological conditions (Folkesson and Matthay, 2006). However, it has been suggested that this localized alveolar fluid resorption results in temporary thinning of the alveolar hypophase which in turn promotes contact between LBPs and the interphase and facilitates adsorption of newly released surfactant into the air–liquid interface (Thompson et al., 2013). Hence, activation of $P2X_4$ and FACE (which in order to embrace the true nature of FACE should now be referred to as "fusion-activated cation entry") facilitates surfactant release via fusion pore opening and contributes to "activation" or "functionalising" of surfactant. Such a temporal and local coordination of surfactant secretion and reduction of alveolar lining fluid could constitute a powerful mechanism for fine-tuning surfactant replenishment – the integrators being vesicular $P2X_4$ receptors and extracellular ATP.

ATP AS INTEGRATOR FOR SURFACTANT SECRETION AND "ACTIVATION"

It is intriguing that extracellular ATP plays multiple functions for surfactant secretion in the alveolus. Apart from $P2X_4$ receptors, ATII cells also express $P2Y_2$ receptors (Garcia-Verdugo et al., 2008; Burnstock et al., 2012) and activation thereof is one of the most potent stimuli for LB exocytosis and surfactant secretion (Rice and Singleton, 1987; Frick et al., 2001; Andreeva et al., 2007; Dietl et al., 2010). Hence, ATP is integrating the entire secretion process from stimulating LB exocytosis to facilitating surfactant release and "activating" surfactant during the post-fusion phase.

Despite this importance of ATP for lung function, the origins of ATP in the alveoli are still elusive. It has been reported that ATP is present in the pulmonary hypophase (Patel et al., 2005), however, the estimated concentration under resting conditions is in the low nM range (Bove et al., 2010), well below the EC_{50} values for $P2X_4$ activation (North, 2002) or $P2Y_2$ activation (Lazarowski et al., 1995; Brunschweiger and Muller, 2006).

Cell stretch during deep inflation is considered the most potent if not only physiologically relevant stimulus for surfactant secretion (Wirtz and Dobbs, 2000; Dietl et al., 2004, 2010; Frick et al., 2004) and stretch-induced ATP release from alveolar epithelial cells (Patel et al., 2005; Mishra et al., 2011) could represent a key regulatory element (Dietl et al., 2010). Several possible pathways for ATP release have been described in the respiratory epithelia. ATP can either be released into the hypophase via regulated exocytosis from secretory cells (Kreda et al., 2010; Okada et al., 2011), or in a conductive way via pannexin hemichannels (Ransford et al., 2009; Seminario-Vidal et al., 2011) or $P2X_7$ receptors (Mishra et al., 2011). In particular, local ATP release within individual alveoli may provide an ideal mechanism to gradually adapt local surfactant secretion to local demands. The alveolar epithelium consists of only two cell types; besides surfactant secreting ATII cells, flat alveolar type I (ATI) cells cover most of the alveolar surface. In contrast to primary ATII cells that only express $P2X_4$ receptors (Miklavc et al., 2011) ATI cells express $P2X_4$ and $P2X_7$ receptors (Weinhold et al., 2010; Burnstock et al., 2012). $P2X_7$ knock-out mice fail to increase surfactant secretion in response to hyperventilation and substantial evidence suggests that ATP release via $P2X_7$ receptors on ATI cells maintains alveolar surfactant homeostasis in response to increased alveolar distension by stimulating $P2Y_2$ receptors on ATII cells (Mishra et al., 2011) and, in light of our recent findings, possible activation of $P2X_4$ (Miklavc et al., 2011; Thompson et al., 2013). In addition to responding to mechanical distension of alveoli, alveolar epithelial cells also respond to increased tension forces at the air–liquid interphase with exocytic release of ATP (e.g., upon local depletion of surfactant or when coming in close proximity to the air–liquid interphase following a decrease in alveolar hypophase height; Ramsingh et al., 2011).

Whether ATII cells also release ATP, to act in an autocrine feedback loop, is still unknown. Many secretory vesicles, including lysosome-related organelles, have been found to contain significant amounts of ATP (Bodin and Burnstock, 2001; Praetorius and Leipziger, 2009; Lazarowski et al., 2011) and it has been reported that ATP is released from ATII-like A549 cells, likely via exocytosis (Tatur et al., 2008; Ramsingh et al., 2011). It is tempting to speculate that LBs contain ATP and hence provide the ligand for the $P2X_4$ receptors themselves. In such a scenario, the high degree of pH sensitivity of this receptor (Clarke et al., 2000; Zsembery et al., 2003; Coddou et al., 2011) could prevent intravesicular activation of the receptor in the presence of vesicular ATP (pH of LB is <6.1; Chander et al., 1986).

Also, under pathophysiological conditions resulting from many chronic lung diseases, release of purine nucleotides from respiratory epithelia is significantly increased (Adriaensen and Timmermans, 2004; Lommatzsch et al., 2010). It has been demonstrated that trauma-induced damage of the alveolus leads to substantial ATP release and that extracellular ATP is a key player to rescue alveolar function following damage, including regulation of surfactant secretion (Riteau et al., 2010; Belete et al., 2011). In addition, several studies have demonstrated up-regulation of P2X receptors in various cell types during pathological conditions including inflammation, tumor growth, and injury (Burnstock and Kennedy, 2011) and it has been hypothesized that chronic extracellular ATP may be responsible (Geisler et al., 2013). Such a mechanism could be particularly relevant in the lung, and P2X receptors may play an even greater role in many pathological conditions with chronically increased extracellular ATP levels. Initial evidence came from studies indicating that smoke-induced lung inflammation leads to increased levels of ATP in broncho-alveolar fluid and up-regulation of $P2X_7$ expression (Lommatzsch et al., 2010; Lucattelli et al., 2011). A similar role for $P2X_4$ receptors under pathophysiological conditions is still to be confirmed. However,

it is becoming increasingly evident that purinergic signaling is taking center stage in regulating secretion of pulmonary surfactant and adapting it to local demands under physiological and diseased conditions. $P2X_4$ receptors on LBs provide an ideal mechanism for fine-tuning surfactant secretion via ATP levels in the alveolar hypophase.

Despite recent advances in our understanding how purinergic signaling in the alveolus, in particular activation of vesicular $P2X_4$ receptors, modulates LB exocytosis, surfactant secretion and activation of released surfactant, several important questions still remain to be answered: What is the physiological relevance of such a complex regulatory mechanism? What are the sources of ATP under physiological and more importantly pathophysiological conditions? And – extending the scope from the lung – is purinergic signaling a general mechanism for secretion of large, macromolecular vesicle contents or is it unique to LB exocytosis and surfactant secretion? The answers to these questions warrant further research and certainly promise an increased understanding of the role of P2X receptors in regulating exocytosis and cellular secretion.

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The trafficking and targeting of P2X receptors

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The functional expression of P2X receptors at the plasma membrane is dependent on their trafficking along secretory and endocytic pathways. There are seven P2X receptor subunits, and these differ in their subcellular distributions because they have very different trafficking properties. Some are retained within the endoplasmic reticulum (ER), while others are predominantly at the cell surface or within endosomes and lysosomes. Changes in recruitment of receptors to and from the plasma membrane provides a way of rapidly up- or down-regulating the cellular response to adenosine triphosphate (ATP). An additional layer of regulation is the targeting of these receptors within the membranes of each compartment, which affects their stability, function and the nature of the effector proteins with which they form signaling complexes. The trafficking and targeting of P2X receptors is regulated by their interactions with other proteins and with lipids and we can expect this to vary in a cell-type specific manner and in response to changes in the environment giving rise to differences in receptor activity and function.

Keywords: P2X receptor trafficking, lipid rafts, receptor endocytosis, receptor regulation, P2X receptor targeting

INTRODUCTION

P2X receptors open an integral ion channel at the plasma membrane in response to binding extracellular adenosine triphosphate (ATP). Some subtypes of P2X receptor are predominantly within intracellular membranes, but there is no compelling evidence that the receptors on intracellular membranes open in response to ATP, at least in mammals. Nonetheless, this remains an intriguing possibility, given that the distantly related P2X-like receptors in *Dictyostelium* are located and function within contractile vacuoles (Fountain et al., 2007; Ludlow et al., 2009; Sivaramakrishnan and Fountain, 2012a,b; Baines et al., 2013). The best-established role of the internal mammalian P2X receptors is, therefore, to regulate the expression and activity of receptors at the cell surface. Here we consider three related issues concerning the targeting and trafficking of P2X receptors: first, primary location, and the amino acid motifs which determine it; second, regulation of mobility both within the plasma membrane and between the plasma membrane and intracellular membranes; third, targeting to lipid rafts and the effects of the lipid environment on receptor signaling.

SUBCELLULAR LOCALIZATION OF P2X RECEPTORS

Trimeric P2X receptor complexes assemble and are core glycosylated within the endoplasmic reticulum (ER) and then traffic via the trans-Golgi network (TGN) to the plasma membrane.

They are subsequently internalized and either recycled back to the surface or targeted to late endosomes and lysosomes. The kinetics of these processes determines receptor distribution.

ER RESIDENT P2X RECEPTORS

P2X receptors are predominantly found within the ER, at the plasma membrane or within late endosomes and lysosomes, dependent upon the subtype (**Figure 1**). The only full-length P2X receptor that is retained within the ER and is therefore non-functional is P2X6 (Ormond et al., 2006). Imaging of P2X6 receptors by atomic force microscopy indicates that the subunits do not assemble to form stable homotrimeric complexes, but they do form stable heterotrimers with either P2X2 or P2X4 (Bobanovic et al., 2002; Barrera et al., 2005, 2007; Ormond et al., 2006). The P2X2/6 and P2X4/6 receptors are expressed as functional receptors at the plasma membrane and have trafficking properties that resemble the P2X2 and P2X4 homomeric receptors respectively. In the category of ER resident P2X receptors there is also the human P2X5 receptor. Although the full-length P2X5 receptor traffics to the cell surface, the predominant allele expressed in most humans gives rise to an exon 10-deleted variant which is retained in the ER (Bo et al., 2003; Kotnis et al., 2010; Compan et al., 2012).

PLASMA MEMBRANE P2X RECEPTORS

Two subtypes that traffic relatively slowly through the secretory pathway and hence often appear to have a predominantly ER distribution are P2X2 and P2X7 receptors. P2X2 receptors are stably expressed at the plasma membrane, but, when heterologously expressed, they accumulate slowly at the cell surface. This slow traffic might be important for facilitating interactions with other proteins along the way (Bobanovic et al., 2002). For example, in spinal cord neurons, intracellular P2X2 receptors interact with $GABA_A$ receptors and co-traffic to the surface (Shrivastava et al., 2011). Another protein that interacts with P2X2 receptors to regulate its targeting to synapses is the beta-amyloid precursor protein-binding protein Fe65 (Masin et al., 2006). There is also the neuronal calcium sensor, visinin-like protein-1 (VILIP-1) that interacts with P2X2 in a calcium-dependent manner (Chaumont et al., 2008). These interacting proteins affect the stability, targeting and function of the receptors at the plasma membrane.

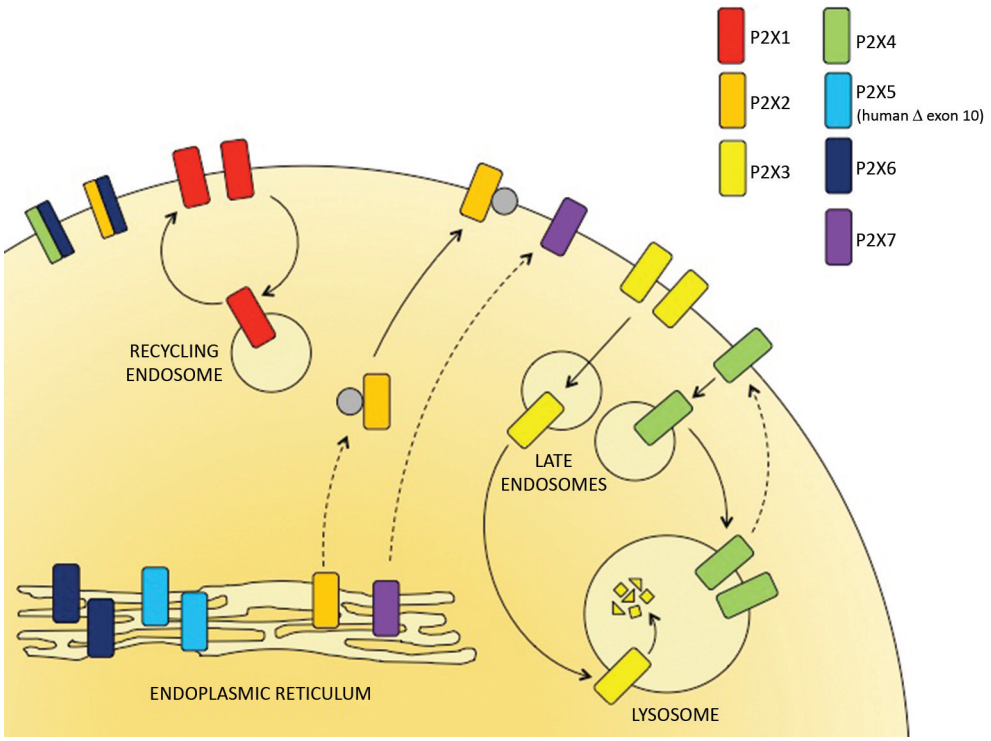

FIGURE 1 | The subcellular distribution of P2X receptors. P2X receptor subtypes differ in their trafficking properties and hence are localized to different subcellular compartments. P2X6 receptors are retained within the ER but can assemble with P2X4 and P2X6 subunits to form heterotrimers that traffic to the cell surface. The predominant human allele of P2X5 lacks exon 10 and is also retained in the ER. P2X2 and P2X7 receptors traffic relatively slowly through the secretory pathway but are stably expressed at the surface. P2X1 receptors are expressed at the cell surface but rapidly cycle to and from recycling endosomes. P2X3 and P2X4 receptors are consitutively internalized and delivered to late endosomes and lysosomes. Within the lysosomes, P2X3 receptors are rapidly degraded but P2X4 receptors resist degradation and can recycle back to the surface.

P2X7 receptor trafficking is dependent upon cell-type and species. For example, in human monocytes and lymphocytes, P2X7 receptors are predominantly intracellular, but upon differentiation of monocytes to macrophages receptors locate to the plasma membrane (Hickman et al., 1994; Gu et al., 2000; Gudipaty et al., 2001). Native P2X7 receptors in rodent microglia and macrophages are also predominantly at the plasma membrane (Boumechache et al., 2009). What regulates the rate at which P2X7 receptors traffic from the ER to the cell surface is unknown, although mutagenesis analysis suggests that it involves the cytoplasmic C-terminal domain of the receptor (Denlinger et al., 2003; Smart et al., 2003).

P2X1 receptors are predominantly at the cell surface, and at least one interacting protein has been identified, namely heat shock protein 90, which further promotes their trafficking and plasma membrane expression (Lalo et al., 2010, 2012). At the plasma membrane, P2X1 receptors differ in trafficking behavior from P2X2 receptors. P2X2 receptors are relatively stable and show little constitutive internalization over a period of an hour (Bobanovic et al., 2002). In contrast, measurements of P2X1 receptor mobility by fluorescence recovery after photobleaching (FRAP) shows that receptors undergo considerable internalization and recycling over a similar time period (Lalo et al., 2010). Their surface localization indicates that recycling is rapid compared to their rate of endocytosis and targeting to late endosomes.

ENDO-LYSOSOMAL P2X RECEPTORS

P2X3 and P2X4 receptors also undergo rapid constitutive internalization from the plasma membrane, but unlike P2X1 they are predominantly localized to late endosomes and lysosomes (Bobanovic et al., 2002; Qureshi et al., 2007; Vacca et al., 2009). For P2X3 receptors this has been shown for heterologously expressed receptors in HEK293 cells and also for native receptors in dorsal root ganglion (DRG) neurons (Vacca et al., 2009). For P2X4 receptors, their localization to endolysosomes has been shown in immune and endothelial cells as well as for the heterologously expressed receptors in neurons (Bobanovic et al., 2002; Royle et al., 2002; Qureshi et al., 2007; Toulme et al., 2010). Constitutive endocytosis of P2X4 receptors is mediated via a dynamin/clathrin-dependent process and can be inhibited using dynasore (Bobanovic et al., 2002). Treatment with dynasore rapidly up-regulates P2X4 receptors at the surface of some but not all cells that express this receptor (Boumechache et al., 2009). In cultured microglial cells, native P2X4 receptors are rapidly up-regulated, indicating that they are continually cycling to and from the surface. In contrast, in bone marrow derived macrophages, total P2X4 receptor expression is high, but expression at the surface is very low and not increased by 1 h incubation with dynasore, suggesting that under basal conditions, receptors remain within endolysosomal compartments.

P2X3 receptors have a high rate of turnover, caused by rapid endocytosis and targeting to lysosomes, where the receptor is degraded (Vacca et al., 2009). In contrast, P2X4 receptors are surprisingly stable: they are resistant to degradation in the lysosome and they show little turnover over a period of 24 h (Qureshi et al., 2007). Their resistance to degradation is dependent upon multiple N-linked glycans which decorate the loop between the first and second transmembrane domains (TMD), which is predicted to face towards the lumen of lysosomes. Glycosylation is thought to play a similar role in protecting other lysosome-targeted proteins, such as Lamp-1, from degradation (Kundra and Kornfeld, 1999).

P2XR MOTIFS THAT DETERMINE RECEPTOR TRAFFICKING

P2X receptors share a YXXXK in the C-terminus which regulates surface expression (Chaumont et al., 2004). This motif is situated eight residues downstream of TMD2, and in P2X7 after an additional 18 amino acid cysteine-rich region. For P2X2 receptors, mutations around this motif reduce the stability of the receptor at the plasma membrane and increase internalization. Mutations within the motif similarly reduce the plasma membrane expression of other P2X receptor subtypes (Chaumont et al., 2004).

The P2X4 receptor has two tyrosine-based endocytic motifs within the C-terminus and one di-leucine-like motif within the N-terminus (Royle et al., 2002, 2005; Qureshi et al., 2007). Mutations of Y378, which forms part of a non-canonical YXXGΦmotif, substantially slow receptor endocytosis, suggesting that this is the more accessible of the two tyrosine-based motifs (Royle et al., 2002, 2005). These mutants still, however, show targeting to lysosomes, but this is further inhibited by mutating the leucine and isoleucine pair within the N-terminus.

Though the P2X3 receptor does not share any endocytic motifs with P2X4, there is a di-leucine motif in its C-terminal tail and also a consensus sequence for ubiquitination (DSGΦXS) that is suggested to be involved in the endocytosis and rapid degradation of the receptor (Vacca et al., 2009).

For P2X7 receptors, several mutations, deletions and naturally occurring single nucleotide polymorphisms (SNPs) within the long C-terminal tail have been shown to disrupt its normal trafficking to the plasma membrane. Attention has mostly focused on the distal end of the C-terminus of P2X7 (573–590), where there is a region with strong amino acid identity to the lipopolysaccharide (LPS) binding region of the LPS binding protein (Denlinger et al., 2001). Truncations and mutations within a region overlapping this site (551–581) disrupt normal receptor trafficking in HEK293 cells (Smart et al., 2003). For example, the I568N SNP in human P2X7 receptors disrupts normal trafficking and function (Wiley et al., 2003), as does substitution of acidic residues for the dibasic R578 and K579 (Denlinger et al., 2003). More recently it was shown that mutations within this region also disrupt the normal targeting of rat P2X7 receptors in polarized epithelial cells (Bradley et al., 2010). Alanine substitutions at P582-Q587 switched receptor targeting from the basolateral to the apical membrane but without disrupting plasma membrane expression or receptor function. Although site-directed mutagenesis has revealed critical residues in P2X7 receptor trafficking, the mechanism(s) underlying their involvement remains unknown.

REGULATION OF RECEPTOR TRAFFICKING AND MOBILITY

The activation of P2X receptors regulates their trafficking to and from the plasma membrane and their mobility within the plasma membrane in a calcium-dependent manner. Agonist-stimulated P2X receptor internalization and recycling back to the plasma membrane was first shown for P2X1 in rat vas deferens (Ennion and Evans, 2001). A later study of P2X1 mobility in HEK293 cells showed an increase in the rate of FRAP at the cell surface in the presence of agonist, which was dependent upon a rise in calcium, upon clathrin-mediated endocytosis and upon trafficking of vesicles back to the surface (Lalo et al., 2010). P2X1 receptors rapidly desensitize in the presence of agonist and inhibiting the internalization and recycling of receptors reduces the rate of recovery from desensitization. The P2X3 receptor also rapidly desensitizes and shows agonist-stimulated internalization (Vacca et al., 2009). P2X4 receptors desensitize more slowly but inhibiting dynamin-mediated endocytosis similarly slows the resensitization process (Murrell-Lagnado, unpublished). Thus, receptor retrieval and recycling appears to be important for maintaining the activity of the surface receptors.

Enhanced translocation of intracellular receptors to the plasma membrane has been shown to be a mechanism for up-regulating receptor function, particularly for those receptors that are predominantly intracellular. P2X4 receptors translocate from endolysosomes back to the surface, whereas for P2X3 receptors it is unclear whether up-regulation involves increased delivery from the secretory or endocytic pathway. ATP produces a transient increase in the number of P2X3 receptors at the surface causing sensitization of the current to repetitive doses (Vacca et al., 2009). Another example of increased trafficking of P2X3 receptors to the plasma membrane is in trigeminal sensory neurons in response to calcitonin gene-related peptide (CGRP; Fabbretti et al., 2006). A 1 h incubation with CGRP increased both the amplitude of P2X3 receptor currents and their rate of recovery from desensitization. For P2X4 receptors in macrophages, surface expression is increased in response to stimuli that promote lysosome exocytosis either by increasing cytosolic calcium or by alkalinization of the lysosomes (Qureshi et al., 2007). In the cerebellar microglial cell line, C8-B4, P2X4 receptor currents are negligible in resting cells, but after activating cells with either LPS or fibronectin, receptors translocate from lysosomes to the surface to enhance receptor-mediated currents (Toulme et al., 2010). Anti-depressants, which inhibit these currents, act by blocking this translocation process rather than by directly inhibiting the opening of the channel pore (Toulme et al., 2010). This mode of action could prove to be a useful way of selectively targeting the different subtypes with new therapeutics.

It is not only the retrieval and recycling of P2X receptors that is sensitive to agonist stimulation: their mobility within the lateral plane of the plasma membrane is promoted by the binding of ATP triggering a local influx of calcium. P2X2 receptor mobility was measured in hippocampal neurons by imaging single molecules using a quantum dot-based approach, and a similar approach was used with P2X4 receptors in microglia (Richler et al., 2011; Toulme and Khakh, 2012). In both cases two populations of receptor were observed, characterized as the

mobile and the slowly mobile pool. Neither population correlated with clusters of receptors or receptors localized in lipid rafts, so the molecular basis for the different mobility is unclear. Both populations showed increased mobility in response to ATP. For P2X2 receptors, mobility was also increased by the co-expression of VILIP-1 (Richler et al., 2011). The implications of this increased lateral mobility for receptor signaling remains to be established.

TARGETING OF P2X RECEPTORS TO LIPID RAFTS

The plasma membrane is an extremely heterogeneous environment and the trafficking and function of P2X receptors are regulated by the proteins and lipids within their immediate environment, with which they interact. Lipid rafts are commonly defined as microdomains rich in cholesterol, sphingolipids and saturated phospholipids, but there is heterogeneity amongst these domains in terms of their protein and lipid composition (Pike, 2004). While some proteins are targeted to rafts, others are excluded, affecting the nature of the signaling complexes formed and their stability within the membrane.

Lipid rafts are often identified biochemically by their low buoyant density in a sucrose density gradient, their resistance to solubilization in Triton-X 100 and the presence of protein markers such as caveolin-1 (Pike, 2004). Several of the P2X receptors have been shown to associate with lipid rafts, but the degree to which they distribute between the raft and non-raft fractions depends upon the cells in which they are expressed and the method used to prepare the rafts (Allsopp et al., 2010). P2X1-4 receptors expressed in HEK293 cells associate with rafts prepared using a detergent-free method (Allsopp et al., 2010). When rafts are instead prepared using Triton-X 100, the receptors shift to non-raft fractions. P2X1 and P2X2 receptors are more resistant to extraction from rafts by Triton-X 100 than are P2X3 and P2X4 receptors, suggesting that P2X1 and P2X2 interact more strongly with the cholesterol enriched domains (Allsopp et al., 2010).

Native P2X receptors have also been shown to target to lipid rafts. For P2X1 receptors, the distribution between raft and non-raft fractions is dependent upon the cell type. For example, P2X1 receptors in smooth muscle preparations from artery, vas deferens and bladder are almost exclusively in rafts, whereas only 20% of P2X1 receptors in platelets are in rafts (Vial and Evans, 2005; Vial et al., 2006). Native P2X3 receptors in trigeminal sensory neurons target to lipid rafts and, in a transgenic migraine mouse model, up-regulation of these receptors correlates with an increase in the abundance of lipid rafts and an increase in the fraction of P2X3 receptors within rafts (Gnanasekaran et al., 2011). Thus regulation of lipid rafts provides a mechanism for changing the functional expression of these receptors. P2X7 receptors are also found within rafts, both for heterologously-expressed receptors in HEK293 cells and for native receptors in rat submandibular glands, peritoneal macrophages and mouse lung alveolar cells (Garcia-Marcos et al., 2006a,b; Barth et al., 2007, 2008; Gonnord et al., 2009). Similar to P2X1-4, the association of P2X7 receptors with rafts is dependent upon the method used for isolating them. The receptors target to rafts prepared in detergent-free conditions, but this is reduced by low concentrations of Triton-X-100. This might reflect a weak association of P2X7 receptors with rafts or a difference in the nature of the rafts isolated by these methods. Rafts prepared in the absence of detergent retain a greater fraction of inner leaflet-membrane lipids, in particular phosphatidylserine (Pike et al., 2002) and this might be involved in stabilizing the association with P2X7 receptors.

How P2X receptors target to rafts is unclear. For P2X7 receptors there is evidence to support the involvement of both caveolin-1 and palmitoylation of the receptor within its cytoplasmic C-terminal domain (Barth et al., 2007, 2008; Gonnord et al., 2009). P2X7 receptors palmitoylated with a radiolabeled palmitate were detected exclusively in lipid rafts, while inhibiting palmitoylation reduced receptor targeting to rafts. In mouse lung alveolar epithelial cells, P2X7R and caveolin-1 were detected in the same native complexes and caveolin-1 co-immunoprecipitated with P2X7 receptors (Weinhold et al., 2010). Also, epithelial cells from the caveolin-1 knock-out mice showed reduced levels of P2X7 immunostaining at the plasma membrane (Barth et al., 2007, 2008). A role for caveolin-1 could explain cell-type dependent differences in P2X7 receptor trafficking and function, such as between fibroblasts, rich in caveolin-1, and some immune cells, deficient in caveolin-1.

Depleting plasma membrane cholesterol with methyl-β-cyclodextrin disrupts lipid rafts and alters the function of some but not all of the P2X receptors. P2X1 receptor currents are strongly inhibited by cholesterol depletion, whereas P2X2 receptor currents are unchanged (Vial and Evans, 2005; Allsopp et al., 2010). A region within the N-terminus of P2X1 proximal to TMD1 was identified as an important determinant of cholesterol sensitivity (Allsopp et al., 2010). Cholesterol sensitivity of P2X1 receptor currents was, however, abolished by a cytoskeletal stabilizing agent, suggesting that lipid rafts regulate P2X1 by affecting its interaction with the cytoskeleton (Lalo et al., 2011). P2X3 receptor currents in trigeminal sensory neurons were inhibited by methyl-β-cyclodextrin treatment and desensitization was accelerated (Gnanasekaran et al., 2011). P2X4 receptor currents in Thp-1 monocytes were similarly inhibited by depleting cholesterol (Li and Fountain, 2012). The role of lipid rafts as regulators of P2X7 receptor signaling is of particular interest because receptor stimulation activates lipid-metabolizing enzymes, including phospholipases and sphingomyelinases, that reside in lipid rafts and whose substrates are also enriched in rafts. In cells from rat submandibular glands, cholesterol depletion reduced ATP-stimulated ceramide generation and phospholipase A2 activation, consistent with the idea that targeting to rafts controls signaling between the P2X7 receptor and its downstream effectors (Garcia-Marcos et al., 2006a).

SUMMARY AND OUTLOOK

For many of the P2X receptors we now have a basic understanding of their trafficking properties and subcellular distributions, and in some cases have identified regulators that can alter their trafficking to and from the plasma membrane. For the P2X4 receptor, a key unanswered question is why it stably resides within endolysosomes. Only for the type II alveolar cells has a role for P2X4 receptors in the lamellar bodies, which resemble secretory lysosomes, been demonstrated, and only upon their fusion with the plasma membrane (Miklavc et al., 2011). It remains to be established whether P2X4 receptors have additional roles within conventional

or secretory lysosomes of other cells. P2X7 receptors are known to be up-regulated in many different cell types under inflammatory conditions, contributing to pathology (Lister et al., 2007). We would like to understand what regulates the trafficking of these receptors to and from the cell surface. For all of the P2X receptors we would like to understand how the lipid environment controls their function and stability. For P2X7 receptors this interaction might also operate in the reverse direction: events downstream of P2X7 receptor activation, including sphingomyelinase activation and ceramide generation directly modify raft structure, thereby providing the potential for cross-talk with other receptors that are modulated by lipid rafts.

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Two open states of P2X receptor channels

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The occupancy of the orthosteric ligand binding sites of P2X receptor (P2XR) channels causes the rapid opening of a small cation-permeable pore, followed by a gradual dilation that renders the pore permeable to large organic cations. Electrophysiologically, this phenomenon was shown using whole-cell current recording on P2X2R-, P2X2/X5R-, P2X4R- and P2X7R-expressing cells that were bathed in N-methyl-D-glucamine ($NMDG^+$)-containing buffers in the presence and/or absence of small monovalent and divalent cations. The pore dilation of P2X4R and P2X7R caused a secondary current growth, whereas that of P2X2R showed a sustained kinetic coupling of dilation and desensitization, leading to receptor channel closure. The pore size of the P2X7R open and dilated states was estimated to be approximately 0.85 nm and greater than 1 nm, respectively. The P2XR pore dilation was also observed in intact cells by measurement of fluorescent dye uptake/release, application of polyethylene glycols of different sizes, and atomic force microscopy. However, pore dilation was not observed at the single channel level. Structural data describing the dilated state are not available, and the relevance of orthosteric and allosteric ligand interactions to pore dilation was not studied.

Keywords: ATP, purinergic receptor channels, gating, pore opening, pore dilation, NMDG, YO-PRO-1

INTRODUCTION

P2X receptors (P2XRs) are adenosine triphosphate (ATP)-gated ion channels expressed in numerous excitable and non-excitable cells from various species, including humans (hP2XRs), rats (rP2XRs), mice (mP2XRs), and zebrafish (zP2X4.1R), where they contribute to the control of many physiological functions (Chizh and Illes, 2001; Vassort, 2001; North, 2002; Burnstock and Knight, 2004; Stojilkovic, 2009; Khakh and North, 2012). Mammalian P2X subunits exist in seven isoforms with the ability to form homo- and heterotrimers *in vitro* and in native tissues (Nicke et al., 1998; Kaczmarek-Hajek et al., 2012). The structure of zP2X4.1R in the closed state revealed that the core domains of the three subunits mutually intertwine, forming a central vertical cavity. The ectodomain is projected 0.7 nm above the plasma membrane and contains three vestibules along its central axis (Kawate et al., 2009). The structure of zP2X4.1R in the open state showed that the key residues for ATP binding are located approximately 0.45 nm from the plasma membrane (Hattori and Gouaux, 2012). Consistent with mutagenesis studies (Ennion et al., 2000; Jiang et al., 2000; Roberts and Evans, 2006; Yan et al., 2006; Zemkova et al., 2007), this structure revealed that the residues K67, K69, N293, R295, and K313 (rP2X4R numbering) are crucial for the recognition of phosphate groups, while the adenine moiety lies deeper in the binding pocket and is stabilized by hydrogen bonding (Hattori and Gouaux, 2012).

When bathed in physiological ion conditions, P2XRs respond to ATP stimulation with amplitude-modulated inward currents (North, 2002). Using whole-cell recording, these currents can be described by four parameters: (i) the activation time; (ii) the peak current amplitude; (iii) the rate of decay of the current amplitude during a sustained receptor stimulation; and (iv) the rate of decay of the current amplitude during agonist washout (Coddou et al., 2011). The activation time decreases and the peak current amplitude and rate of receptor desensitization increase with elevation in agonist concentration, whereas the rate of current deactivation is independent of agonist concentration; this phenomenon is well documented for rP2X4R using an ultra-rapid perfusion system (Yan et al., 2006). At the single channel level, activation is defined as the transition from the closed state to the open state, desensitization as the transition from the open state to the closed-desensitized state, and deactivation as the transition from the open state to the closed state (Egan et al., 2006). The existence of naïve (not previously exposed to ATP) and experienced (previously exposed to ATP) receptor states has also been proposed, which may reflect the phosphorylation state of P2XRs or other mechanisms accounting for short-term (less than 30 min) memory (Yan et al., 2010).

P2XRs are permeable to Na^+, K^+, and Ca^{2+} (Ding and Sachs, 1999), but the permeability to Ca^{2+} varies widely depending on the isoform (Evans et al., 1996; Soto et al., 1996; Virginio et al., 1998; Egan and Khakh, 2004). Some homomeric and heteromeric P2XRs also display a time-dependent modulation of ion selectivity by developing a new open state that permits relatively large cations to traverse the pore of the channel (Khakh et al., 1999; Virginio et al., 1999b). This phenomenon is called pore dilation, with the dilated pore representing a second open state with higher ion conductance. Our review focuses on the biophysical and biochemical aspects of pore dilation and transition to closed states, noting the isoform specificities.

HISTORICAL PERSPECTIVES

Extracellular ATP was initially suggested to affect permeabilization of the plasma membrane by two mechanisms: through its interaction with P2 receptors and by using a P2-independent mechanism to make pores large enough to allow for the permeation of substances with molecular weights (MW) ranging between 300 and 900 (Cockcroft and Gomperts, 1979; Steinberg et al., 1987; Tatham and Lindau, 1990). Other authors suggested that both actions were mediated by P2 receptors and introduced the term "P2Z" to describe a putative permeabilizing P2 receptor that required for activation high concentrations of ATP, lower concentrations of Benzoylbenzoyl adenosine-5′-triphosphate (BzATP), and the removal of Ca^{2+}/Mg^{2+} from the bath medium (Gordon, 1986). A receptor with a pharmacological profile typical of the P2Z receptor was cloned in 1996 and named P2X7R (Surprenant et al., 1996) and was able to permeabilize membranes (Virginio et al., 1999a). We now know that the rP2X7R transition from open to dilated states accounts for the permeabilizing action of ATP (Yan et al., 2008) and that the allosteric nature of Ca^{2+}-dependent inhibition accounts for the stimulatory effects of divalent cation removal on ATP's potency for activation (Yan et al., 2011). Initial experiments with rP2X2R (Virginio et al., 1999b) and rP2X4R (Khakh et al., 1999) have shown that the transition from the open to dilated state is not a unique characteristic of P2X7R. Experiments with other channels also revealed that pore dilation is not a P2XR-specific phenomenon (Khakh and Lester, 1999; Chung et al., 2008).

METHODS FOR STUDYING PORE DILATION

In whole-cell recording, large organic cations such as *N-methyl-D-glucamine (NMDG$^+$;* MW 195) are commonly used to evaluate changes in the permeability of P2XR pores during sustained receptor activation. $NMDG^+$ is used alone or as a substitute for bath Na^+ in the presence of other inorganic cations. Prior to ATP application, cells expressing P2XR are impermeable to $NMDG^+$. The uptake of nucleic acid-binding dyes, including YO-PRO-1 and TO-PRO-1, ethidium bromide (MW 394), and propidium iodide (MW 668), is also a standard tool for measuring cell permeabilization. YO-PRO-1 has been shown to permeate through rP2X2R, rP2X4R, rP2X7R, mP2X7R, and hP2X7R (Khakh et al., 1999; Virginio et al., 1999a; Hibell et al., 2000; Chessell et al., 2001; Yan et al., 2008). Ethidium bromide, most commonly used in the visualization of DNA and RNA in electrophoresis gels, was used to show changes in the permeability of human and mouse P2X7R (Stokes et al., 2010; Tran et al., 2010). This application is also the case with propidium iodide (Sun et al., 2010) and TO-PRO-1 (Mankus et al., 2011). The uptake of Lucifer Yellow (MW 457) was used to study the permeation of native mP2X2R and mP2X7R in taste bud cells (Hayato et al., 2007). Cellular leakage of the Ca^{2+} probes Fura-2 (MW ranging between 636 and 1001) and Fura-FF (MW ranging between 658 and 1023) has provided further useful information about the dilation of rP2X2R and rP2X7R (Yan et al., 2008; Khadra et al., 2012). In addition, the size of the dilated pore was measured by the application of differently sized polyethylene glycols; those having a MW greater than or equal to 5000 blocked the increase in cation permeability in cells expressing rP2X7R, suggesting that the dilated pore is greater than 1 nm in diameter (Virginio et al., 1999a). Finally, rP2X4R (expressed in human 1321N1 cells) pore dilation was observed by atomic force microscopy (Shinozaki et al., 2009).

PORE DILATION ACCOUNTS FOR BIPHASIC CURRENTS

The current generated by naïve rP2X7R expressed in Human Embryionic Kidney 293 (HEK293) cells (Roger et al., 2008; Yan et al., 2008) and by hP2X7R expressed in *Xenopus* oocytes (Klapperstuck et al., 2001) features a biphasic response in whole-cell recordings; the initial rapid rise in current (I_1) is accompanied by a secondary slow current growth (I_2), the rate of which increases with agonist concentration (Yan et al., 2010). The I_2 is also evident when intracellular Ca^{2+} measurements are used to indicate the receptor activity in both amphotericin-perforated and intact cells (Yan et al., 2010). This phenomenon suggests that neither the expression system nor the washout of secondary messengers or intracellular ions accounts for this P2X7R behavior.

Substituting bath Na^+ with $NMDG^+$ permits current recordings in cells clamped at −60 mV and the detection of changes in reversal potential, as estimated by repetitive voltage-ramp pulses from −80 mV to +80 mV. This finding was used to support the hypothesis of rP2X7R pore dilation (Yan et al., 2008). The substitution of 90% (**Figure 1A**) and 100% (**Figure 1B**) of the extracellular Na^+ with $NMDG^+$ does not alter the biphasic response pattern of rP2X7R generated by the prolonged application of BzATP when Ca^{2+}, Mg^{2+}, and K^+ are all present in the bath medium. Under repetitive voltage-ramp pulses from −80 to +80 mV, a positive shift was also found in the reversal potential (indicated by horizontal arrows in **Figures 1D, E**). The rate of reversal potential shift was highly comparable to the rate of I_2 current growth (**Figure 1G**).

However, the time-course of rP2X7R agonist induced current was substantially different in cells bathed in $NMDG^+$-containing medium lacking other cations (**Figure 1C**). The time-course consisted of an initial outward current, reflecting the movement of intracellular Na^+ through the channel pore, followed by a shift to an inward current, reflecting the gradually developing permeability to $NMDG^+$ and the lack of receptor deactivation after agonist washout (Jiang et al., 2005). This finding indicates that $NMDG^+$ cannot substitute for Na^+ in receptor deactivation (Yan et al., 2008), in contrast to Ca^{2+} (**Figure 1B**). Under these experimental conditions, a shift also occurred in the reversal potential (**Figure 1F**), indicating that the kinetics of decay from an outward to an inward current reflects pore dilation. Certainly, $NMDG^+$ only partially substituted for inorganic cations as the conducting ion. This phenomenon is indicated by the peak amplitude of the inward current in **Figures 1A–C**. The time needed for the development of pore dilation was also observed in experiments where $NMDG^+$ was substituted for physiological cations during early and sustained agonist application (**Figures 1H, I**).

When expressed in *Xenopus* oocytes, rP2X4R exhibited a similar biphasic current response, with I_2 developing slowly over several minutes. The biphasic current was also observed when using $NMDG^+$-containing medium. The I_2 growth occurred

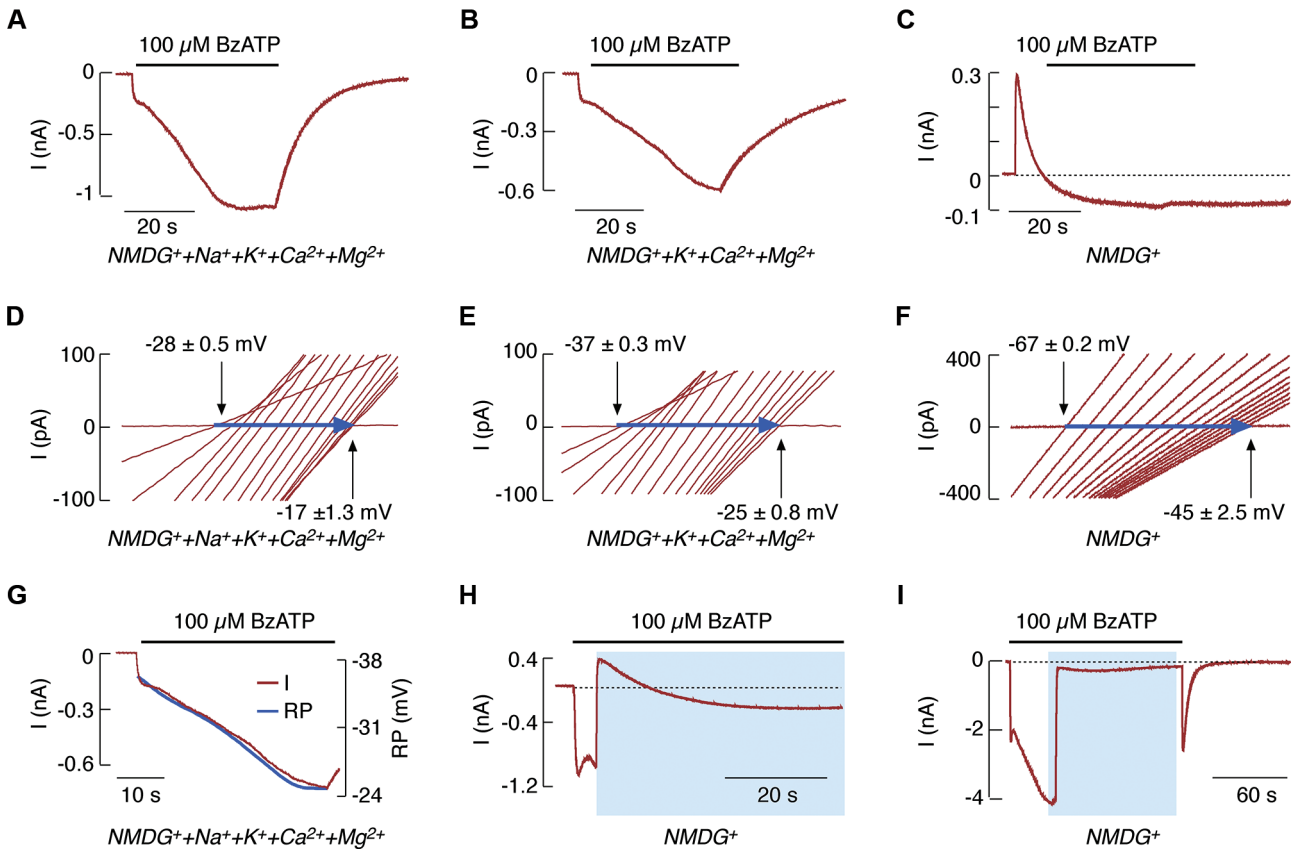

FIGURE 1 | NMDG$^+$ permeability of rP2X7R expressed in HEK293 cells. **(A–F)** Patterns of BzATP-induced currents **(A–C)** and reversal potentials **(D–F)** in cells continuously bathed in media containing NMDG$^+$, Na^+, K^+, and divalent cations (**A** and **D**); NMDG$^+$, K^+, and divalent cations (**B** and **E**); and NMDG$^+$ only (**C** and **F**). This figure (as well as the one following) shows that whole-cell current recordings were performed at a holding potential of −60 mV, and agonist was applied for 40 s. Voltage ramps were delivered twice per second during the 40 s agonist application to record positive shifts in reversal potential (horizontal blue arrows); only 15 traces (out of a total of 100) for the current-voltage relationship with equal time intervals are shown. (**G**) Comparison of the kinetics of secondary current growth (red trace) and of changes in reversal potential (RP; blue trace) in cells bathed in media containing NMDG$^+$ and divalent cations during the 40 s application of BzATP. (**H** and **I**) 100 μM BzATP-induced current profiles in cells bathed in a normal physiological solution for 4 s **(H)** and 40 s **(I)**, and in NMDG$^+$-containing media for the rest of the agonist application time. Horizontal black lines above the traces indicate the duration of BzATP application, and blue areas indicate the duration of NMDG$^+$ application. Derived from Yan et al. (2008, 2010).

simultaneously with a positive shift in reversal potential and an uptake of YO-PRO-1 (Khakh et al., 1999). Other authors also observed pore dilation in rP2X4R expressed in *Xenopus* oocytes that were bathed in a low Ca^{2+} medium (Toulme et al., 2006).

Thus, the P2X4R and P2X7R permeation path is not a single step transition from a small to a large size pore. Instead, the receptor undergoes progressive dilation. Furthermore, the process of rP2X7R dilation was Ca^{2+}-independent, in contrast to P2X7R gating, which was allosterically influenced by the presence of Ca^{2+} (Yan et al., 2011). However, rP2X4R pore dilation was blocked by bath Ca^{2+} (Khakh et al., 1999). A model of rP2X7R kinetics was proposed in accordance with these findings, suggesting the coupling of kinetic transitions from the open state to either the closed-desensitized state or to an additional open (dilated) state, which is also the sensitized/facilitated state. The transition to this open sensitized state is kinetically favored over the transition to the desensitized state, leading to the generation of a biphasic current response during the initial agonist application (Yan et al., 2010; Khadra et al., 2013).

PORE DILATION IS MASKED BY RECEPTOR DESENSITIZATION

When expressed in HEK293 cells, the full size rat receptor (rP2X2aR) and the splice variant missing 69 C-terminal amino acids (rP2X2bR) (Brandle et al., 1997; Koshimizu et al., 1998) each rapidly generated outward currents followed by slowly developing inward currents. Each also exhibited shifts in reversal potential when bathed in NMDG$^+$- containing media and uptake of YO-PRO-1 when bathed in physiologically normal buffer. Together, these findings indicate pore dilation in both rP2X2Rs (Virginio et al., 1999b). However, when bathed in a Ca^{2+}-containing medium, both forms of rP2X2Rs, as well as rP2X4R, generated desensitizing currents during sustained agonist application; notably, rP2X2bR was desensitized more rapidly than rP2X2aR (**Figures 2A–C**). The kinetics of pore dilation

revealed the splice variant-dependent specificities of this process (Khadra et al., 2012). Cells clamped at −60 mV and stimulated with 100 μM ATP showed an initial rapid outward current, reflecting an efflux of intracellular Na^+ through the pore. This activity was accompanied by a current decline, which also shifted directionality from outward to inward, reflecting the development of permeability to $NMDG^+$ (**Figures 2D, E**). In contrast, there was no shift from outward to inward current in HEK293 cells expressing rP2X4R (**Figure 2F**). In cells expressing rP2X2aR and rP2X2bR, but not in those expressing rP2X4R, the substitution of extracellular Na^+ with $NMDG^+$ also resulted in a strong time-dependent shift in reversal potential after the application of voltage-ramp pulses from −80 to +80 mV (**Figures 2G–I**). Finally, a correlation was found between the development of the $NMDG^+$-induced current and the temporal changes in reversal potential (**Figure 2D**, inset).

Thus, the kinetics of desensitization for rP2X4R, but not for rP2X2aR or rP2X2bR, is identical in cells bathed in physiological and $NMDG^+$-containing buffers. Furthermore, rP2X2aR and rP2X2bR, but not rP2X4R, dilate when bathed in $NMDG^+$-containing media. Recently, we showed that, in contrast to rP2X2R pore dilation, the removal of bath Ca^{2+} accounts for the difference in rP2X2aR and rP2X2bR desensitization kinetics in cells bathed in physiological and $NMDG^+$-containing media (Coddou et al., 2012). Both P2X2R homomers and rP2X2/X5R heteromers expressed in HEK293 cells dilate during sustained receptor activation (Compan et al., 2012). Channel clustering is not obligatory for P2X2R pore dilation, which most likely reflects permissive motions at the interface between the first and second transmembrane domains of neighboring subunits (Khakh and Egan, 2005). Finally, an experimentally supported mathematical model provides a rationale for the lack of sustained current growth in dilating rP2X2Rs by showing that dilated receptors also desensitize in the presence of Ca^{2+} (Khadra et al., 2012). The Ca^{2+}-dependent transition from dilated to desensitized states does not occur in rP2X7R-expressing cells. This receptor only shows Ca^{2+}-independent desensitization, resulting in a biphasic current response (Khadra et al., 2013).

PANNEXIN-1 AND P2XR SIGNALING

A comparative study of $NMDG^+$ and YO-PRO-1 uptake with wild type and mutant rP2X7Rs raised the possibility that these two molecules do not enter the cell by the same permeation pathway (Jiang et al., 2005). The search for a pathway accounting for YO-PRO-1 uptake led to the hypothesis that pannexin-1 (Panx1) could mediate pore formation and interleukin-1β release by rP2X7R (Pelegrin and Surprenant, 2006). Consistent with this hypothesis, Panx1 was found to co-immunoprecipitate with

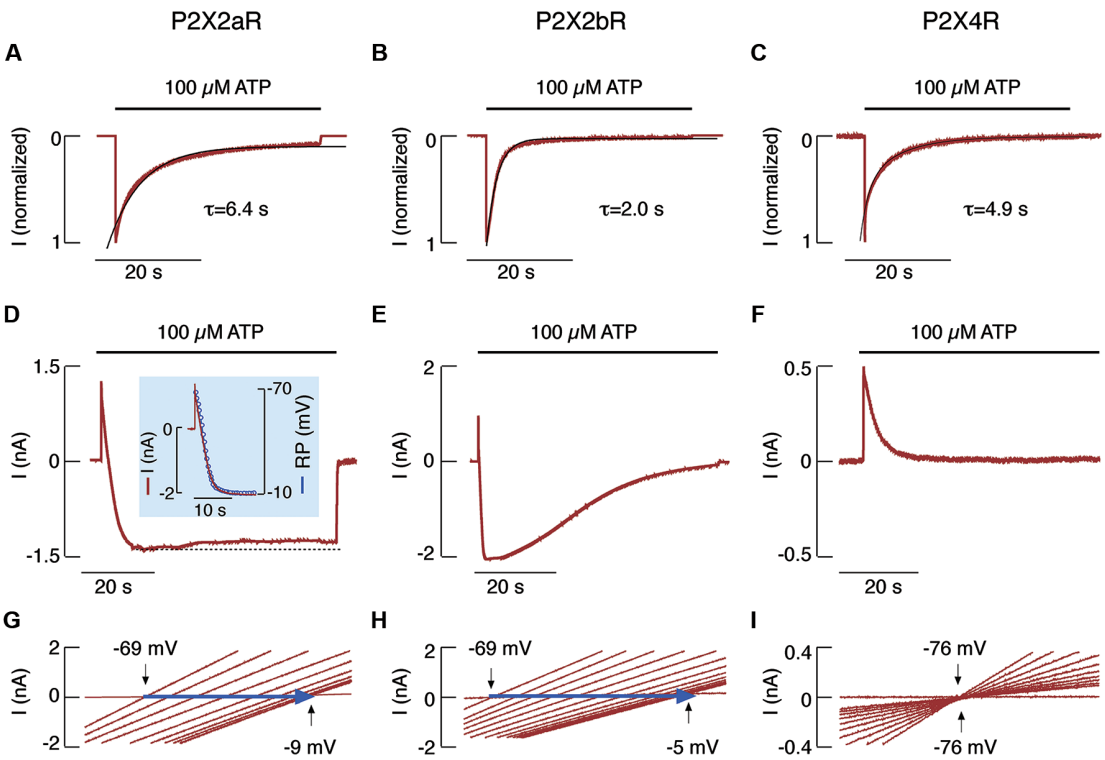

FIGURE 2 | $NMDG^+$ permeability of rP2X2aR, rP2X2bR and rP2X4R expressed in HEK293 cells. **(A–C)** Patterns of ATP-induced currents in cells bathed in a normal physiological medium. Traces shown were obtained during the first (P2X4R) and third (P2X2aR and P2X2bR) ATP application. Black lines indicate exponential fittings, with τ_{des} shown below the traces. **(D–I)**. Patterns of ATP-induced current **(D–F)** and reversal potential **(G–I)** in cells that have been continuously bathed in a medium containing only $NMDG^+$. (**D**, inset) Temporal correlation between P2X2aR current (red trace) and reversal potential (blue circles). Note the lack of inward current **(F)** and the positive shift in reversal potential **(I)** in cells expressing P2X4R. Derived from Khadra et al. (2012).

rP2X7R (Pelegrin and Surprenant, 2006; Li et al., 2011b) and other rP2XRs (Li et al., 2011b) when co-expressed in HEK293 cells.

However, several lines of evidence suggest that pore dilation is an intrinsic property of P2XR channels, independent of Panx1 expression and function. First, C6 astroglioma cells lack endogenously expressed Panx1, but the rP2X7R expressed in these cells dilates (Yan et al., 2008). Second, rP2X2R and rP2X7R dilation was not affected by overexpression of Panx1 or by blockading this channel with carbenoxolone (Chaumont and Khakh, 2008; Yan et al., 2008). Third, dilation of native mP2X4R bathed in a low Ca^{2+} medium was unaffected by carbenoxolone (Bernier et al., 2012). Fourth, RNAi targeting Panx1 did not affect native mP2X7R pore dilation (Alberto et al., 2013). Fifth, recent simultaneous measurement of membrane currents, fluorescent dye uptake, and permeation pathway sizing revealed that the dilated channel of rP2X7R allows the passage of molecules as large as 1.4 nm (Browne et al., 2013). Sixth, recent literature suggests that Panx1 may contribute to ATP release (Locovei et al., 2006; Huang et al., 2007; Iglesias et al., 2009; Li et al., 2011a); the role of Panx1 in ATP release but not in P2X7R pore dilation was further supported by experiments using Panx1 knockout bone marrow-derived macrophages (Qu et al., 2011).

THE ROLE OF CYTOSOLIC DOMAINS IN P2XR GATING

The initial observation of the role of the C-terminus of rP2X7R in YO-PRO-1 uptake was reported by Surprenant et al. (1996). hP2X7R expressed in *Xenopus* oocytes also exhibited C-terminus-dependent gating properties (Becker et al., 2008), but there were some differences in responses of rP2X7R and hP2X7R when expressed in HEK293 cells, probably reflecting the receptor-specific C-terminal domain structure (Rassendren et al., 1997). Studies comparing the wild type and C-terminal truncation forms of rP2X7R expressed in HEK293 cells and *Xenopus* oocytes further indicated the importance of the C-terminus for receptor dilation (Smart et al., 2003). Rat, but not mouse, P2X2aR showed pore dilation when expressed in *Xenopus* oocytes, thus suggesting the role of specific cytosolic domains as determinants of permeation in a state-specific manner (Eickhorst et al., 2002). The removal of a cysteine-rich segment of the intracellular juxta-membrane region of rP2X7R was reported to cause the loss of $NMDG^{+}$ permeability without affecting YO-PRO-1 uptake (Jiang et al., 2005). This discrepancy can be explained by the instantaneous opening of the mutant receptor into the dilated state (Yan et al., 2008). The N-terminal T15 mutants also opened instantaneously into the dilated state in a protein kinase C-independent manner (Yan et al., 2008, 2010), suggesting that N-terminal contributes to the control of transition from open to dilated state.

FUTURE DIRECTIONS

Although the crystal structures of the zP2X4.1R channel in both the ATP-bound open state and the apo-closed state have been solved (Kawate et al., 2009; Hattori and Gouaux, 2012), the structure of the dilated state is missing, presumably due in part to the lack of N- and C-termini in crystallization studies. The structural correlate of the P2X7R sensitization/facilitation state (Roger et al., 2008; Yan et al., 2010) is also missing. The dilated state is strongly ATP sensitized (Yan et al., 2010), which could imply structural changes in the ATP binding site and/or different orientations of bound ATP (Jiang et al., 2011). Pore dilation was not observed in single channel recording (Tatham and Lindau, 1990; Ding and Sachs, 1999; Riedel et al., 2007a,b) and the open-1 state was estimated to be 0.85 nm (Riedel et al., 2007b). The reason for this discrepancy is not clear. Mutagenesis studies have identified specific regions and residues of P2XRs that influence pore dilation (Khakh and Egan, 2005; Yan et al., 2008; Sun et al., 2013), but further crystallization and mutagenesis studies are needed to better understand the transition from open to dilated states. It is also unclear which physiologically relevant metabolites from the cellular microenvironment permeate through the dilated P2XR pore, or whether there are any allosteric up- or down-regulatory mechanisms that could convert the receptor to the dilated or naïve closed states. P2X7R exhibits a significant number of gene polymorphisms with strong pathophysiological implications in hP2XRs (Wesselius et al., 2011); further investigations should clarify whether the loss- or gain-of-function variants shows pore dilation. The development of pharmacological tools for altering the transition of P2XR pores from open to dilated states could help in dissecting the physiological significance of the two open states of P2XRs.

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AUTHOR CONTRIBUTIONS

Both authors contributed to the writing of this review.

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Structural and functional properties of the rat P2X4 purinoreceptor extracellular vestibule during gating

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P2X receptors are ATP-gated cation channels consisting of three subunits that are mutually intertwined and form an upper, central, and extracellular vestibule with three lateral portals and the channel pore. Here we used cysteine and alanine scanning mutagenesis of the rat P2X4R receptor V47–V61 and K326–N338 sequences to study structural and functional properties of extracellular vestibule during gating. Cysteine mutants were used to test the accessibility of these residue side chains to cadmium during closed-open-desensitized transitions, whereas alanine mutants served as controls. This study revealed the accessibility of residues E51, T57, S59, V61, K326, and M336 to cadmium in channels undergoing a transition from a closed-to-open state and the accessibility of residues V47, G53, D331, I332, I333, T335, I337, and N338 in channels undergoing a transition from an open-to-desensitized state; residues E56 and K329 were accessible during both transitions. The effect of cadmium on channel gating was stimulatory in all reactive V47–V61 mutants and inhibitory in the majority of reactive K326–N338 mutants. The rat P2X4 receptor homology model suggests that residues affected by cadmium in the closed-to-open transition were located within the lumen of the extracellular vestibule and toward the central vestibule; however, the residues affected by cadmium in the open-to-desensitized state were located at the bottom of the vestibule near the pore. Analysis of the model assumed that there is ion access to extracellular and central vestibules through lateral ports when the channel is closed, with residues above the first transmembrane domain being predominantly responsible for ion uptake. Upon receptor activation, there is passage of ions toward the residues located on the upper region of the second transmembrane domain, followed by permeation through the gate region.

Keywords: ATP, cadmium, gate, ion access, lateral portals, purinergic receptors

INTRODUCTION

Providing the crystal structure of the zebrafish purinergic P2X4.1 receptor (zP2X4.1R) in its closed state (Kawate et al., 2009) was a landmark achievement that confirmed previous findings about the trimeric organization of these ATP-gated channels (Nicke et al., 1998). Each P2XR subunit is composed of two transmembrane domains (TM1 and TM2) that are separated by a large extracellular loop and N- and C-termini, which are located intracellularly (Brake et al., 1994; Valera et al., 1994). The crystallization study also confirmed that three TM2 α-helices form the P2XR pore (Rassendren et al., 1997; Egan et al., 1998), as well as a hydrophobic barrier to ion flow, called a gate, and an extracellular vestibule on the ion channel, located above the gate (Khakh and Lester, 1999). The study also revealed the existence of two additional vestibules (central and upper) and lateral fenestrations, which raised the possibility that cations travel through a central pathway that spans the entire length of the ectodomain and/or through three lateral portals that are formed at the interfaces of the adjoining subunits. Furthermore, amino acid residues that comprise the ion access portals were shown as natively unfolded regions of the zP2X4.1R molecule (Kawate et al., 2009). The subsequent crystal structure study of this receptor with and without bound ATP showed that the lateral fenestrations are encompassed by amino acid residues above the TM domains in a β-sheet conformation (Hattori and Gouaux, 2012).

These findings have prompted investigations of the pathway through which ions traverse the extracellular domain of P2XRs to enter/exit the TM pore. One study that addressed this problem suggested the importance of residues I332, T336, and T339 for forming an ion gate in the rat P2X2R (rP2X2R), and it established that the opening of the gate is accompanied by movement of the pore-lining regions, which narrow toward the cytosolic end of TM2 (Kracun et al., 2010). These conclusions are in agreement with those of earlier studies that showed the relevance of residues I328, I332, and T336 in ion gating (Stoop et al., 1999; Jiang et al., 2001; Li et al., 2008). The second study (Kawate et al., 2011) also suggested that ions access the pore by using the lateral fenestrations, which breathe as the gate opens. Their experiments raised the possibility of ions accessing the upper vestibule, which could play a regulatory function. The ion access point was also studied

in the human P2X4 receptor (hP2X4R). The main conclusions of this study were that lateral portals are preferentially used because of their favorable diameters and that residues E56 and D58 are crucial for ion access to the extracellular vestibule (Samways et al., 2011).

In line with these investigations, here we focused on structural and functional properties of extracellular vestibule during gating by identifying the amino acid residues that are important for the interaction with the ion during closed-to-open and open-to-desensitized state transitions. Alanine and cysteine scanning mutagenesis was performed on the rP2X4R extracellular vestibule region encompassing the V47–V61 and K326–N338 sequences. Because cadmium ion is widely used in screening of surface accessibility of amino acids from membrane proteins (Li et al., 2008; Kracun et al., 2010; Samways et al., 2011), but is also acting as an allosteric modulator of P2X4R (Coddou et al., 2011), here we dissected the native allosteric effects of cadmium ion from cysteine binding effects. In addition, a homology model of rP2X4R in closed and open states was done to discuss topological characteristics of cadmium-sensitive mutants and to propose a model of ion access and conformational changes of extracellular vestibule during gating.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

Recombinant rP2X4R channels were expressed in human embryonic kidney 293T cells (American Type Culture Collection, Rockville, MD, USA), which were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were grown in a humidified 5% CO_2 atmosphere at 37°C. Transfection was performed using the jetPRIME TM polymer-based transfection reagent, according to the manufacturer's instructions (PolyPlus-transfection, Illkirch, France).

DNA CONSTRUCTS

Complementary DNA sequences of wild type (WT) rP2X4R were subcloned into the pIRES2-EGFP vector (Clontech, Mountain View, CA, USA). To generate the mutants, oligonucleotides (synthesized by VBC-Genomics, Vienna, Austria and Sigma Chemical Company, USA) that contained specific point mutations were introduced into the rP2X4/pIRES2-EGFP template by using PfU Ultra DNA polymerase (Agilent Technologies Inc., USA). To isolate the plasmids for transfection, a High-Speed Plasmid Mini Kit (Geneaid, Shijr, Taipei, Taiwan) was used. Dye terminator cycle sequencing (ABI PRISM 3100, Applied Biosystems, Foster City, CA, USA) was used to identify and verify the mutagenesis outcomes. The sequencing was performed by the DNA Sequencing Laboratory, Institute of Microbiology, ASCR, Prague.

PATCH CLAMP RECORDINGS

ATP-induced whole-cell currents were recorded at −60 mV using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA, USA). The recordings were captured and stored using the Digidata 1322A and pCLAMP9 software. The cell culture was perfused with an extracellular solution that contained the following: 142 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM D-Glucose, adjusted to pH 7.3 with 1 M NaOH. The patch electrodes were filled with a solution containing the following: 154 mM CsCl, 11 mM EGTA, and 10 mM HEPES, adjusted to pH 7.2 with 1.6 M CsOH. The control and ATP-containing solutions were applied via a perfusion system (RSC-200, BIOLOGIC, Claix, France).

EXPERIMENTAL PROTOCOLS

To probe the surface accessibility of particular amino acid residues within the extracellular vestibule region of rP2X4R, 20 μM cadmium was applied, a concentration that was based on previous efficacy reports involving this cadmium concentration in experiments with rP2X2R (Kracun et al., 2010) and hP2X4R (Samways et al., 2011). Two experimental protocols were used in our study. Protocol 1: cadmium was applied for 1 min, followed by a transient (2 s) application of ATP in the presence of cadmium. Because allosteric binding sites for cadmium ions are believed to be numerous and could stimulate and/or inhibit the channel activity (Coddou et al., 2011), ATP was applied at the EC_{50} concentration to permit estimates of both stimulatory and inhibitory effects. Protocol 2: cadmium was perfused during the application of 100 μM ATP. Specifically, cadmium was applied at 50 ± 10% of the current rundown for 2–15 s, depending on the rate of receptor desensitization. The effect of cadmium was measured as the change in current amplitude (in %) immediately after ion application compared with the current amplitude before cadmium application. The same application protocols have been used for measuring cadmium effects on the WT receptor and alanine and cysteine mutants. Only the first (naïve) response was considered to exclude the impact of receptor internalization on gating (Bobanovic et al., 2002; Royle et al., 2002, 2005).

HOMOLOGY MODELING

Because the sequence identities of rP2X4R (P51577) and zP2X4.1R (Q6NYR1) are 62.4% homologous, a homology model of the rP2X4R was developed using the automated mode of the SWISS-MODEL server (Schwede et al., 2003). A tertiary structure template was extracted from the Brookhaven Protein Data Bank under the accession number 4DW1 for the zP2X4.1R in the ATP-bound open state and 4DW0 for the receptor in the apo-closed state. Model quality was estimated by a SWISSMODEL through estimation of a QMEAN4 score, which was 0.593 (Benkert et al., 2008). All graphical representations of the protein structure were prepared using PyMOL software (DeLano Scientific LLC, USA). The rP2X4R homology models of lateral portals and extracellular vestibule in closed and open states are shown in **Figure 1B** and described in the section "Model Prediction of the Position of Residues of Interest."

CALCULATIONS

The concentration-response data were taken from (Rokic et al., 2013), and the ATP-induced current data points were fitted with the equation $y = I_{max}/[1 + (EC_{50}/x)^h]$; y is the amplitude of the ATP-induced current, I_{max} is the maximum current amplitude induced by supramaximal doses of ATP, h is the Hill

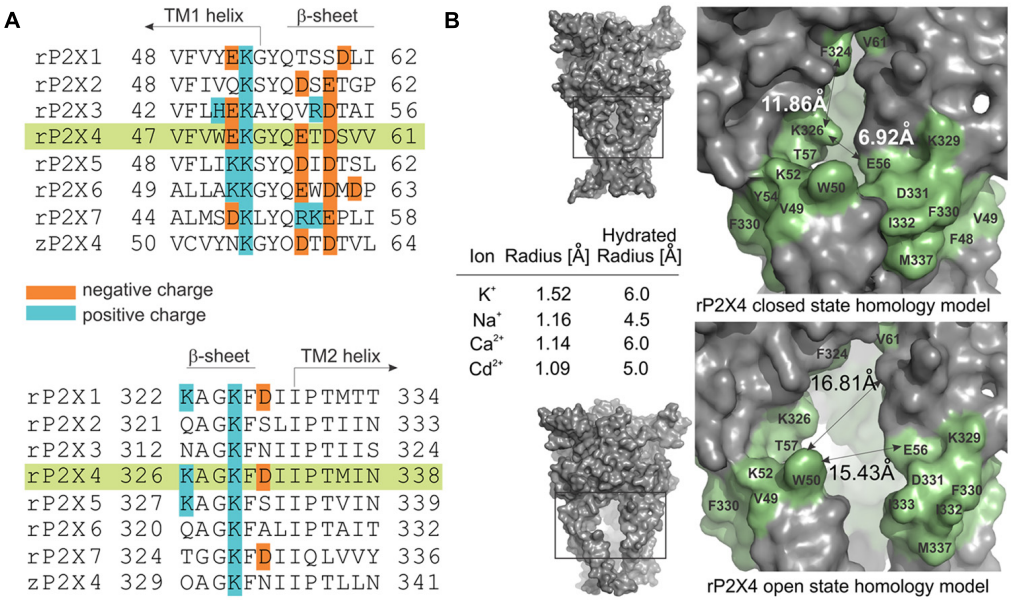

FIGURE 1 | Characteristics of the ion access point of P2XRs. (A) Multiple sequence alignment of seven rat P2XRs compared to zP2X4.1R. The sequence alignments contain the amino acid residues that are homologous to the V47–V61 and K326–N338 segments of rP2X4R (green shade), and the positively and negatively charged residues are indicated. **(B)** The surface landscape of the homology model for rP2X4R in a closed and open state; the entire molecule (left panels) and the selected regions (right panels). The residues comprising the peptide segments from V47–V61 and K326–N338 are presented in green; double arrows and numbers show average distances encompassing the ion entrance point. The Table within the insets summarizes the ion radii of physiologically permeable ions through P2X4R and cadmium ions in hydrated and non-hydrated states.

coefficient (fixed to 1.3 in all cases), and x is the concentration of ATP (SigmaPlot 2000 v9.01; SPSS Inc., Chicago, IL, USA). All numerical values in the text are reported as the mean ± SEM. Significant differences ($p < 0.05$) between means for the WT receptor, cysteine mutants, and alanine mutants were determined by an ANOVA test followed by *post hoc* t-test analysis with Bonferonni correction for three sets of data using SigmaStat 2000 v9.01.

RESULTS

EXPERIMENTAL MODEL

Figure 1A illustrates amino acid diversity of extracellular vestibule sequences among rat P2X subunits and different content of positively and negatively charged residues of these sequences. The low evolutionary conservation (Kaczmarek-Hajek et al., 2012) and the different content of positively and negatively charged amino acid residues of these sequences among receptor subtypes prompted us to examine the hypothesis that the mechanism of ion access is isoform-specific. As a receptor model, we selected the rP2X4R and as an expression system human embryonic kidney 293T cells. We used cysteine and alanine scanning mutagenesis of the extracellular vestibule's V47–V61 and K326–N338 sequences; cysteine mutants were used to test the accessibility of these residue side chains to reporters during closed-open-desensitized transitions, whereas alanine mutants served to exclude the possible effects caused by mutation-induced changes in cadmium binding at native allosteric sites.

The amino acid surface accessibility reporters commonly used in P2XR research include methanethiosulfonate (MTS) reagents (Egan et al., 1998; Stoop et al., 1999; Haines et al., 2001; Jiang et al., 2001; Li et al., 2008; Roberts et al., 2009; Allsopp et al., 2011; Kawate et al., 2011), silver ion (Egan et al., 1998; Haines et al., 2001; Li et al., 2008; Jindrichova et al., 2011), and cadmium ion (Li et al., 2008; Kracun et al., 2010; Samways et al., 2011; Heymann et al., 2013). Because MTS reagents are not of the appropriate size and the molecular properties as native cations, and silver ions are photosensitive requiring the use of a chloride-free extracellular solution, we used the cadmium–cysteine interaction to study ion accessibility of the receptor vestibule. The ionic radius of a cadmium ion in its hydrated and anhydrous state is comparable to both ionic radii of physiologically gated ions passing through the rP2X4R pore (**Figure 1B**, inset; Kielland, 1937; Shannon, 1976). The permeability of P2XRs to calcium (Egan and Khakh, 2004) further suggests that the divalent property of cadmium should not represent an obstacle for studies on gating function.

CADMIUM EFFECTS ON WT-rP2X4R

As stated in detail in the section "Materials and Methods," two protocols were used in our experiments. **Figure 2A** summarizes data using the protocol-1. We initially determined the ATP EC_{50} value for the WT receptor in our experimental conditions (2.3 ± 0.1 μM). Left panel shows representative traces from two different cells, control (blue trace) and a cell pretreated with cadmium for 60 s and cadmium plus ATP (red trace). These data clearly indicate a lack of cadmium influence on the peak current amplitude during the short (2 s) application of 2.3 μM ATP.

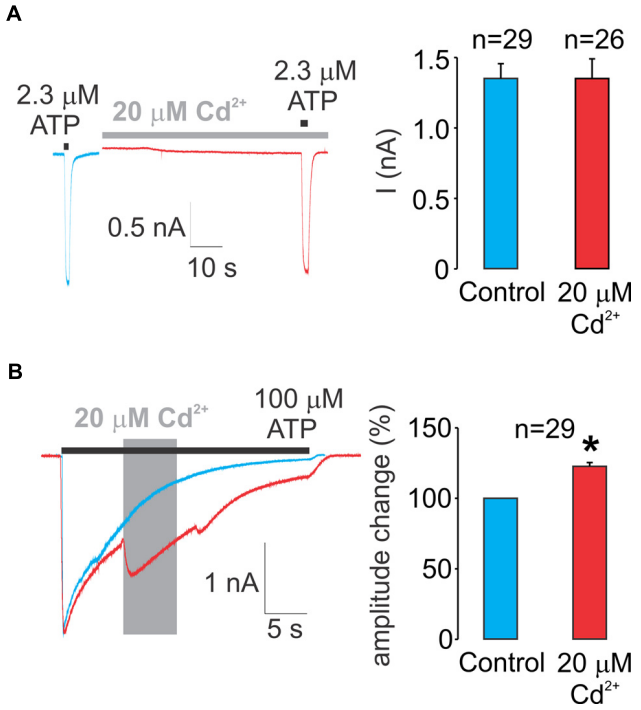

FIGURE 2 | Effects of cadmium on WT rP2X4R gating. (A) A lack of pre-application during 1 min and a subsequent 2-s co-application of 20 μM cadmium on ATP-induced peak current response. 2.3 μM ATP (applied for 2 s) is the EC_{50} dose for the WT receptor. **(B)** Effect of the application of 20 μM cadmium ion on 100 μM ATP-induced sustained current. Cadmium was applied within 50 ± 10% desensitization decay (gray area) and significantly amplified the current amplitude, on average 22 ± 3%. Left panels show representative traces; control traces are blue, and traces with cadmium treatment are red. Right panels illustrate mean ± SEM values, with n shown above bars; * illustrates significant difference, $P < 0.01$. To avoid the impact of desensitization and the subsequent internalization on the current amplitude and patterns, control, and experimental traces were obtained in separate cells during a single ATP application.

Figure 2B summarizes experiments with the WT receptor using protocol-2. In the absence of cadmium, the receptor desensitized almost completely during the sustained application of supramaximal (100 μM) ATP (blue trace). In contrast to protocol-1, the allosteric effect of cadmium on rP2X4R gating was visible when using protocol-2; the application of cadmium during the ATP treatment transiently reversed the process of desensitization by amplifying the current (red trace). On average, this amplification was 22 ± 3% (right panel). Note that in the presence of cadmium, the receptor continues to desensitize. Because cadmium shows allosteric effects when applied by protocol-2 but not protocol-1, all experiments with protocol-2 were performed in alanine mutants as well to distinguish between native cadmium allosteric effects and cysteine binding effects.

CADMIUM EFFECTS ON MUTANTS

To study the accessibility of rP2X4R residue side chains to cadmium ions, we used previously generated single residue alanine (controls) and cysteine mutants of V47–V61 and K326–N338 sequences (Rokic et al., 2013). Most of mutants were functional, which is consistent with analysis conducted previously on hP2X1R (Allsopp et al., 2011), rP2X2R (Rassendren et al., 1997; Li et al., 2004; Friday and Hume, 2008; Jiang et al., 2010), and rP2X4R (Popova et al., 2010). **Table 1** lists the functional mutants. The EC_{50} (column two) and I_{max} values (column three) for WT and functional mutants were adopted from (Rokic et al., 2013). Column four shows mean values ± SEM of the cadmium effect on current induced by EC_{50} dose ATP during 1 min cadmium pre-application. Column five summarizes the effects of cadmium transiently applied during stimulation with 100 μM ATP for 2–15 s (protocol-2).

In contrast to the WT receptor (**Figure 2A**), protocol-1 revealed that a cadmium pre-application resulted in a statistically significant current amplitude potentiating effect on the EC_{50} dose pulse of ATP for the following mutants: E51C, E56C, T57C, S59C, V61C, K326C, and M336C, whereas the K329C mutant was significantly inhibited by cadmium. **Figure 3** shows representative traces for these mutants, and **Table 1** (column four) shows mean ± SEM values.

Cadmium application during 100 μM ATP treatment (protocol-2) revealed four types of responses when compared to the WT receptor: (i) we observed statistically significant current augmentation in six mutants: V47C, W50C, G53C, E56C, I333C, and N338C; (ii) five mutants responded to cadmium application with the inhibition of current: K329C, D331C, I332C, T335C, and I337C; (iii) the effect of cadmium was lost in five mutants: F48C, D58C, S59C, V60C, and P334C mutants; (iv) the residual mutants responded with the facilitation of current, which was not significantly different from that observed in cells expressing the WT receptor (**Table 1**).

The alanine scanning mutagenesis also revealed that the stimulatory effect of cadmium was lost in eight mutants: F48A, D58A, S59A, V60A, I332A, I333A, P334A, and M336A. The cadmium response was significantly amplified in the W50A mutant, whereas the sustained current was significantly inhibited in the E51A mutant (**Table 1**). In the remaining mutants, the stimulatory effect of cadmium was comparable with the WT receptor. The same direction of changes in alanine and cysteine mutants for F48, W50, D58, S59, V60, and P334 residue mutants suggests that cysteine mutants should not be considered as cadmium hits. Hereafter, the following residues are considered as directly affected by cadmium: V47, G53, E56, K329, D331, I332, I333, T335, I337, and N338.

The cadmium-hit residues exhibited two types of responses: stimulation or inhibition of sustained current. **Figure 4** shows the example traces for cysteine mutants exhibiting facilitation (**Figure 4A**, left) and inhibition (**Figure 4B**, left) of current by cadmium as well as the pattern of response by the corresponding alanine mutants (**Figure 4B**, right). Note that all (three of three) affected V47–V61 mutants exhibited facilitation, and the majority of K326–N338 mutants (five of seven) exhibited inhibition of the sustained ATP-induced current. **Figure 4** also illustrates that single residue mutations affect the rates of receptor desensitization, an issue that we have not further addressed. Among mutant receptors, there was no significant correlation between the EC_{50} values for ATP and cadmium potentiation/inhibition effects ($R = 0.48$, $p > 0.05$).

Table 1 | Effect of pre-application and co-application of cadmium ion on alanine and cysteine rP2X4R mutants.

Receptor	EC_{50} (μM)	I_{max} (nA)	Protocol 1 (%)	Protocol 2 (%)
WT	2.3 ± 0.4	2.3 ± 0.2	100 ± 14	122 ± 3
V47A	5.2 ± 0.7	2.1 ± 0.2	–	#133 ± 3
V47C	2.3 ± 0.7	2.8 ± 0.5	114 ± 15	199 ± 3*
F48A	2.4 ± 0.3	2.3 ± 0.2	–	99 ± 1*
F48C	2.0 ± 0.9	2.9 ± 0.5	111 ± 13	104 ± 1*
W50A	3.6 ± 0.3	1.9 ± 0.2	–	147 ± 6*
W50C	4.4 ± 0.8	2.2 ± 0.8	100 ± 16	157 ± 5*
E51A	1.6 ± 0.6	1.2 ± 0.2*	–	#41 ± 6*
E51C	3.5 ± 0.5	2.1 ± 0.5	150 ± 20*	134 ± 5
K52A	3.1 ± 1.1	3.0 ± 0.4	–	128 ± 4
K52C	2.4 ± 0.6	2.6 ± 0.4	111 ± 23	132 ± 3
G53A	2.8 ± 0.6	2.9 ± 0.3	–	#121 ± 4
G53C	3.8 ± 0.5	2.2 ± 0.5	100 ± 16	162 ± 3*
E56A	2.0 ± 0.9	1.6 ± 0.2	–	#134 ± 4
E56C	3.9 ± 0.7	1.9 ± 0.2	262 ± 45*	222 ± 18*
T57A	1.9 ± 0.6	2.2 ± 0.3	–	#109 ± 2
T57C	2.0 ± 0.3	1.9 ± 0.2	162 ± 20*	130 ± 3
D58A	3.2 ± 1.3	0.6 ± 0.1*	–	100 ± 1*
D58C	2.3 ± 0.9	0.9 ± 0.1*	84 ± 30	91 ± 2*
S59A	2.9 ± 1.3	2.6 ± 0.4	–	102 ± 2*
S59C	2.0 ± 0.5	2.5 ± 0.3	220 ± 32*	96 ± 1*
V60A	2.4 ± 0.6	2.3 ± 0.4	–	101 ± 1*
V60C	2.1 ± 0.8	2.6 ± 0.5	116 ± 18	100 ± 1*
V61A	2.9 ± 0.4	2.1 ± 0.3	–	116 ± 2
V61C	3.7 ± 1.5	2.5 ± 0.3	166 ± 22*	117 ± 2
K326A	1.7 ± 0.6	1.9 ± 0.3	175 ± 34*	126 ± 2
K326C	2.4 ± 0.6	1.3 ± 0.2		129 ± 2
G328A	2.6 ± 0.9	1.9 ± 0.5	107 ± 16	114 ± 2
G328C	2.7 ± 0.6	2.2 ± 0.3		111 ± 4
K329A	4.9 ± 1.5	1.4 ± 0.1	43 ± 20*	#125 ± 3
K329C	4.0 ± 1.2	1.8 ± 0.3		55 ± 4*
F330A	3.2 ± 1.2	1.5 ± 0.3	100 ± 13	118 ± 2
F330C	4.0 + 1.2	0.8 ± 0.1*		121 ± 2
D331A	1.5 ± 0.2	1.8 ± 0.3		#120 ± 2
D331C	2.1 ± 0.2	2.3 ± 0.4	100 ± 15	64 ± 3*
I332A	1.3 ± 0.3	1.5 ± 0.4	110 ± 21	#100 ± 1*
I332C	1.6 ± 0.3	2.2 ± 0.3		75 ± 2*
I333A	2.9 ± 0.8	1.6 ± 0.1	100 ± 22	#101 ± 1*
I333C	2.8 ± 1.1	1.9 ± 0.3		196 ± 15*
P334A	1.0 ± 0.3*	1.3 ± 0.3	100 ± 35	101 ± 1*
P334C	1.4 ± 0.4	0.6 ± 0.1*		105 ± 1*
T335A	1.8 ± 0.6	2.2 ± 0.2	110 ± 21	#121 ± 2
T335C	2.6 ± 0.5	1.8 ± 0.2		73 ± 2*
M336A	2.5 ± 0.3	2.2 ± 0.5	150 ± 20*	104 ± 2*
M336C	1.2 ± 0.2	2.3 ± 0.5		110 ± 1
I337A	2.9 ± 1.3	2.0 ± 0.4	100 ± 31	#128 ± 3
I337C	4.1 ± 0.6	1.0 ± 0.2*		68 ± 5*
N338A	2.0 ± 0.2	3.5 ± 0.4*	100 ± 25	#125 ± 3
N338C	1.1 ± 0.2	3.2 ± 0.5*		155 ± 4*

The data are expressed as the mean ± SEM from 12 to 40 measurements per mutant and 96 measurements for the wild type (WT) receptor. The statistical significance was estimated by ANOVA followed by post hoc two-way t-test with Bonferroni correction, comparing the level of cadmium effect between WT and mutant receptors ($p < 0.05$) and between alanine and cysteine mutants of the same residue (#$p < 0.05$). Fifth and fourth columns depict the percentage of ATP induced amplitude change elicited by 20 μM cadmium acquired by protocol 1 and protocol 2.*

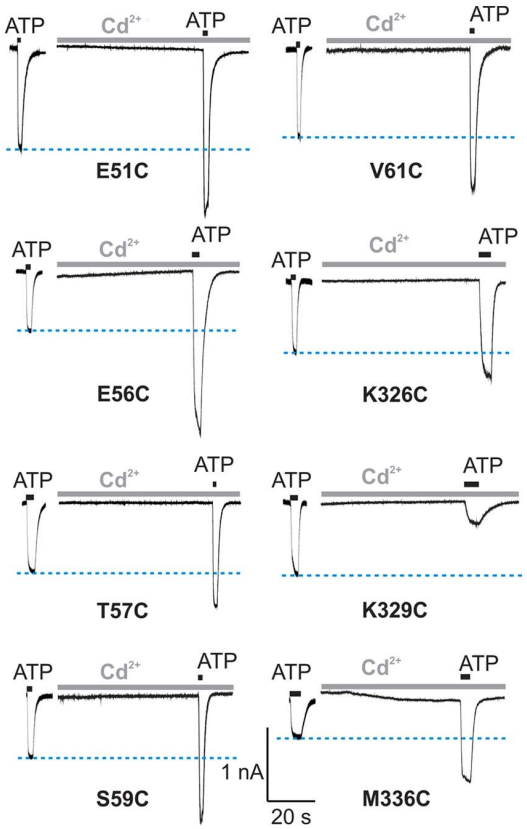

FIGURE 3 | Protocol 1: the effect of cadmium ion pre-application on peak amplitude of current response by mutant rP2X4Rs. The whole-cell current traces represent the effect of 20 μM cadmium pre-applied for 1 min and subsequently co-applied with EC_{50} doses of ATP for 2 s. Horizontal blue lines represent the peak amplitude of currents in cadmium-non-treated cells. Traces shown are representative and mean ± SEM values are shown in **Table 1**. The holding potential was −60 mV.

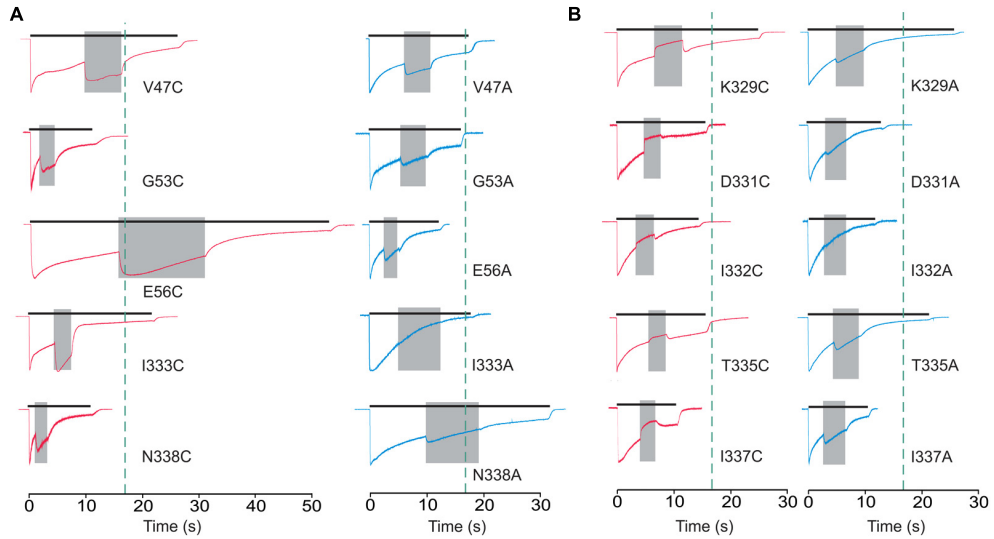

FIGURE 4 | Protocol 2: the effect of cadmium on sustained current response by mutant rP2X4Rs. The whole-cell current recordings from −60 mV represent the effects of 20 μM cadmium transiently co-applied (gray field) with 100 μM ATP. Cadmium application was initiated at 50 ± 10% of current desensitization decay. **(A)** Enhancement of cadmium effects in cysteine mutants (red traces) and the corresponding alanine mutants (blue traces). **(B)** A transient inhibition of sustained current by cadmium (red traces). Right panels show the response of the corresponding alanine mutants. Vertical dotted lines illustrate the duration of ATP application in cells expressing the WT receptor. Traces shown are representative and mean ± SEM values for these mutants are shown in **Table 1**.

MODEL PREDICTION OF THE POSITION OF RESIDUES OF INTEREST

We develop the rP2X4R homology model (see Homology Modeling) to identify the position of residues of the extracellular vestibule in open and closed states. Amino acid residues that have side chains pointing toward the central cavity of the extracellular vestibule when the channel is closed are V47, G53, Q55, T57, D58, S59, V60, V61, K326, A327, G328, P334, I337, and N338. The side chains of F48, V49, W50, K52, Y54, K329, F330, I332, and M336 are pointed exclusively toward the water environment and away from the central axis of the receptor channel. Amino acid residues that could be found at the interface between the interior of the vestibule and the outside include E51, E56, D331, I333, and T335, which comprise the inverted cone-shaped access portal of the receptor and the upper segment of the pore. With exception of D58, all negatively charged residues of the rP2X4R homology model are found within the structure of the portal. The Van der Waals distances between particular extracellular vestibule residues in closed state were found to range between 6.9 and 15 Å (**Figure 1B**, top panel). The closed rP2X4R channel homology model was used to identify residues detected in experiments with protocol-1; residues affected are situated along the central axis of the vestibule (**Figure 5A**).

In contrast to the closed state, the homology model in the open state reveals slightly different distribution patterns of amino acid side chains that are found on the level of the lateral portal. The E51 residue side chain points toward the interior of the vestibule in open state, while the adjacent K52 residue points from the outside toward the level between compartments. The Van der Waals distances between particular extracellular vestibule residues in open state were found to range between 16 and 21.7 Å (**Figure 1B**, bottom panel). The open rP2X4R channel homology model was

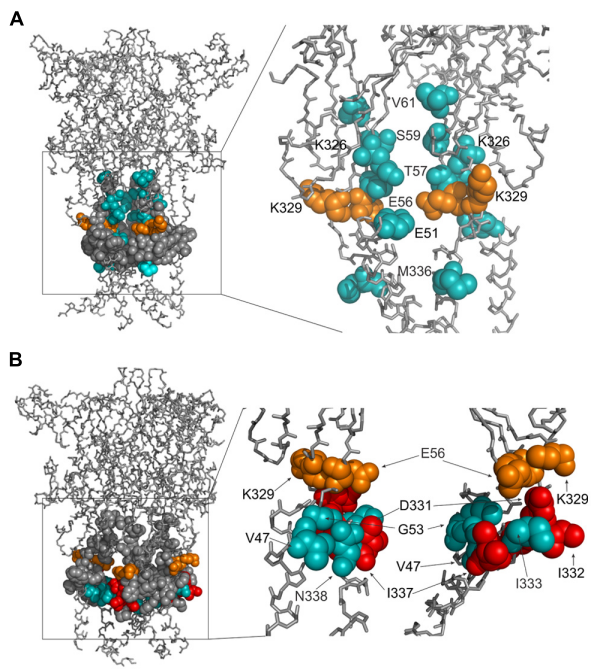

FIGURE 5 | Topology of cadmium-affected residues in the homology model of rP2X4R. Left panel represents a wire frame model of the rP2X4R polypeptide backbone with residues from V47–V61 and K326–N338 (spheres) in closed **(A)** and open **(B)** state. Orange spheres depict the residues in cysteine mutants that were affected by cadmium in both states. Cadmium-inhibited residue mutants are depicted by red spheres and potentiated residue mutants are depicted by blue spheres. On the right panels the front subunit was removed for better visibility of the vestibule interior.

used to identify residues detected in experiments with protocol-2; the residue affected situated at the bottom of the vestibule near pore forming region as an inverted cone-shaped structure (**Figure 5B**).

Homology model of rP2X4R in open and closed state together with cadmium accessibility data has given us an insight on how the ion accesses the extracellular vestibule. Amino acid residues that were predominantly affected during a closed-to-open state transition by cadmium were identified with their side chains pointed toward the central cavity of the extracellular vestibule (T57, K326), and on the level between the extracellular and central vestibules (S59 and V61; **Figure 6A**). M336 was the only residue found outside the central cavity with its side chain facing the extracellular environment on the level of the water-lipid interface (**Figure 6A**). This suggests that the ion uptake is facilitated by peptide segments above TM1 and that ion can pass to the central vestibule before receptor activation. All affected residues during open-desensitized state transition, with the exception of K329, T335, and I332, point their amino acid side chains towards the lumen of the vestibule (**Figure 6B**) indicating that upon ATP binding ion gets channeled toward the upper part of TM2. The K329 and E56 residues are cadmium reactive by both protocols, which indicates their role in an interaction with ions during the transition from closed-to-open-to-desensitized state. These residues are found in close proximity to each other at the entrance point to the vestibule, however, both open and closed states of our rP2X4R homology model and zP2X4.1R do not show the formation of a salt bridge (**Figures 6A,B**).

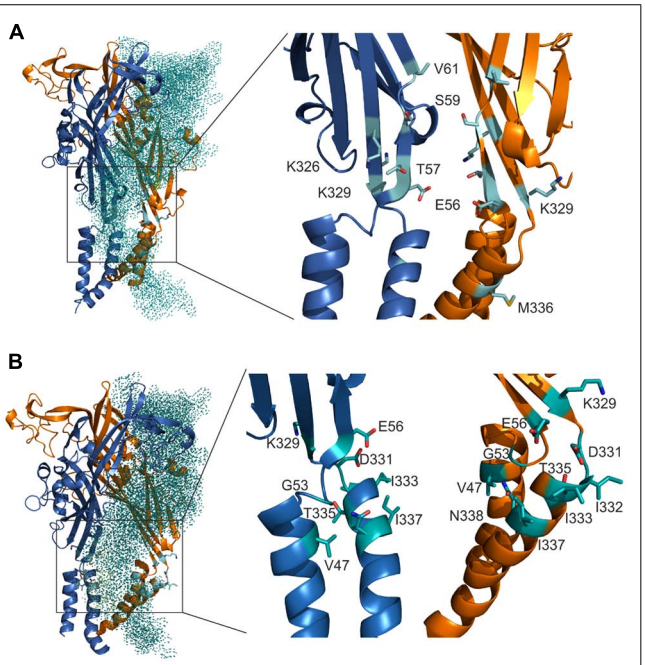

FIGURE 6 | Orientation of the side chain of residues are important for interaction with ions during gating. The positions of cadmium-affected residues are in closed **(A)** and open **(B)** states. In the left panels, the teal colored dotted subunit was removed for better visibility of the vestibule interior. Cadmium-affected residues in rP2X4R the homology model in closed and open states are presented in the right panels. Particular cadmium hits are presented as light blue sticks on light blue ribbon segments, and different subunits are presented in orange and dark blue.

DISCUSSION

This study focuses on the ion accessibility of amino acid residues from the extracellular vestibule of rP2X4R in a closed and open state. As a reporter, we used cadmium ions for reasons stated in the section "Experimental Model." However, cadmium is not an ideal reporter for rP2X4R because it acts as an allosteric modulator of this receptor (Coddou et al., 2005); the extracellular zinc-binding histidine residues appears to serve as cadmium allosteric sites at P2XRs (Coddou et al., 2005; Lorca et al., 2005; Acuna-Castillo et al., 2007). We also observed the facilitatory effect of cadmium on an ATP-induced current when this metal was applied during the ATP pulse. However, this effect was observed in cells treated with 100 μM ATP, a supramaximal ATP concentration for rP2X4R, suggesting that no leftward shift in the concentration response could account for the observed effect. Thus, the most probable explanation for the results shown in **Figure 2B** is that cadmium increases the probability of the open state in the channels.

In general, the substitution of a native residue with cysteine generates an additional allosteric binding site for cadmium if the residue is accessible to this ion (Huber and Freisinger, 2013; Kunihiro et al., 2013). This in turn may or may not affect the ATP-induced current, depending on the position of the residue and protocol used for cadmium application. The affected residue could amplify or attenuate the current, and both effects indicate that the residue is accessible to cadmium, i.e., it represents the cadmium-sensitive hit. Cadmium dissociation kinetics was not studied in P2XRs, but it was addressed in relation to calmodulin and calmodulin fragments and the rate of dissociation was found to be 445 s^{-1} or faster (Martin et al., 1986). However, the kinetics of cadmium alteration of receptor gating was studied in WT and mutant rP2X2Rs and it has been found that cadmium modifies the receptor gating at the rates ranging from 10 to 10^5 $M^{-1}s^{-1}$ (Kracun et al., 2010).

In our experiments, two protocols were used to identify different mutants as cadmium-sensitive. Protocol 1 was designed to avoid the possible impact of the dissociation of cadmium on the ATP response; cadmium was present during 1 min pre-application and 2-s stimulation with 2.3 μM ATP, a time sufficient to initiate the transition from closed to open state and to reach the peak current response but not the decay of the current. The τ_{on} time for cadmium to modulate rP2X2R in an open state was estimated to be approximately 0.5 s (Kracun et al., 2010), which further indicated that a fraction of channels were in an open state for a sufficient time to bind cadmium. Thus, protocol-1 reflects cadmium binding to engineered cysteine residues when the receptor is undergoing a transition from a closed-to-open state. In contrast, protocol-2 clearly detected the residues that are accessible to the ion when the channel is transiting from an open-to-desensitized state.

Protocol-1 revealed that E51, E56, T57, S59, V61, K326, K329, and M336 cysteine mutants have substantial reactivity to cadmium, whereas protocol-2 identified V47, G53, E56, K329,

D331, I332, I333, T335, I337, and N338 cysteine mutants as cadmium-sensitive. Thus, out of the 16 cadmium-sensitive mutants that we observed, only E56C and K329C mutants responded to cadmium application in both protocols. These results clearly indicate different positions (conformation) of the majority of residues during exposure to cadmium in the two protocols. It is also interesting that combined experiments with two protocols suggest that all seven cadmium sensitive V47–V61 mutants show stimulatory cadmium effects, whereas five out of nine K326–N338 affected mutants show inhibitory cadmium effects, suggesting that two segments may play opposite roles in the control of closed-to-open transition.

We have developed the rP2X4R homology model and utilized the model together with available crystal structures to propose a putative ion access mechanism for the rP2X4R pore, while considering current and previous findings of amino acid side chain accessibilities in other P2XRs and the limitations of cadmium accessibility screening imposed by allosterism. Amino acid residues that were affected by cadmium during close-to-open transition were identified within the lumen of the extracellular vestibule and between the extracellular and central vestibule. Amino acid residues affected by cadmium during open to desensitized state transition were identified as the inverted cone shaped structure at the bottom of the vestibule near the pore-forming region. These striking cadmium modification patterns indicate that upon receptor activation, the vestibules widen and the E56 residue begins interacting with the ions and direct them downwards to the gate of the receptor where they interact with D331, I332, I333, T335, I337, and N338 residues.

The topological analysis of residues affected by cadmium during closed-to-open state transition reveals the possibility of ion access to the receptor through the interaction with E51, E56, T57, S59, V61, K326, K329, and M336 residues. Because the E51A mutant shows an inhibition of ATP action by cadmium in protocol-2, we estimated that cadmium could not be used to probe the accessibility of this residue. However, E51 plays a role in calcium permeability (Samways and Egan, 2007), and therefore, the accessibility of this residue in an open state is substantial. Polar non-charged residues T57 and S59 could interact with cadmium through the coordination of cadmium ions by their hydroxyl groups. The role of the hydrophobic V61 residue in ion access is still unclear, but the S59 and V61 residues are found between the middle and extracellular vestibule, which clearly confirms that the ions can access the middle vestibule. Because the channel vestibules can be charged with ions before opening, they may have a role as ion reservoirs that contribute to fast activation kinetics, which was previously described (Yan et al., 2006). The M336 residue, which is crucial for ethanol binding by the rP2X4R (Popova et al., 2010), is found at the lipid-water interface, and its interaction with cadmium in a closed state may affect receptor activation.

The K329 and E56 residues are cadmium-reactive in both protocols, which suggest roles in interactions with ions during a closed-to-open transition. Our data showed prolonged desensitization rates for both residues, which implies that they are functionally coupled. These residues are found in close proximity to each other, but both open and closed states of our rP2X4R homology model and the zP2X4.1R do not show the formation of salt bridges. Notably, most of cadmium hits for probing the open state were found below the K329 and E56 residues along the central channel axis, while most hits found in a predominantly closed state were situated above the K329 and E56 residues. However, the presence and role for a number of positively charged amino acid residues within this region remains unclear because the K52, K326, and K329 residues presented different accessibility potential to cadmium by the protocols that were used. These positively charged residues may play a role in depleting anions from the cation hydration sphere and thus facilitate cation permeability, but their exclusive role as selectivity filters has not been elucidated. Finally, the most prominent facilitatory effect of cadmium was identified in I333 and V47 mutants and our homology model has predicted a spatial proximity of these residues (not shown). This issue was also addressed in rP2X2R where V48 and I328 interaction stabilizes the closed state and facilitates lipid intercalation during channel gating (Rothwell et al., 2014).

Figure 7 summarizes a comparison of our findings from three major studies in this field. The focus in a study with rP2X2R

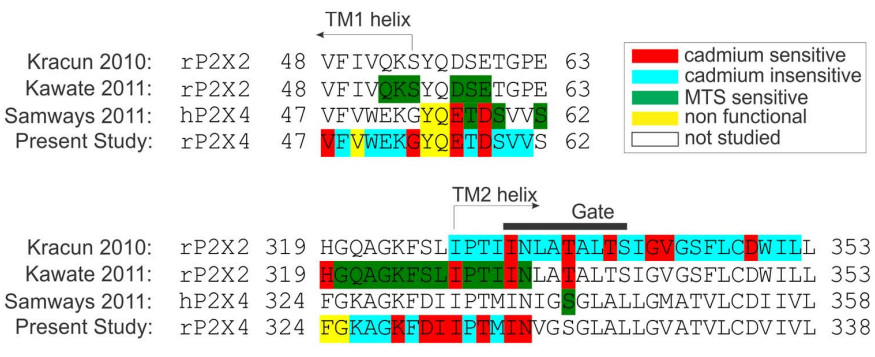

FIGURE 7 | Amino acid sequence alignment of P2XRs used in studies for probing surface accessibility of TM and ectodomain residue side chains in channels in an open state. The alignment represents the comparison of amino acid side chain modifications with cysteine modifying MTS agents (Kawate et al., 2011; Samways et al., 2011) or cadmium (Kracun et al., 2010; Samways et al., 2011) during co-application with supramaximal doses of ATP. The gate region is presented with a black horizontal line above the TM2 domain [derived from (Kracun et al., 2010), while TM helices are marked with arrows. Non-functional rP2X4R mutants were described in Rokic et al. (2013)].

was the I328–L353 sequence, which covered the TM2 region and accompanied five ectodomain residues (Kracun et al., 2010). It was observed that the rP2X2-T336C and I332C mutants had gating modification rates that were the same order of magnitude when cadmium was pre-applied or co-applied, which implies that these residues are fully accessible to cadmium in open and closed states. In contrast to this result, most of the TM2 residue mutants that line the permeation pathway of rP2X2R exhibited a different order of magnitude in the modification rate during the pre-application and co-application of cadmium, which suggests that the residues are accessible only during the open state. This study also helped in defining the gating rP2X2R region (indicated by the horizontal black line in **Figure 7**). Within the gating region, the I332C mutant is identified as cadmium-sensitive, which was confirmed later by (Kawate et al., 2011). We also identified the corresponding rP2X4R mutant (I337C) as cadmium hit; however we could not confirm its accessibility when channel transits from closed-to-open state. The homology model reveals a tight packing of this residue side chain triplet in the rP2X4R, and the possible formation of short distance hydrogen bonds between engineered sulfhydryl side chains at the 337 position (Rajagopal and Vishveshwara, 2005). This indicates that I337 plays a role as a hydrophobic plug.

In our study, but not in other studies (Kracun et al., 2010; Kawate et al., 2011), the N338 mutant was also cadmium-sensitive in an open state. In rP2X2R, the co-application of MTS also affected cysteine substitutions at H319 and I328 residues; the later was also detected in our experiments as a cadmium-sensitive hit. In contrast to rP2X2R, we also identified the K329C, D331C, I332C, and T335C mutants as cadmium-sensitive during the open-to-desensitized transition. Experiments with hP2X4R showed the accessibility of E56C and D58C mutants to cadmium ions during co-application. Furthermore, single channel experiments have shown that these residues do not function as selection filters (Samways and Egan, 2007). In our study, cadmium has shown insignificant effect on both D58C and D58A mutants revealing that this residue does not play a role during gating. Experiments on P2X2R that employed MTS reagents showed no effect of these reagents on analogous residues D57 and E59 (Kawate et al., 2011).

In conclusion, our findings indicate the possibility of ion entry in extracellular vestibule though lateral portals while the rP2X4R channel is closed. We also identified amino acid residues of the extracellular vestibule that interact with ion during closed-open-desensitization transition. The residues above TM1 are predominantly responsible for ion uptake into the extracellular vestibule lumen, whereas TM2 predominantly facilitates access to gate and permeation. These findings provide further insight on rP2X4R gating, which is helpful in understanding common, receptor-specific, and species-specific functions of extracellular vestibule.

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Principles and properties of ion flow in P2X receptors

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P2X receptors are a family of trimeric ion channels that are gated by extracellular adenosine 5′-triphosphate (ATP). These receptors have long been a subject of intense research interest by virtue of their vital role in mediating the rapid and direct effects of extracellular ATP on membrane potential and cytosolic Ca^{2+} concentration, which in turn underpin the ability of ATP to regulate a diverse range of clinically significant physiological functions, including those associated with the cardiovascular, sensory, and immune systems. An important aspect of an ion channel's function is, of course, the means by which it transports ions across the biological membrane. A concerted effort by investigators over the last two decades has culminated in significant advances in our understanding of how P2X receptors conduct the inward flux of Na^+ and Ca^{2+} in response to binding by ATP. However, this work has relied heavily on results from current recordings of P2X receptors altered by site-directed mutagenesis. In the absence of a 3-dimensional channel structure, this prior work provided only a vague and indirect appreciation of the relationship between structure, ion selectivity and flux. The recent publication of the crystal structures for both the closed and open channel conformations of the zebrafish P2X4 receptor has thus proved a significant boon, and has provided an important opportunity to overview the amassed functional data in the context of a working 3-dimensional model of a P2X receptor. In this paper, we will attempt to reconcile the existing functional data regarding ion permeation through P2X receptors with the available crystal structure data, highlighting areas of concordance and discordance as appropriate.

Keywords: P2X, ATP, permeability, selectivity, gating, mutagenesis, SCAM

INTRODUCTION

The P2X receptors are a family of seven (P2X1R–P2X7R) cation permeable ligand-gated ion channels (LGICs) that open in response to binding by the extracellular ligand, adenosine 5′-triphosphate (ATP). In contrast to tetrameric ionotropic glutamate receptors and pentameric Cys-loop receptors, the P2XRs are assembled from three peptide subunits (Nicke et al., 1998; Stoop et al., 1999; Jiang et al., 2003; Barrera et al., 2005). Each subunit is comprised of intracellular amino and carboxyl termini linked via two transmembrane-spanning helices (TM1 and TM2) to a large extracellular ligand-binding domain (Newbolt et al., 1998; Torres et al., 1998a,b). Binding of ATP to a site in the extracelluar domain elicits a global conformational change that ultimately leads to the opening of a pore through which cations freely move into and out of the cell (Baconguis and Gouaux, 2012; Hattori and Gouaux, 2012). With the exception of P2X6R, all the subunits assemble into functional homomeric ion channels. In addition, several heteromeric assemblies have been identified and characterized, including the P2X2/3R functionally expressed in pain-processing neurons (Lewis et al., 1995; Le et al., 1998; Torres et al., 1998c, 1999; King et al., 2000; Aschrafi et al., 2004; Compan et al., 2012). P2XRs exhibit little discrimination between Na^+ and K^+ but, at resting membrane potentials, currents are chiefly carried by movement of Na^+ down its electrochemical gradient and into the cell. The result is membrane depolarization. All P2XRs also conduct Ca^{2+}, with the permeability of Ca^{2+} relative to Na^+ (P_{Ca}/P_{Na}) varying depending on the subunit make-up of the functional channel (Egan and Khakh, 2004). Thus, the two initial consequences of P2XR activation to cellular signaling are a Na^+-mediated depolarization of the plasma membrane, and an increase in the concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$). These two results subsequently influence action potential propagation and affect a myriad of Ca^{2+}-sensitive processes, including secretion (Khakh and Henderson, 2000; Norenberg et al., 2001), muscle contraction (Lamont and Wier, 2002; Brain et al., 2003), and cell survival (for review see Di Virgilio, 2012; Volonte et al., 2012).

Over the last two decades, an exhaustive effort to relate P2XR structure to the function of these channels has provided a good understanding of how ATP transduces current across the plasma membrane. Multiple laboratories have successfully utilized molecular biological techniques and patch clamp electrophysiology to identify amino acids necessary for ligand binding, signal transduction and ion permeation. Nevertheless, the successful crystallization of a zebrafish P2X4.1R, in both the closed

Abbreviations: ATP, adenosine 5′-triphosphate; SCAM, scanning cysteine accessibility method; LGIC, ligand-gated ion channel.

(Kawate et al., 2009; PDB ID 4DW0) and ATP-bound open (Hattori and Gouaux, 2012; PDB ID 4DW1) conformations was a dramatic advance for the field. On the one hand, these structures serve as the basis of a slew of new testable hypotheses about the relationship between P2XR structure and function. On the other, they provide an all-important 3-dimensional template upon which to review and interpret previously obtained functional data. Many of the most important advances are described in numerous reviews (Egan et al., 2006; Khakh and North, 2006; Roberts et al., 2006; Burnstock and Kennedy, 2011; Coddou et al., 2011; Kaczmarek-Hajek et al., 2012; North and Jarvis, 2013). This review focuses entirely on the following two questions: (1) How do ions enter the pore of a P2XR and subsequently transition from one side of the plasma membrane to the other? (2) How do P2XRs discriminate between ions, selecting and permeating some to a greater degree than others? With these questions in mind, the principle objective of this review is to view the data obtained from functional studies over the last two decades within the context of the now available 3-dimensional crystal structures, particularly that of the open channel state, in order to gauge the degree of concordance and potentially identify areas of inconsistency. As a visual aid, a number of figures are included which serve to simply map the results of various systematic functional studies onto the relevant P2XR structure. We have also included a sequence alignment showing examples of human and rat P2XRs for reference (**Figure 1**). Most of the studies investigating ion permeation and selection in this family of ion channels were conducted on the P2X2R and P2X4R. Homology models for these receptors were generated based on the available crystallographic data obtained for the truncated zebrafish P2X4.1R.

EXTRACELLULAR ACCESS TO THE TRANSMEMBRANE CHANNEL PORE

The initial publication of the closed channel structure for the zebrafish P2X4R revealed the presence of three lateral portals, or fenestrations, situated in the extracellular domain proximal to the outer leaflet of the lipid bilayer (Kawate et al., 2009). The diameter of these lateral portals, equal to ∼12Å, is sufficient to allow for the passage of water and fully hydrated ions, and provoked the compelling hypothesis that they might serve as the primary access points for extracellular ions to approach the mouth of the transmembrane pore itself. The closed structure also indicated the presence of three cavities within the extracellular domain of the receptor, which Kawate et al. (2009) designated the upper, central and extracellular vestibules (**Figure 2**). These form a broken chain along the central axis of the receptor, and present an alternative ion conduction hypothesis in which the central pathway widens during gating to form a single pore running the entire length of the protein. Further, Kawate et al. saw a gadolinium ion (Gd^{3+}) coordinated in the central vestibule of zebrafish P2X4.1R, and used patch clamp electrophysiology to show that Gd^{3+} inhibited ATP-gated current. However, it did

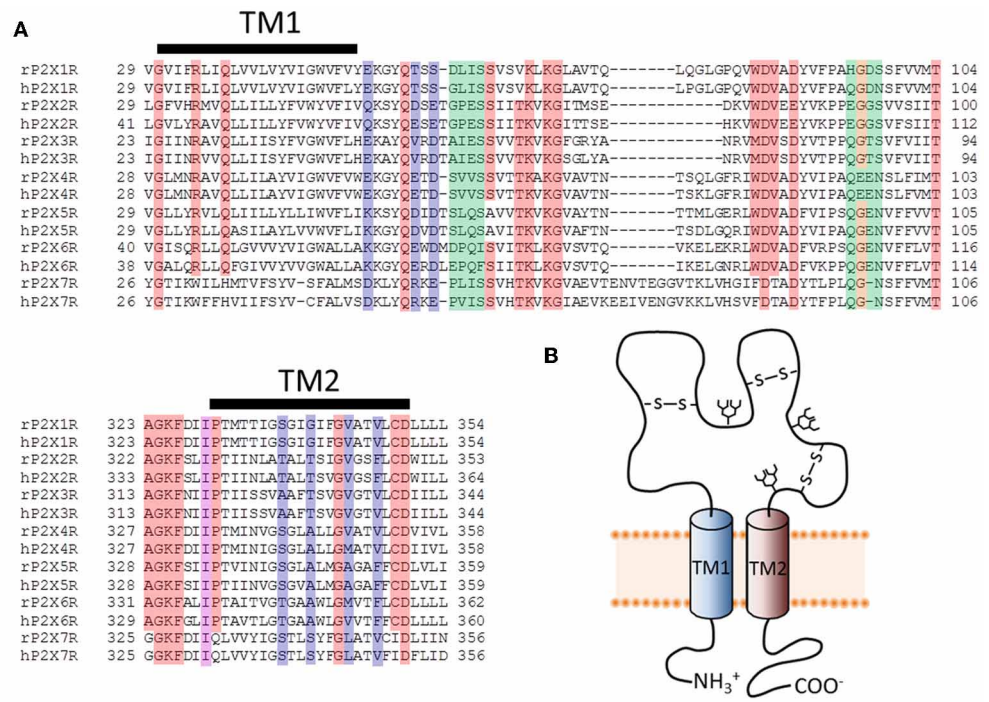

FIGURE 1 | Primary structure of putative pore-forming domains of P2XR subunits. (A) Sequence alignments for select stretches of P2XR subunit polypeptides that include the two transmembrane domains (TM1 and TM2). Both human and rat forms of the P2XRs are shown. Red, orange, and purple boxes indicate highly conserved amino acids that appear in at least six of the seven P2XRs. Blue and purple boxes indicate amino acids likely to form the principal ion permeation pathway of the lateral portals and transmembrane spanning pore. Green and orange boxes indicate residues lining the central and upper vestibules, but which do not appear to form the principal ion permeation pathway. **(B)** Schematic representation of a single P2XR subunit, showing the intracellular amino and carboxyl termini linked by two TM domains and a large ligand-binding extracellular domain.

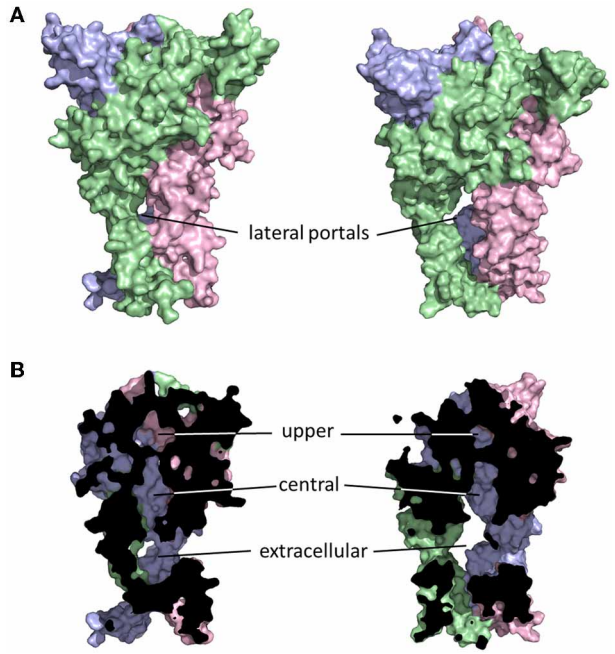

FIGURE 2 | Ion access to the transmembrane pore of a P2XR. Shown are closed (left) and open (right) P2X2R homology models built based on the resolved structures for zebrafish P2X4R (Kawate et al., 2009; Hattori and Gouaux, 2012). Subunits are presented in different colors. The lateral portals are labeled in the complete structure panels presented in **(A)**. The three internal cavities within the extracellular domain of the P2XR are shown in the cutaway models of **(B)**.

so in a manner that was surmountable by increasing the concentration of ATP, suggesting that the inhibition of current was not caused by Gd^{3+} occluding an ionic conduction pathway. Indeed, the fact that current still flowed through the P2X4.1R even when the central vestibule was inhibited by a Gd^{3+} ion suggested that ion entry occurs below this point in the structure. Furthermore, the closed structure shows at least two barriers to ion movement along the central pathway that would have to open in order to allow permeation. The presence of extracellular gates that impede ion flow was not predicted from the results of numerous experiments that used the Scanning Cysteine Accessibility Method (SCAM) to identify differences in solvent accessibility of open and closed channels (Egan et al., 1998; Jiang et al., 2001; Kracun et al., 2010; Li et al., 2010). Indeed, the use of the SCAM in P2X2R and P2X4R revealed that cysteine substitutions introduced into the upper vestibule were not rapidly accessible to modification by thiol-reactive methanethiosulfonate (MTS) compounds (Kawate et al., 2011; Samways et al., 2011). Kawate et al. (2011) produced a double-cysteine mutant P2X2R containing a disulfide bond across the central axis of the central vestibule, which did nothing to impair ion flow. To the contrary, currents through the channel were inhibited rather than potentiated by reduction of the S-S bond with dithiothreitol (DTT) suggesting that while movement in this region might be important for gating, it is highly unlikely that it contributes to the ion conductions pathway.

The lateral pore hypothesis was largely validated by the publication of the ATP-bound open channel structure of zfP2X4R (Hattori and Gouaux, 2012). As the rat P2X2R homology model in **Figure 2** suggests, these lateral portals expand during gating, in agreement with predictions made on the basis of functional experiments (Kawate et al., 2011; Samways et al., 2011). In the open structure state, the upper vestibule seems to remain isolated from the bulk solution, but the central and extracellular vestibules appear to merge to form a single large inner cavity that extends into the opened transmembrane ion conducting pathway. The merging of the central and extracellular vestibules concords with functional data, with cysteine substitutions introduced at positions within the central vestibule in P2X1R, P2X2R, and P2X4R being accessible to water-soluble thiol-reactive agents in the open state. Specifically, currents through P2X1R-G60C, P2X2R-I317C, and P2X2R-H319C, and P2X4R-S62C and P2X4R-N97C, were all modified by the positively charged thiol-reactive MTS reagent, $MTSET^+$ (Kawate et al., 2011; Samways et al., 2011). However, the currents through four of these mutants, P2X1R-G60C, P2X2R-H319C, P2X4R-E56C, and P2X4R-D58C, were potentiated rather than suppressed by $MTSET^+$ modification.

The central vestibule has a negative electrostatic surface potential (Kawate et al., 2009, 2011). It also shows some charge discrimination, as seen in its ability to discriminate between cationic and anionic MTS compounds. Brief 3 s exposure to the anionic MTS reagent, $MTSES^-$, had no effect on currents mediated by P2X4R-S62C and P2X4R-N97C, and more importantly did not prevent subsequent $MTSET^+$ exposure from potentiating currents (Samways et al., 2011), indicating that only the positively charged molecule could access and modify these sites. On the other hand, much longer applications (∼60 s) were sufficient for $MTSES^-$ to modify cysteines introduced in the same region of P2X2R (Jiang et al., 2010). Nevertheless, $MTSES^-$ can readily and rapidly modify cysteines introduced intracellular to this region within the lateral portals and as far down into the transmembrane pore as positions Ser^{341} in P2X4R (Samways et al., 2011) and Thr^{336} in P2X2R (Rassendren et al., 1997), adding weight to the idea that thiol-reactive agents enter the extracellular vestibule through the lateral portals.

The possibility that P2XRs might inhabit conformational states in which ions can intrude into the central pathway cannot be completely ruled out. SCAM studies of P2X1R showed that longer (5–60 min) incubations with thiol-reactive reagents were sufficient to cause modification of upper vestibule positions (Allsopp et al., 2011; Roberts et al., 2012a), indicating that these areas are not completely sealed from the surrounding aqueous environment. Based on free energy calculations and the closed zebrafish P2X4.1R crystal structure, Kawate et al. (2011) estimated that a modest widening of the central axis pore during gating could feasibly provide a sufficient permeation pathway favorable for Na^+ conduction, but found that this was not so for the P2X2R homology model due to differences in primary amino acid sequence within this region of the receptor. Along similar lines, Roberts et al. (2012a,b), showed that the cysteines introduced into the upper vestibule of P2X1R were more easily accessed and modified by N-Biotinoylaminoethyl-MTSEA in the absence of ATP than in the presence, suggesting that, although

this region might be weakly accommodating of water and ions in the closed state, it becomes much less so in the open state, further ruling out this region as a key ion conducting pathway in the open channel. Thus, the lateral portals remain the most obvious route of entry for ions based on both the available crystallographic data and results from functional studies.

The relatively large size of the lateral portals in the open state crystal structure has invited some surprise, particularly due to the degree to which these portals appear to invade the lipid bilayer, and the seeming lack of contact between intersubunit helices (**Figure 2**). There would appear to be little to prevent the intrusion of lipids into the ion conducting pathway, and this contention is supported in a recent study from Heymann et al. (2013). Here, molecular dynamics simulations of the open P2X4R incorporated into a lipid bilayer revealed rapid intrusion of lipid molecules into the permeation pathway, resulting in a hydrophobic barrier to ion permeation that was complete within less than 80 ns. The authors noted that a minor reorientation of the TM2 domains was sufficient to produce an open state model that retained a stable water soluble pathway through the lipid bilayer, without producing a conformation at odds with previously published functional data.

Direct functional evidence for ion entry through the lateral portals has been shown by several groups utilizing SCAM. In P2X4R, cysteines introduced at positions Glu^{56} and Asp^{58} lining the lateral portals were found to be accessible to modification by $MTSET^{+}$, which significantly inhibited transmembrane currents in these mutants in a manner that was only reversed by exposure to the reducing agent, DTT (Samways et al., 2011). This inhibition of current was observed whether ATP was present during modification or not, implying that thiol-reactive reagents can penetrate these lateral portals even in the closed state. A cysteine substituted at position Thr^{57} of P2X1R, a lateral portal-lining residue analogous to Glu^{56} in P2X4R, was likewise found to be accessible to the large thiol-reactive molecule, N-Biotinoylaminoethyl-MTSEA, in both the closed and open states (Roberts et al., 2012a). Curiously, MTS-modification of cysteines introduced at positions Thr^{57} and Ser^{59} in P2X1R had no significant functional effect on the ATP-gated current amplitude (Allsopp et al., 2011), and the same lack of effect was reported for the analogous experiments in P2X2R (Kawate et al., 2011). Nevertheless, cysteines introduced at other lateral portal-lining positions in P2X1R and P2X2R have been shown to be accessible to and modified by thiol-reactive agents, including Gly^{60}, Gly^{321}, and Ile^{328} of P2X1R (Roberts and Evans, 2007; Allsopp et al., 2011), and Lys^{53}, Ser^{54}, Leu^{327}, and Ile^{328} of P2X2R (Rassendren et al., 1997; Egan et al., 1998; Haines et al., 2001). Based on the P2X2R homology model, Ile^{328} lies between the extracellular vestibules and the lateral portals and, in agreement with the suggestion from the available crystal structural that the lateral portals enlarge substantially during gating, the mutant receptor P2X2–I328C was only modified by the large bulky thiol-reactive agent, Texas Red-MTSEA, when the channel was in the open state (Kawate et al., 2011).

The relatively large size of the lateral portals in the closed state (~12Å) suggests that they do not form an appreciable barrier to permeation (Kawate et al., 2009), a fact supported by the ability of thiol-reactive agents to access and modify cysteines introduced as deep down into the TM domain as Thr^{336} (P2X2R) even when the channel is closed (Li et al., 2010). As a result, hydrated ions and water can likely diffuse freely between the bulk solution and the extracellular vestibule regardless of whether the P2XR is gated by ATP or not. When the ATP-bound P2XR opens, the ions can then immediately enter the external mouth of the channel pore and begin their journey across the opened transmembrane pore inwards toward the cytosolic space.

TRANSMEMBRANE ION CONDUCTION PATHWAY

By necessity, actual ion conduction across the plasma membrane proper must involve the parts of a channel protein embedded in the lipid bilayer. For most ion channels, part or all of the permeation pathway is lined by fully transmembrane spanning α-helices, with a subset of ion channels having an additional re-entrant pore loop dipping into the membrane from the extra- or intracellular side (MacKinnon, 2003). Although the initial cloning and sequence analysis of a P2XR suggested the presence of a re-entrant pore loop just extracellular to TM2 (Brake et al., 1994; Valera et al., 1995), subsequent functional studies, and now the available crystal structures, clearly demonstrate that this is not the case. The conventional wisdom has been that more than three TM helices are required in order to assemble a transmembrane pore of sufficient diameter to allow passage of ions (Spencer et al., 2002). Given that each P2XR subunit only has two putative transmembrane domains, there was a distinct possibility that both the TM1 and TM2 domains might to some extent contribute to the pore. This is in contrast to the tetra- and pentameric LGICs, the ionotropic glutamate receptors and Cys-loop channels, in which each of the four or five subunits is only required to contribute a single α-helical TM domain to the formation of permeation pathway (Keramidas et al., 2004; Traynelis et al., 2010). Over a decade prior to the successful crystallization and structural resolution of the zfP2XR, significant progress was made in determining the approximate role of these TM domains in ion conduction through this family of ion channels. Once the approximate amino acid sequences contributing to the transmembrane domains were identified (Newbolt et al., 1998; Torres et al., 1998b), SCAM was employed to identify the specific parts of these domains likely to contribute to the lining of the ion conduction pathway.

It is of particular importance to ascertain the degree of concordance between functional studies conducted on TM mutant P2XRs and the 3-dimensional crystal structures for two reasons. First, it is recognized that the lipid bilayer plays an important role in the packing and stability of membrane-spanning proteins, and so the absence of lipid in the crystal structures may have resulted in an abnormal arrangement of the TM domain helices relative to the P2XR in its native environment (see Zhou and Cross, 2013; Heymann et al., 2013). Second, there was a practical necessity to use a truncated version of the zfP2X4R.1 that lacked the cytoplasmic termini for crystallization (Kawate et al., 2009). Functional experiments suggest that the intracellular domains are of considerable importance to the normal functioning of P2XRs, and so it is important to confirm that their removal has not unduly affected the normal arrangement of the TM domains of these proteins within the lipid bilayer (Boue-Grabot et al., 2000; Ennion and Evans, 2002; Yan et al., 2008; Nicke et al., 2009; Roberts et al.,

2012b; and see Costa-Junior et al., 2011). This said, as we believe will be clear from the following discussion, the available functional data actually correlates relatively well with the available crystal structure data.

LIMITED CONTRIBUTION OF TM1

If a part of TM1 lines the ion conduction pathway, then we would predict that cysteine substitutions introduced into at least some of the positions in this domain would be rapidly accessed and modified by water-soluble thiol-reactive agents when the channel is in the ATP-bound open state. Cysteines introduced at five positions in and near the TM1 domain of P2X2R, His^{33}, Arg^{34}, Ile^{50}, Lys^{53}, and Ser^{54}, produced mutant receptors with currents sensitive to modification by short, 5 s co-applications of Ag^+ and ATP (Haines et al., 2001) (**Figure 3**). Ag^+ can coordinate thiol-groups, and its small size allows it to penetrate into small, potentially pore-lining, cavities (Lu and Miller, 1995). These side chains are located on the intra- and extracellular extremes of TM1, with Lys^{53} and Ser^{54} actually residing outside the plain of the plasma membrane, and provides little evidence that TM1 contributes significantly to the transmembrane permeation pathway proper. That cysteines introduced into TM1 are not rapidly accessible to thiol-reactive agents was later confirmed by a subsequent study which failed to observe open channel current modification in all but one mutant, P2X2R-V48C, in response to a brief 10 s exposure to the larger thiol-reactive agent, $MTSET^+$ (Li et al., 2010) (**Figure 3**, green).

Nevertheless, although the TM1 domains do not have a primary role in forming the ion conduction pathway, they do not appear to be sealed from the water-soluble pore completely, because longer exposures to thiol-reactive agents were found to be sufficient to uncover a few additional hits within this region. Cysteines substituted into P2X2R positions Gly^{30}, Gln^{37}, Tyr^{43}, and Phe^{44} produced mutants with currents modified by a minute long exposure to thiol-reactive agents (Jiang et al., 2001; Samways et al., 2008a) (**Figure 3**, cyan). The limited role of the TM1 domain in forming the narrow part of the transmembrane ion conduction path has largely been confirmed by the open channel P2XR crystal structure, which as we explain next is mostly formed by TM2 helices. That said, the extracellular extremes of TM1, including Val^{48}, do appear to line the wider part of the extracellular pore opening. Indeed, from the structure it is possible to imagine how cysteines introduced at positions Tyr^{43} and Phe^{44} might have some limited accessibility to water-soluble thiol-reactive agents. These residues are located at the base the enlarged lateral portal in the open state and, although positioned far from the central axis of the conduction pathway, they may be near or possibly in contact with the water-filled cavity (**Figure 3**, but also see the refined P2X2R model of Heymann et al., 2013).

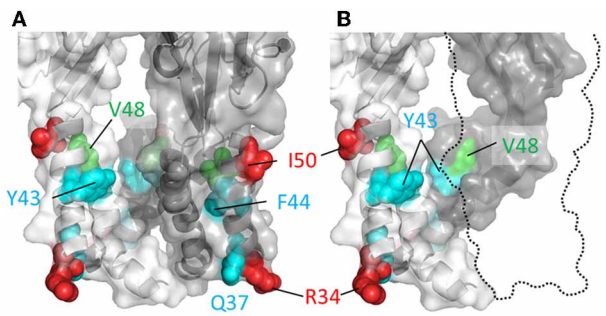

FIGURE 3 | Mapping P2X2R TM1 side-chains accessible to water-soluble thiol-reactive agents using SCAM. TM domain regions shown with all subunits present **(A)** or the third subunit omitted **(B)**. Side chains at which cysteine substitution renders the mutant receptor sensitive to current modification by thiol-reactive agents are shown as ball models. Color coordination is as follows: Red, mutant currents modified by brief 5 s application of Ag^+ only; Green, mutant currents modified by brief 10 s application to $MTSET^+$; Cyan, mutant currents modified by prolonged (>1 min) exposure to thiol-reactive reagents.

ROLE OF TM2

The TM2 domain was the initial focus of attempts to define the transmembrane ion permeation pathway for P2XRs, because early sequence analysis suggested that this region might connect with an extracellular re-entrant pore loop, not unlike that found in potassium channels (Brake et al., 1994; Valera et al., 1994). Although subsequent functional studies did not provide supporting evidence for the pore-loop hypothesis, systematic probing of the TM2 domain using SCAM presented highly compelling evidence that this was the primary pore-forming part of the P2XR subunit, which concords completely with the available crystal structures (Kawate et al., 2009; Hattori and Gouaux, 2012). In contrast to the TM1 of P2X2R, SCAM uncovered numerous hits along the length of TM2, including contiguous stretches that were confusing due to being seemingly inconsistent with this domain possessing a static helical structure (Rassendren et al., 1997; Egan et al., 1998) (**Figure 4**). Indeed, cysteines introduced at every single side chain between Thr^{336} and Phe^{346} have been reported to be accessible to modification by thiol-reactive compounds by one study or more (Rassendren et al., 1997; Egan et al., 1998; Li et al., 2008, 2010). However, the size of the thiol-reactive agent used and/or long durations of exposure could lead to thiol-modification of side chains that are either located in small protein cavities removed from the permeation pathway, or that are only accessible in rarely visited structural conformations.

Rassendren et al. (1997), tested the effects of three thiol-reactive agents, $MTSEA^+$, $MTSET^+$ and $MTSES^-$ on current elicited through mutant P2X2Rs containing cysteine substitutions within TM2. Using an experimental approach in which the thiol-reactive reagents were constantly present during pulsatile ATP applications, and thus exposed to the open and closed channel conformations, it was found that >30 s long applications of $MTSEA^+$ significantly modified currents through the mutant receptors, I328C, N333C, T336C, L338C, S340C, G342C, and D349C. With the exception of S340C and G342C, in which currents were potentiated by $MTSEA^+$, the effect of $MTSEA^+$ was inhibitory. Only three of these mutants, I328C, N333C, and T336C, also showed sensitivity to $MTSET^+$ and $MTSES^-$, both of which evoked inhibitory effects on channel current in each mutant (**Figure 4**, blue and cyan). In a later study, the acute effects of a 10 s application of $MTSET^+$ on open channel currents through TM2 cysteine mutants confirmed that I328C and

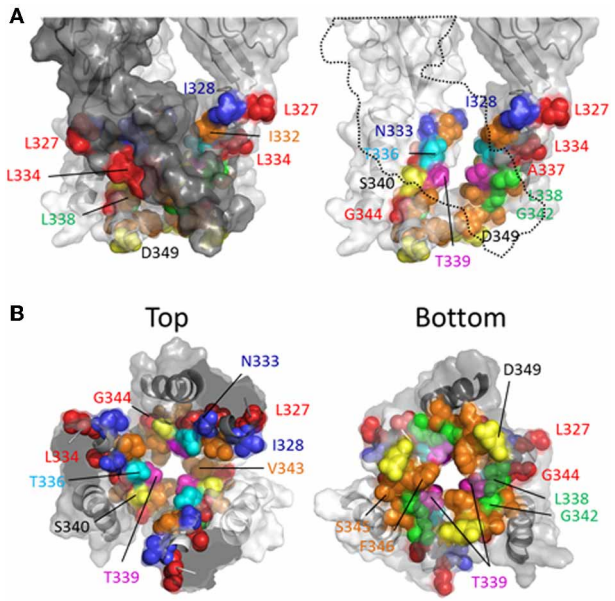

FIGURE 4 | Mapping P2X2R TM2 side-chains accessible to water-soluble thiol-reactive agents in the open state using SCAM. Side chains at which cysteine substitution renders the mutant receptor sensitive to current modification by thiol-reactive agents applied in the open channel state are shown as ball models. **(A)** Side view of TM domains shown with all subunits present (left panel) or the third subunit omitted for clarity (right panel). **(B)** TM domains viewed from extracellular (left panel) and intracellular perspectives (right panel). Color coordination is as follows: Red, mutant currents modified by Ag^+ only; Yellow, mutant currents modified by $MTSEA^+$ only; Orange, mutant currents modified by $MTSET^+$ and Ag^+; Green, mutant currents modified by $MTSEA^+$ and Ag^+; Blue, mutant currents modified by Ag^+, $MTSEA^+$, $MTSET^+$, and $MTSES^-$; Cyan, mutant current modified by Ag^+, $MTSET^+$, $MTSES^-$, MTS-TPAE$^+$, and Texas Red-$MTSEA^+$; Magenta, Ag^+, $MTSEA^+$, $MTSET^+$, MTS-TPAE, but not Texas Red-$MTSEA^+$ or $MTSES^-$.

T336C were readily accessible to this thiol-reactive reagent (Li et al., 2008). This latter study identified several additional positions that, upon cysteine substitution, were readily accessible to $MTSET^+$ in the open state within this short time frame, Ile^{332}, Thr^{339}, Ile^{341}, Val^{343}, Ser^{345}, and Phe^{346}. Consistent with the identification of Gly^{342}, Ser^{345}, and Asp^{349} in P2X2R as facing the aqueous environment, the substitution of cysteines for the analogous residues of P2X7R, Gly^{345}, Thr^{348}, and Asp^{352}, also yielded mutants accessible to thiol-modification (Browne et al., 2013).

Investigating the stretch of TM2 between Leu^{327} and Met^{356} in P2X2R, the Egan laboratory (Egan et al., 1998) probed the accessibility of cysteines substituted into TM2 with Ag^+, focusing on the open state of the channel in the presence of ATP. They found that ATP-gated currents through the following P2X2R mutants were rapidly modified by Ag^+ (<5 s): L327C, I328C, N333C, L334C, T336C, A337C, L338C, T339C, G342C, V343C, G344C, S345C, L352C, and L353C. Fast Ag^+ modification of currents mediated by N333C, T336C, A337C, T339C, V343C, and S345C were also observed in another laboratory, in addition to hits for I332C, A335C, I341C, and F346C (Li et al., 2008, 2010). It is perhaps not surprising that Ag^+ could access and modify so many more side chains, as this relatively small ion can potentially enter narrow gaps between protein interfaces that are otherwise inaccessible to the larger thiol-reactive reagents such as $MTSET^+$. We must, of course acknowledge the caveat of using SCAM that a lack of effect of a thiol-reactive agent on the current evoked through a mutant bearing an introduced cysteine is not necessarily evidence that the side chain is inaccessible to that thiol-reactive agent. Nevertheless, mapping the various hits for the different thiol-reactive agents presents a convenient pattern that seems mostly consistent with the crystal structure data (**Figure 4**). Thus, the three mutant P2X2Rs with currents modified by Ag^+, $MTSEA^+$, $MTSET^+$, and $MTSES^-$, are I328C, N333C, and T336C, which line the wide outer part of the transmembrane pore (**Figure 4**). The side chain of Thr^{336} is also accessible to the very large thiol-reactive agents 2-tripentylaminoethyl MTS (MTS-TPAE$^+$) and Texas Red-$MTSEA^+$ (Li et al., 2010). In contrast, substituting a cysteine for Thr^{339} (**Figure 4**, magenta) renders channel currents sensitive to Ag^+, $MTSEA^+$, $MTSET^+$, and MTS-TPAE$^+$, but not $MTSES^-$ and Texas Red-$MTSEA^+$ (Rassendren et al., 1997; Li et al., 2010), suggesting that, consistent with the open channel crystal structure, the pore narrows at this point. Cysteines substituted for Leu^{338} and Gly^{342} rendered currents sensitive only to the small thiol-reactive agents Ag^+ and $MTSEA^+$ (**Figure 4**, green), which fits with their off-axis orientation toward the narrow opening between intersubunit TM2 domains. Leu^{334}, Ala^{337} and Gly^{344} are located in tight gaps forming the interfaces between transmembrane helices, and so it perhaps makes sense that cysteines substituted at these positions render mutant currents sensitive only to Ag^+ (**Figure 4**, red).

It is interesting that thiol-modification of S340C and G342C potentiates rather than inhibits ATP-gated currents through these mutant P2X2Rs. A similar observation was made for G342C when Cd^{2+} was used as the thiol-reactive probe (Kracun et al., 2010). Both side chains are positioned slightly off the pore axis in the open crystal structure, so it is conceivable that introduction of a covalent or coordinate bonded molecule here does not obstruct the ion permeation pathway, but instead might disrupt the normal gating equilibrium of the ligand-bound receptor. In P2X7R, substitution of Gly^{345} (analogous to Gly^{342}, P2X2R) produced a mutant with ATP-gated currents that were modestly inhibited by $MTSEA^+$ and MTSEA-biotin applied in the open state (Browne et al., 2013), possibly indicative of subtle differences in TM domain configuration between P2XRs.

More recent studies have employed SCAM in combination with Cd^{2+} as the thiol-reactive probe. Cd^{2+} ions can form coordinate interactions with Cys and His side chains (Kurz et al., 1995; Krovetz et al., 1997; Liu et al., 1997; Holmgren et al., 1998). Ion channel mutants with Cys substituted at positions that orientate toward the central pore axis can potentially coordinate permeating Cd^{2+}, leading to current block. This can assist in identifying side chains forming the narrow regions of the transmembrane pore. Two studies identified TM2 positions at which cysteine substitutions rendered the resulting P2X2R mutant sensitive to Cd^{2+} block: I332C, T336C, T339C, G342C, V343C, D349C, and L353C (Kracun et al., 2010; Li et al., 2010), although there were some discrepancies. The mutant F346C was non-functional in Kracun et al's study (Kracun et al., 2010), whereas Li et al. successfully

recorded currents from this receptor and demonstrated Cd^{2+} sensitivity (Li et al., 2010). Li et al. also reported Cd^{2+} sensitivity in the S345C mutant, which was not found to be inhibited by Cd^{2+} in the other study. Finally, Kracun et al. identified only one mutant that was irreversibly inhibited by Cd^{2+}, D349C, whereas Li et al., observed irreversible Cd^{2+} block in the mutants, V343C, S345C, and F346C. Minor discrepancies aside, the combined observations of Kracun et al. and Li et al. are largely consistent with the open channel crystal structure (**Figure 5**). The sulfhydryl groups of neighboring Cys side chains must be ∼5Å apart in order to be bridged effectively by Cd^{2+} (the coordinate bond formed between Cd^{2+} and each Cys side chain being ∼2.5Å) (Dokmanic et al., 2008). Taking into account the length of the Cys side chain, ∼3Å, one would predict that the backbone α-carbons of amino acid positions in which Cys substitution would favor coordination of Cd^{2+} in the central axis of the pore would need to be within ∼10Å of each other. The closed P2X2R homology model shows the distance between the intersubunit α-carbons of position Val^{343} (∼17Å) and Phe^{346} (∼24Å) to be excessive for Cd^{2+} bridge formation. However, as the channel gates into the open conformation, these side chains come into much closer proximity, as predicted by Li et al. (2010). For Val^{343}, the distance between α-carbons in the P2X2R homology models closes to ∼12Å, and for Phe^{346} the distance closes to ∼14Å (see **Figure 7**, pink). This is still not quite optimal for Cd^{2+} bridge formation, but one must consider the limitations of homology modeling, potential inaccuracies in TM arrangement as represented by the open state crystal structure (Heymann et al., 2013), and the possibility that the P2X receptor can visit open state conformations in which these side chains come into closer proximity (Kwan et al., 2012).

The α-carbons at positions Ser^{345} and Asp^{349} are positioned relatively distant from their intersubunit counterparts even in the open channel pore, at 21 and 19Å, respectively (**Figure 5**). However, in the case of these mutants, additional experiments conducted by Li et al. and Kracun et al., respectively, showed that Cd^{2+} coordination here might occur removed from the central pore axis, and involve additional native side chains present in the transmembrane domains. Thus, Cd^{2+} block of D349C was abolished when the native Cys^{348} adjacent to Asp^{349} was mutated to threonine (Kracun et al., 2010) (see **Figure 1**), whereas Cd^{2+} block of S345C was abolished by substitution of the nearby His^{33} of TM1 with tyrosine (Li et al., 2010). The effects of Cd^{2+} at these mutants, then, is not due to direct, steric blockade of ion flux due to Cd^{2+} occupying the channel pore, but may be due to an effect on channel gating.

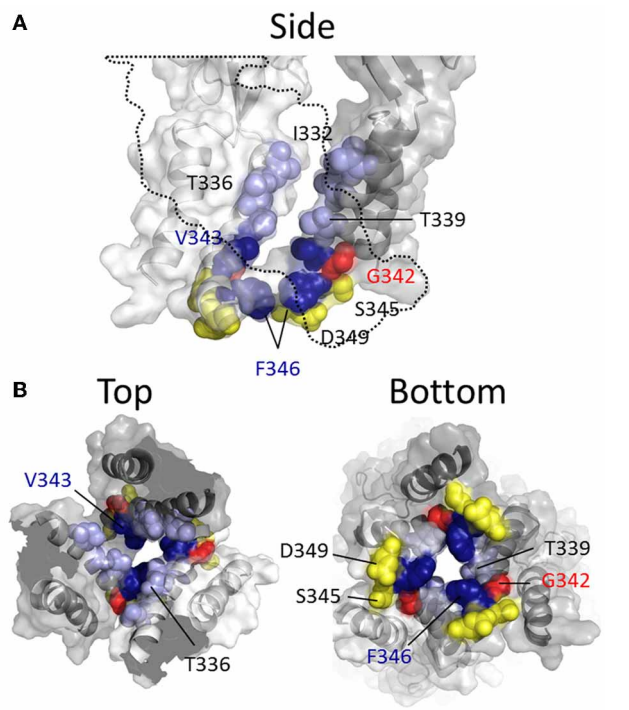

FIGURE 5 | Mapping P2X2R TM2 substituted cysteine side chains capable of coordinating Cd^{2+} in the open channel state. Side chains at which cysteine substitution renders the mutant receptor sensitive to current modification by Cd^{2+} applied in the presence of ATP are shown as ball models. **(A)** Side view of TM domains with third subunit omitted for clarity (dotted line). **(B)** TM domains viewed from extracellular (left panel) and intracellular perspectives (right panel). Color coordination is as follows: Light blue, mutant currents reversibly inhibited by Cd^{2+}; Dark blue, mutant currents irreversibly inhibited by Cd^{2+}; Yellow, mutant currents irreversibly modified due to Cd^{2+} coordination between introduced Cys side chain and native amino acid side chains; Red, mutant currents reversibly potentiated by Cd^{2+}.

Closed state specific SCAM hits

Thiol-reactive agents have also been used to probe the accessibility of TM2 substituted cysteines specifically in the closed channel state of P2X2R. This involved exposure of mutants to thiol-reactive agents in the absence of ATP, and then comparing the amplitudes of subsequent ATP-gated currents to control currents evoked prior to MTS exposure. In early studies, two laboratories reported that currents through the following mutants were observed to be altered by prolonged exposure to $MTSEA^{+}$ applied in the absence of ATP: I328C, N333C, L334C, L338C, T336C, T339C, L338C, G342C, S345C, and D349C (Rassendren et al., 1997; Egan et al., 1998) (**Figure 6**). However, Egan et al., observed that inclusion of free cysteine in the patch pipette solution, thereby sequestering intracellular $MTSEA^{+}$, was found to prevent thiol-modification of currents evoked through the mutants, L334C, L338C, T339C, G342C, and S345C, suggesting that $MTSEA^{+}$ accesses these side chains from the cytosol by first passively diffusing through the lipid bilayer in the uncharged state (Rassendren et al., 1997; Egan et al., 1998). Consistent with this, a later study also conducted in P2XR cysteine mutants revealed that the rate of current modification by Ag^{+} and $MTSET^{+}$ was markedly reduced in the closed channel state for the mutants T336C and T339C (Li et al., 2008). In contrast, I328C and I332C were as rapidly modified by Ag^{+} and $MTSET^{+}$ in the closed as in the open state of the channel. I332C was also found to be accessible to Cd^{+} modification in the closed channel state, as was the mutant T336C (Kracun et al., 2010) (**Figure 6**, green and blue). That I328C currents are modified is not surprising given their apparent location near to the lateral portals, which are wide in both the closed and open channel state. That positions Leu^{338}, Thr^{339}, Gly^{342}, and Ser^{345} are accessible to intracellular MTS reagents is mostly consistent with the closed channel crystal

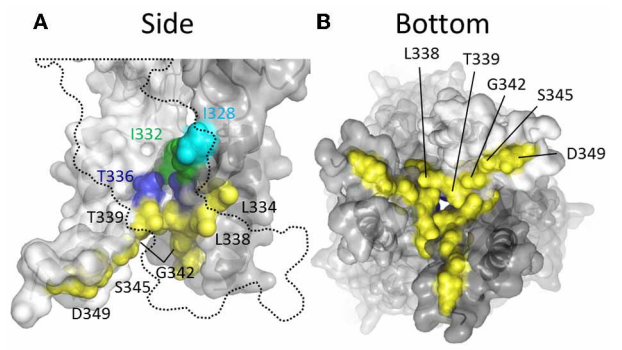

FIGURE 6 | Mapping P2X2R TM2 side-chains accessible to water-soluble thiol-reactive agents in the closed state using SCAM. **(A)** Side view of TM domains. Two subunits are shown with the third omitted for clarity (dotted outline). **(B)** TM domains viewed from the intracellular side of the membrane. The colored residues indicate positions at which substitution with cysteine produces a mutant that mediates currents sensitive to modification by thiol-reactive agents applied in the closed channel state. Color coordination is as follows: Cyan, mutant currents modified by external $MTSEA^+$ and $MTSET^+$ applied in the closed state; Green, mutant currents modified by external Ag^+, Cd^{2+}, and $MTSET^+$ applied in the closed state; Blue, mutant currents modified by external Cd^{2+} applied in the closed state; Yellow, mutant currents modified by intracellular $MTSEA^+$ applied in the closed state.

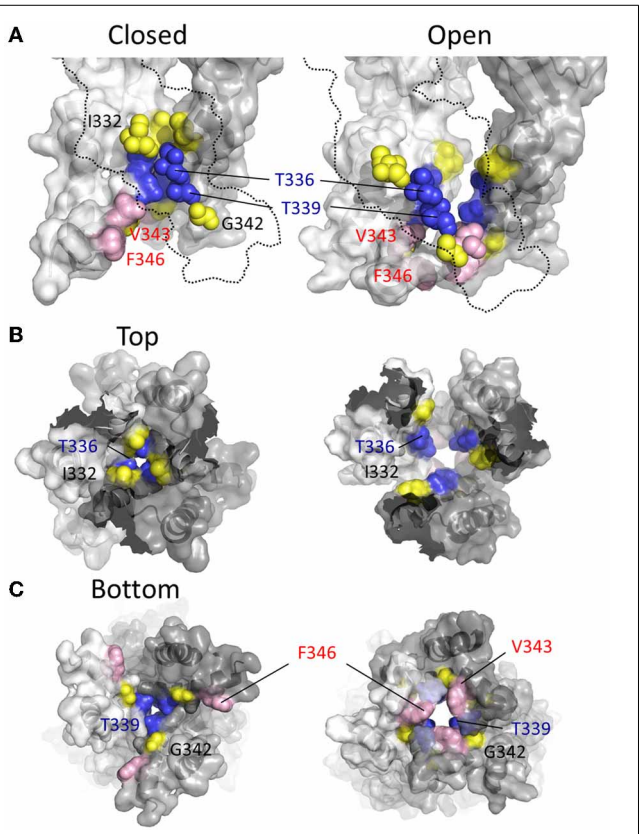

FIGURE 7 | Location of channel gate in P2X2R. **(A)** Side views of TM domains in closed (left panel) and open (right panel) conformation. Residues near and within the channel gate are shown in blue and yellow for all three subunits, with the remainder of the missing subunit outlined (dotted line). The blue residues highlight the putative channel gate. Residues immediately flanking this region are shown in yellow. Val^{343} and Phe^{346}, which converge upon the permeation pathway in the open state, are shown in pink. **(B)** TM domains viewed from the extracellular vestibule in the closed and open configurations. **(C)** TM domains viewed from the intracellular side in the closed and open configurations.

structure, in which these positions can be seen to be exposed to the cytosol within the inverted cone opening out from the putative channel gate (**Figure 6**, yellow).

LOCATION OF THE CHANNEL GATE

SCAM studies conducted prior to the publication of the closed state crystal structure for the P2XR revealed divergent hypotheses on the exact position of the channel gate in members of the ion channel family. Egan et al. (1998) observed that only intracellular $MTSEA^+$ could access a Cys introduced at position Leu^{334} of P2X2R in the closed state, implying that this residue might form the intracellular extreme of the gate constriction. However, in the same study, Gly^{342} was found to be accessible by $MTSEA^+$ from both sides of the membrane rendering a firm conclusion on gate position difficult. Rassendren et al. (1997) argued for a gate location between Leu^{338} and Asp^{349}, finding that, in contrast to Egan et al. (1998) extracellular $MTSEA^+$ could access and modify Leu^{338} in the closed channel state.

In two later studies, Li et al. (2008) concluded that the closed state pore narrowed to an ion impermeable constriction within the stretch of amino acids between Ile^{332} and Thr^{339} (Li et al., 2008; Kracun et al., 2010). Cysteines introduced here required progressively longer applications of $MTSET^+$ for functional modification of the channel. It was observed that Ag^+ and Cd^{2+} access to a Cys substituted for Ile^{332} was unaffected by whether the channel was opened or closed, but that Cys introduced at positions intracellular to Thr^{336} were almost completely inaccessible in the closed state, supporting the idea that the main barrier to small monovalent cations began in this region of the pore (Li et al., 2008; Kracun et al., 2010). Looking to the crystal structures, according to the closed state homology model for P2X2R the barrier to ion permeation is formed by Thr^{336} and Thr^{339} (**Figure 7**, blue). This agrees with the predictions of Kracun et al. (2010) and Li et al. (2008) that the extracellular side of the gate occurs just below Ile^{332} (**Figure 7**, yellow) and is in accordance with SCAM studies investigating the effect of Cd^{2+} and Ag^+ on mutants P2X2Rs containing Cys substitutions within TM2 (Kracun et al., 2010; Li et al., 2010).

ION SELECTION AND PERMEATION

P2XRs are commonly described as non-selective cation channels, being chiefly permeable to Na^+, K^+ and Ca^{2+} under physiological conditions, although at least one family member has significant permeability to Cl^- (North, 2002). Like other cation-permeable LGICs, at negative membrane potentials the ionic electrochemical gradients chiefly favor influx of Na^{2+} and Ca^{2+} through P2XRs, causing depolarization and an elevation in $[Ca^{2+}]_i$. The depolarization is sufficient to initiate action potential propagation (Cook et al., 1997; Dowd et al., 1998;

Kirkup et al., 1999; Rong and Burnstock, 2004) and the elevation in $[Ca^{2+}]_i$ can affect a diverse array of Ca^{2+}-dependent signaling processes, including synaptic transmission (Khakh and Henderson, 1998; Boehm, 1999; von Kugelgen et al., 1999; Khakh and Henderson, 2000), smooth muscle contraction (Smith and Burnstock, 2004), and cell survival (Adinolfi et al., 2005).

This review will confine itself to a very brief and simplified general introduction to the study of ion selectivity and flux through ion channels, before reviewing the current understanding of these matters with respect to P2XRs. A more thorough discussion of the general mechanisms underlying ion selectivity and flux ion channels can be obtained from other sources (Eisenman and Horn, 1983; Eisenman and Dani, 1987; Hille, 2001). The reader is also directed to reviews covering ion selectivity and flux through specific ion channels, such as the voltage-gated K^+ (Roux, 2005), Na^+ (Catterall, 2012), and Ca^{2+} (Sather and McCleskey, 2003) channels, and the ligand-gated Cys-loop (Sine et al., 2010), and ionotropic glutamate receptors (Traynelis et al., 2010).

The study of ion permeability and flux has a rich and prestigious history (Hille, 2001 and see Catterall et al., 2012), and the topic can be approached using a number of experimental techniques. The most common approaches involve the estimation of the "relative permeability" of ions, either from determination of current reversal potentials or from the comparison of single channel conductances in solutions of varying ionic composition. Relative permeabilities are usually reported for an ion, X, with respect to Na^+, P_X/P_{Na}, or Cs^+, P_X/P_{Cs}, and are calculated from experimental reversal potential and/or single channel conductance data using the Goldman-Hodgkin-Katz (GHK) voltage and current equations, respectively (Hille, 2001). Despite the fact that ion permeation through most, if not all, ion channels violates the assumptions of independence implicit to the GHK model (that permeating ions neither interact with the walls of the channel pore nor with other ions in the permeation pathway), these methods have nevertheless provided a good approximation of ion permeability for a number of cation non-selective LGICs. Nevertheless, in the case of assessing relative Ca^{2+} permeability, an alternative model-independent method is becoming more commonly used, the "fractional Ca^{2+} current" or "dye overload" method developed by Neher (1995) and Rogers and Dani (1995). This involves performing whole cell patch clamp fluorimetry with a pipette containing a saturating concentration of the Ca^{2+} dye, fura-2, and measuring both ionic current and Ca^{2+} influx simultaneously. From this, direct determination of the contribution of Ca^{2+} to the total inward current is obtained in the presence of physiological concentrations of extracellular Ca^{2+}. By contrast, to determine P_{Ca}/P_{Cs} using the reversal potential method commonly requires the use of non-physiological ionic solutions, and only allows permeability to be determined for a single membrane potential (the E_{Rev}). This technique has proved particularly useful, then, for estimating Ca^{2+} flux in a physiologically normal ionic environment and at a range of membrane potentials most likely encountered by an animal cell *in vivo* (Burnashev, 1998; Jatzke et al., 2002; Egan and Khakh, 2004; Fucile, 2004; Samways et al., 2008b).

The entry of an ion into a channel pore and subsequent conductance across the biological membrane partition is influenced by a number of ion channel pore properties (see Hille, 2001). First, the maximum diameter of the channel pore will provide a steric size limit on permeating ions, allowing selection by "molecular sieving". Second, the electrostatic environment near the openings of the pore can produce local surface potentials that contribute to selectivity by attracting and repelling ions based on charge. Lastly, the presence of charged and polar side chains, or backbone carbonyls, oriented toward the axis of narrow portions of the ion permeating pathway can directly interact with the permeating ions, assisting with dehydration, if necessary, for the ion to pass through completely.

UPPER PORE SIZE LIMIT FOR P2XRs

An early rough estimate based on single channel cation conductance predicted the P2X2R pore diameter to be ∼20Å at its narrowest point (Ding and Sachs, 1999a), much larger than the ∼7Å predicted by the crystal structure of the open P2X4R (Hattori and Gouaux, 2012). Functional studies of cation permeability and uptake of fluorescent cationic dyes suggest that the pore diameter of P2XRs can accommodate cation species as large as the cationic dye YO-PRO1 and the large thiol-reactive agent MTS-TPAE$^+$, predicting a maximum pore diameter closer to 12–14Å (Khakh et al., 1999a; Li et al., 2010; Browne et al., 2013). An explanation for the discrepancy is that the open pore has a considerable degree of flexibility, a notion supported by the phenomenon of "pore dilation" (see below), which is proposed to allow certain members of the P2XR family to increase their permeability to larger cation species (Khakh et al., 1999a; Virginio et al., 1999a). For comparison, the pore diameters of other LGICs are as follows: the nAChR, ∼8Å (Albuquerque et al., 2009); NMDAR, 6–7Å (Villarroel et al., 1995); TRPV1, 10-12Å (Chung et al., 2008); and ASIC1, ∼4Å (Carattino and Della Vecchia, 2012).

CATION vs. ANION SELECTION

P2XRs are broadly described as non-selective cation channels that favor the conduction of positively charged ions, such as Na^+, K^+, and Ca^{2+}, vs. negatively charged ions such as Cl^- (North, 2002). The family members P2X1R, P2X2R, P2X4R, and P2X7R all exhibit a Cl^- permeability relative to monovalent cations of less than 0.1 (Virginio et al., 1999b; Samways and Egan, 2007; Browne et al., 2013) (**Table 1**). Nevertheless, ATP-gated currents with significant Cl^- permeability have been recorded previously in native cells (Thomas and Hume, 1990), and some recombinant P2X5R have a P_{Cl}/P_{Na} of 0.5–0.7 (Ruppelt et al., 2001; Bo et al., 2003; Samways and Egan, 2007). Exactly how the predominantly cation-selective P2XRs discriminate between cations and anions has yet to be adequately determined. Resolution of the P2X4R crystal structure revealed that the extracellular vestibule carries a net negative surface charge, primarily due to the presence of Glu^{56} and Asp^{58} (human P2X4R numbering), and free energy calculations conducted using the zfP2X4R closed structure as a model support the view that Na^+ and Ca^{2+} entry into this domain is favored relative to Cl^- (Kawate et al., 2009, 2011). However, a number of observations are inconsistent with this hypothesis. Firstly, there is little correlation between the conservation of charge at positions analogous to Glu^{56} and Asp^{58} and the

Table 1 | Relative permeability and fractional Ca^{2+} current (Pf%) data for recombinant homomeric P2XRs.

P2XR	P_{Cl}/P_{X+} Derived from E_{rev}	P_{Ca}/P_{X+} Derived from E_{rev}	Ca^{2+} flux Pf%	References
P2X1R	0.09	3.6–3.9	12	Evans et al., 1996; Egan and Khakh, 2004; Samways and Egan, 2007
P2X2R	0.02	2.2–2.9	5.7	Evans et al., 1996; Virginio et al., 1998; Migita et al., 2001; Egan and Khakh, 2004; Samways and Egan, 2007
P2X3R	n.d.	1.2	3–4.8	Egan and Khakh, 2004; Samways and Egan, 2007; Ma et al., 2012
P2X4R	0.09	4.2–4.6	16	Garcia-Guzman et al., 1997; Egan and Khakh, 2004; Samways and Egan, 2007
P2X5R	0.5–0.66	n.d.	4.5	Ruppelt et al., 2001; Egan and Khakh, 2004; Samways and Egan, 2007
P2X7R	<0.1	35	4.6	Bretschneider et al., 1995; Virginio et al., 1999b; Egan and Khakh, 2004; Browne et al., 2013

X^+ represent either Na^+ or Cs^+ depending on experimental method used.

cation vs. anion selectivity exhibited by P2XRs. Thus, whereas the strongly cation selective channel P2X1R has Ser at both of these positions, and no neighboring acidic side chains to compensate for the loss of charge, P2X5R has an Asp at both positions but exhibits a much weaker cation vs. anion selectivity (see **Figure 1**). Secondly, in trying to elucidate the structural basis of Cl^- permeation in P2X5R, Bo et al. (2003) observed that neutralizing the positive charge at position Lys^{52}, which is occupied by a neutral Gln in P2X2R and an acidic Glu or Asp in P2X1R, P2X3R, P2X4R, and P2X7R, had no effect on P_{Cl}/P_{Na}. Further, the complimenting mutation in P2X2R, substitution of Lys for Gln^{52}, had no effect on this channel's P_{Cl}/P_{Na}. Although these results are curious in light of evidence that this position regulates Ca^{2+} selection and flux relative to Na^+ in P2XRs (Samways and Egan, 2007 and see below), it seems to imply that charge selection is not a major function of the lateral portals/extracellular vestibule of the P2XR structure. A third observation that challenges the view that charge selection might happen extracellular to the transmembrane conducting pathway is that, in SCAM studies of P2X4R, negatively charged $MTSES^-$ was found to access and modify substituted cysteines introduced into the extracellular vestibule at positions Glu^{56}, Thr^{57}, Asp^{58}, and Ser^{59} of P2X4R, and as deep into the transmembrane pore as Thr^{336} and Ser^{341} of P2X2R and P2X4R, respectively (Rassendren et al., 1997; Samways et al., 2011). The modification rates were not that dissimilar to those observed for the positively charged but similarly sized $MTSET^+$, suggesting that any charge selection barrier within the typical P2XR is likely to be within, or intracellular to, the channel gate. Interestingly, charge selection was observed in the central vestibule, above the extracellular vestibule, where $MTSET^+$, but not $MTSES^-$, was found to access and modify cysteines introduced at positions Ser^{62} and Asn^{97} of P2X4R (Samways et al., 2011). Nevertheless, the proximity of the central vestibule is clearly not preventing $MTSES^-$ from intruding deep into the transmembrane pore, likely because the pore opening is too far removed from the influence of the central vestibule's surface potential.

It seems more plausible, then, that cation vs. anion selection occurs further along the ion conducting pathway, perhaps deep within the transmembrane spanning pore as has been suggested for the Cys-loop family of ion channels (Sine et al., 2010). Although this has not been investigated systematically as yet, it has been shown that substitution of Thr^{339} of P2X2R for Lys, thereby creating a symmetrical lining of positive charge deep in the open channel pore, renders the channel non-selective for Na^+ vs. Cl^- (Browne et al., 2011). Further, substitution of Arg at position Thr^{339} conferred a reversal of selectivity, with this mutant displaying a modest P_{Cl}/P_{Na} of $\sim$2. However, although Thr^{339} has been previously implicated in selection between mono- and divalent cations (Migita et al., 2001; Egan and Khakh, 2004), it is very weakly conserved position among P2XR family members, being occupied by Gly in P2X1R and Ala in P2X4R for example. It is difficult, then, to draw general conclusions about its role in selection and permeation. In P2X7R, substitution of residues intracellular to the gating region also affected cation vs. anion selection, with substitution of Lys at positions Thr^{348} (Ser^{345}, P2X2R) and Asp^{352} (Asp^{349}, P2X2R) significantly enhancing P_{Cl}/P_{Na} (Browne et al., 2013), but again it is not clear whether these positions play an important role in charge discrimination, particularly for extracellular cations. The lack of concrete data regarding the structural underpinning of cation vs. anion selectivity is a significant deficit in our understanding of P2X function and one that would be timely to address.

DISCRIMINATION BETWEEN MONOVALENT CATIONS

P2XRs are considered "non-selective" cation channels in large part because they do not discriminate well between small monovalent alkali ion species. Studies using the whole cell reversal potential method reported that neither P2X1R nor P2X2R could substantially discriminate between Na^+, K^+, Cs^+, or Rb^+ (Evans, 1996). These data are largely consistent with relative permeability data obtained from single channel measurements of monovalent cation conductance, where K^+, Rb^+, and Cs^+ were more-or-less equally permeable, and Na^+ only marginally less so (Ding and Sachs, 1999a). Interestingly, Li^+ exhibited a significantly higher permeability relative to Na^+, K^+, Cs^+, and Rb^+ as assessed from whole cell reversal potentials (Migita et al., 2001), but a lower relative permeability as assessed from single channel conductance studies (Ding and Sachs, 1999a). This is consistent with the presence of an intrapore binding site with higher selectivity for Li^+ relative to the other alkali metal ions, with the stronger interaction slowing Li^+ conductance through the channel (Hille, 2001). There has been some difficulty in resolving the cation conductances for all the recombinant P2XRs due to the channels exhibiting very short, flickery openings. Estimates

of Na^+ conductance at membrane potentials between −100 and −150 mV are available for P2X1R [∼12 pS; (Evans, 1996)], P2X2R [21–35 pS (Evans, 1996; Ding and Sachs, 1999b)], P2X4R [9–18 pS (Evans, 1996; Priel and Silberberg, 2004; Samways et al., 2011)] and P2X7R [9–13 pS (Riedel et al., 2007)]. For comparison, under similar conditions the conductances of other non-selective cation channels including the nicotinic acetylcholine receptors, glutmate-gated NMDA receptors, and capsaicin-sensitive TRPV1 receptors are 25–50 pS (Mathie et al., 1991), 20–40 pS (Stern et al., 1992), and 50–60 pS (Premkumar et al., 2002; Samways and Egan, 2011), respectively.

Ca^{2+} SELECTIVITY AND FLUX

All of the P2X receptor subunits confer Ca^{2+} permeability, with the selectivity of the functional channel depending on the constituent subunits (Egan and Khakh, 2004). The homomeric P2X1R and P2X4R receptors have the highest relative Ca^{2+} permeability, with reversal potential-derived P_{Ca}/P_{Cs} values of ∼4–5 (Evans et al., 1996; Garcia-Guzman et al., 1997; Samways and Egan, 2007) (**Table 1**). The use of the superior fluorimetric flux method (Neher, 1995) confirmed the ability for these receptors to conduct an appreciable Ca^{2+} influx, showing that Ca^{2+} carries 12% and 16% of the total inward currents through P2X1R and P2X4R, respectively, at −60 mV in the presence of 2 mM extracellular Ca^{2+} (Egan and Khakh, 2004; Samways and Egan, 2007) (**Table 1**). P2X3R has the lowest recorded relative Ca^{2+} permeability of the family, with a reversal potential-determined P_{Ca}/P_{Cs} of 1.6 (Virginio et al., 1998) and a fractional Ca^{2+} current of 3–5%. This method also allowed a more accurate estimate of Ca^{2+} flux through recombinant P2X7 receptor, which are inhibited by the high external Ca^{2+} concentrations required to obtain reversal potential-based measurements of P_{Ca}/P_{Na}. Indeed, a previous estimate of P2X7 relative Ca^{2+} permeability from reversal potentials reported a P_{Ca}/P_{Na} of 35 (Bretschneider et al., 1995), which is far in excess of what is predicted for a channel with a Pf% reading of ∼5% (Egan and Khakh, 2004) (**Table 1**).

As expected, the variability in relative Ca^{2+} permeability exhibited between P2XR subunits correlates with a variability in the Ca^{2+} permeabilities of ATP-gated currents in native tissues. For example, in smooth muscle cells, reversal potential measurements allowed an estimation of $P_{Ca}/P_{Na} = 3$ for P2XR-mediated currents, which is in agreement with the highly Ca^{2+} permeable P2X1R being a predominant subtype in this tissue (Benham et al., 1991). Reversal potential experiments conducted in ATP-sensitive neurons have yielded Ca^{2+} permeability values of $P_{Ca}/P_{Cs} = \sim 1.5$ for nodose ganglion neurons (Virginio et al., 1998) and $P_{Ca}/P_{Cs} = \sim 2$ for retinal ganglion neurons (Taschenberger et al., 1999), both cell types of which likely express homo- and heteromeric P2X2R and P2X3R. In addition, early Pf% experiments conducted in sympathetic neurons reported that, at −60 mV in the presence of 2.5 mM extracellular Ca^{2+}, approximately 7% of the total ATP-gated current was carried by Ca^{2+} (Rogers and Dani, 1995). In activated, P2X4R expressing mammalian microglial cells, ATP-gated currents exhibited a Pf% value of 17% (Toulme et al., 2010), comparable to the 16% calculated for the recombinant mammalian receptor (Egan and Khakh, 2004; Samways and Egan, 2007).

In contrast to our relative lack of understanding with regard to how P2XRs discriminate between cations and anions, we have at least some idea of which amino acid side chains are important in regulating Ca^{2+} selectivity and flux through this family of ion channels. Studies using both the reversal potential and Pf% methods initially revealed an important role of polar side chains within TM2 in regulating Ca^{2+} selection in P2X2R (Migita et al., 2001; Egan and Khakh, 2004). Specifically, it was found that substitution of the polar residues Thr^{336}, Thr^{339}, and Ser^{340} with hydrophobic residues of similar size almost abolished the selectivity between Ca^{2+} and Na^+ (Migita et al., 2001) and substantially reduced the Pf% (Egan and Khakh, 2004). The homology model of P2X2R is consistent with this, showing that the three residues are located in the narrow part of channel pore and that two of them, Thr^{336} and Thr^{339}, are oriented directly into the permeation pathway in the open channel state (see **Figures 4**, **7**). Indeed, increasing the side chain volume at these positions via substitution with Tyr actually formed a barrier to Ca^{2+} permeation, causing the reversal potential-based P_{Ca}/P_{Cs} to be ∼0.3 for the mutant T339Y, and <0.1 for S340Y (Migita et al., 2001), and the respective Pf%s recorded as ∼1 and 0.2 % (Egan and Khakh, 2004) (T336Y was nonfunctional). From this, it would be tempting to speculate that the hydroxyl groups on these side chains might act as surrogate water-like ligands, assisting in the dehydration of permeating Ca^{2+} ions. For Thr^{336}, this hypothesis has merit, as a hydroxyl-bearing Ser or Thr side chain resides at this relative position in all the P2X receptors with the sole exception of P2X3R (where it is occupied by Ala), P2X3R having the lowest Ca^{2+} permeability. The hydroxyl group of Thr^{339}, on the other hand, is only retained in the moderately Ca^{2+} permeable P2X2R (Thr) and P2X7R (Ser), but is absent in the highly Ca^{2+} permeable P2X1R (Gly) and P2X4R (Ala). The properties of the side chain at position Ser^{340} in P2X2R are very poorly conserved, with this position most commonly being occupied by a hydrophobic residue, such as Leu (P2X4, P2X5), Ile (P2X1), Tyr (P2X7), or Trp (P2X6).

Only one negatively charged side chain is present within the actual TM domains of P2XRs, and this is a conserved Asp on the intracellular extreme of TM2 at the position analogous to Asp^{349} of P2X2R (**Figure 4**) and Asp^{354} (P2X4R). It is highly conserved throughout the vertebrate P2XRs, but substitution of this residue had no effect on either the reversal potential-derived P_{Ca}/P_{Cs} or the Pf% of P2X2R (Migita et al., 2001; Egan and Khakh, 2004). However, Fountain et al. (Fountain et al., 2008) observed that a P2XR isoform found in green algae, which possesses an Asn at this position, exhibited a 50% increase in the reversal-potential-derived P_{Ca}/P_{Na} upon substitution for Asp.

Even if a hydroxyl-bearing side chain at the position analogous to Thr^{336} of P2X2R plays a role in Ca^{2+} selectivity, it cannot easily explain the diversity in relative Ca^{2+} permeabilities between the different P2XRs. Why do P2X1R and P2X4R have such a high Ca^{2+} permeability? Inspection of the primary amino acid sequences reveals that at positions located just extracellular to TM1 and TM2, analogous to positions Glu^{51} and Asp^{331} of P2X4, some P2XRs possess fixed negative charge. Samways and Egan (2007), hypothesized that this formal charge might serve to attract divalent cations, and indeed neutralizing both charges

significantly attenuated Ca^{2+} selectivity and relative conductance in P2X1R and P2X4R receptors. Complimentary mutations that substituted these charges into P2X2R, the wild type of which has Gln and Ser at these positions, significantly increased the Pf% of this receptor. Further, these acidic side chains could be titrated by reducing extracellular pH, subsequently reducing Pf% for P2X1R and for the P2X2R-Q52E/S326D mutant.

P2X3R retains a Glu at the position analogous to Glu^{51} in P2X4R and possesses an Asn at Asp^{331}. Interestingly, only one of the side chains needed be present for enhanced Ca^{2+} permeability in P2X1R, P2X2R, and P2X4R receptors, and yet P2X3R receptors have the lowest Ca^{2+} permeability of the family despite retaining the Glu analogous to Glu^{51} in P2X4R. However, here the Glu is closely flanked by the positive charge of a neighboring His (as well as that of the highly conserved Lys on the other side). In addition, the lack of a hydroxyl-bearing residue at the position analogous to Thr^{336} of P2X2R might also impair Ca^{2+} permeability in P2X3R. Nevertheless, substitution of the neighboring His residue with Tyr was observed to significantly elevate Ca^{2+} permeability (Samways and Egan, 2007).

Glu^{51} and Asp^{331} are now known to line the lateral portals of P2X4R, and it is tempting to speculate that the presence of fixed negative charge here could present a sufficient surface potential to concentrate the activity of cations, favoring the charge dense Ca^{2+} over Na^+ (**Figure 8**). However, as compelling as these data are, there are a few caveats to drawing general conclusions from them and applying them to the P2XR family as a whole. First, P2X7R has fixed charge at both of these positions, but the Ca^{2+} permeability is not quite as high as P2X1R and P2X4R (**Figure 1** and **Table 1**). Second, P2X5R has a Lys at this position, and although this has previously been ruled out as a determinant of cation vs. anion discrimination, it seems unusual that P2X5R would maintain a Ca^{2+} permeability not far removed from that of P2X2R (Egan and Khakh, 2004) (**Table 1**). Third, and related to the second caveat, is the question of why lateral portal residues are sufficient to assist in discrimination between mono- and divalent cations, but not sufficient to strongly influence discrimination between anions and cations.

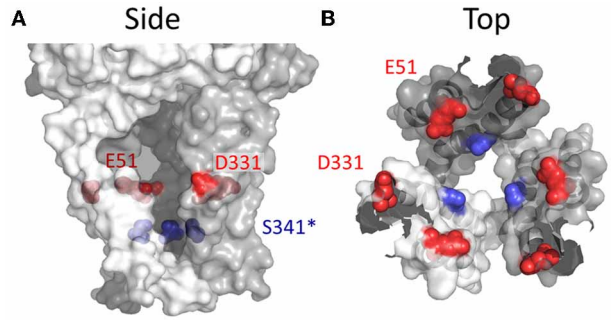

FIGURE 8 | Side chains affecting Ca^{2+} selection and flux in P2X4R. **(A)** Side view of open state P2X4R homology model depicting residues known to regulate ion permeability. **(B)** P2X4R viewed from the extracellular vestibule looking intracellularly in the open configuration. Acidic side chain positions implicated in the regulation of Ca^{2+} permeation, Glu^{51} and Asp^{331} are shown in red. The position highlighted in blue is Ser^{341}, a conserved hydroxyl bearing side chain predicted to regulate Ca^{2+} permeability based on data for the analogous position, Thr^{336}, in P2X2R.

It might be the case that, unlike the selectivity filters of other ion channels (Sather and McCleskey, 2003; Roux, 2005; Sine et al., 2010; Traynelis et al., 2010; Catterall, 2012), P2X receptors lack a single, discreet structural locus for ion selectivity. That instead, selectivity and conductance are regulated more diffusely, and involves multiple parts of the permeation pathway. Further experiments are required to test this hypothesis. A last interesting point to make about the relative Ca^{2+} permeability of P2XRs is that it is sensitive to tuning via allosteric modulation. This was first shown for P2X1, where reducing the extracellular pH was shown to attenuate Pf%, likely as a result of charge shielding of the acidic side chains that we know confer the higher Ca^{2+} permeability in P2X1R and P2X4R (Samways and Egan, 2007). More recently, it was shown that the drug ivermectin, previously known to enhance the open probability of P2X4Rs in the presence of ATP (Khakh et al., 1999b; Priel and Silberberg, 2004), has the additional effect of reducing the Pf% of this receptor (Samways et al., 2012). Thus, far from being fixed properties of P2XRs, ion selection and conductance by these channels appears to be flexible and potentially amenable to fine tuning. In the last part of this review we will discuss a particularly unusual example of this flexibility with regard to the phenomenon described as "pore dilation".

PORE DILATION

A curious property of P2XRs was hinted at by early studies conducted on leukocytes, where it was revealed that prolonged exposure to ATP had the effect of permeabilizing the membrane to much larger ionic species than those usually conducted by LGICs, including 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), N-methyl-D-glucamine ($NMDG^+$) (Nuttle and Dubyak, 1994), and the cationic dye ethidium (Tatham et al., 1988; Wiley et al., 1993). In some mast cells and macrophages it was even observed that sustained ATP exposure promoted the uptake or leakage of large anionic dyes, including the Ca^{2+}-sensor Fura-2 and Lucifer yellow (Steinberg et al., 1987; Yan et al., 2008). It was subsequently revealed that the purinergic receptor being acted upon by ATP in these cells was largely P2X7 (Surprenant et al., 1996). Since this discovery, sustained ATP activation of two other members of the family, P2X2R and P2X4R, has been shown to produce a similar biphasic effect on membrane permeability, causing a progressive change from a primarily Na^+, K^+, Ca^{2+} conducting current (I_1) to one that conducts passage of larger cations such as $NMDG^+$ and the propidium dye, YO-PRO1 (I_2) (Khakh et al., 1999a; Virginio et al., 1999a). A fact even more curious, but one that will not be discussed further in this review, is that two members of the structurally unrelated TRP family of ion channels, TRPV1 and TRPA1, has been observed to exhibit a strikingly similar form of dynamic permeability (Chung et al., 2008; Banke et al., 2010), hinting at a broader significance of this ion channel phenomenon.

Three basic hypotheses have been put forward with regard to the mechanism by which this time-dependent change in permeability to large ionic species occurs. The first posits that the phenomenon represents an intrinsic gating property of the functional P2XR channel, and that sustained ATP exposure causes the

channel pore to literally widen as the commonly used term "pore dilation" describes, thereby mediating the progressive increase in permeability to large ionic species (Khakh and Lester, 1999; Khakh et al., 1999a; Virginio et al., 1999a). The switch from I_1 to I_2 might be spontaneous or regulated by second messenger-mediated modification of the channel protein (e.g., phosphorylation/dephosphorylation). The second hypothesis proposes that an agonist-dependent redistribution and oligomerization of P2XRs leads to the formation of macropores. These could potentially arise from the fusion of two or more trimeric P2XRs and an enlargement of the main functional channel pore, or as a result of a separate but larger pore formed between aggregating trimeric assemblies (Khakh and Egan, 2005; Khakh et al., 1999a). The third proposes that the permeability to large cations is mediated by a structurally separate transport pathway stimulated downstream of P2XR activation (Virginio et al., 1999a). The second hypothesis has been largely ruled out by a study utilizing total internal reflection fluorescence (TIRF) imaging to monitor the lateral movement of P2X2R within the plasma membrane during ATP stimulation, which found no compelling evidence of the redistribution and clustering that would be expected if these channels were oligomerizing into a higher stoichiometric pore structure (Khakh and Egan, 2005). Which of the two remaining hypotheses holds true has been more difficult to determine, with evidence for and against both mechanisms, and the possibility remaining that the observed change in membrane permeability during sustained ATP exposure may involve both intrinsic "pore dilation" and recruitment of secondary transport pathways, and that the contribution of these two mechanisms may differ depending on cell type (for review, see North, 2002; Pelegrin, 2011).

Large ion permeation through the P2XR pore: I_1 to I_2 transition

There is a substantial body of evidence that the intrinsic pores of P2X2R, P2X4R, and P2X7R receptors can potentially accommodate large cations, including fluorescent dyes. In addition to SCAM studies showing that large MTS reagents like MTS-TPAE and even Texas Red-MTSEA can gain access to side chains deep within the permeation pathway (Li et al., 2010; Browne et al., 2011; Samways et al., 2011), site-directed mutagenesis experiments have shown that substituting side chains in the permeation pathway and intracellular domains can alter the ability of the P2XR to transition between the I_1 and I_2 permeability states (Khakh et al., 1999a; Khakh and Lester, 1999; Virginio et al., 1999a; Khakh and Egan, 2005; Yan et al., 2008). For P2X7R, substitutions introduced in the N-terminal domain can lock the receptor in an immediately $NMDG^+$ permeable I_2 state (Yan et al., 2008), supporting the idea that the accommodation of larger polyatomic cations is within the size limits of the intrinsic pore of these ion channels. This argument is further supported by the observation that YO-PRO1 exhibits the characteristics of a permeant blocker of Na^+ currents when applied to the ATP-gated P2X7R (Browne et al., 2013), a result similar to one for the putatively pore dilating TRPV1R (Li et al., 2011).

Accepting that P2XRs have the capability of accommodating larger ionic species of up to 12Å in diameter (Browne et al., 2013), an important question is whether the transition from I_1 to I_2 during sustained activation is automatic and readily occurs in the absence of large ionic species, or whether the presence of these large species is required to induce I_2 formation, perhaps via a "foot-in-the-door" mechanism. Two studies have published data relevant to this question, but with conflicting results. With $NMDG^+$ as the chief extracellular charge carrier, Jiang et al. (2005) observed that complete pore dilation of P2X7R expressed in HEK293 cells usually occurred within 30 s of sustained ATP exposure, consistent with previous studies (Khakh et al., 1999a; Virginio et al., 1999a). However, when they exposed the cells to 30 s of ATP in the presence of normal extracellular saline, and then switched the extracellular Na^+ for $NMDG^+$ in the continued presence of ATP, they observed that the channel's permeability state started in I_1 and then slowly shifted to I_2. One interpretation is that $NMDG^+$ needs to be present to induce the transition from I_1 to I_2. However, a very similar experiment was conducted in GT-1 cells expressing recombinant P2X7Rs, but in this case the substitution of $NMDG^+$ for Na^+ after 60 s of ATP exposure showed the channel to already be in I_2 (Yan et al., 2008), suggesting that the pore widened in the absence of large cations. Establishing the mechanism by which P2XRs transition from the I_1 to the large cation permeable I_2 state is a worthy objective for future investigation, particularly in light of the apparent potential for using LGICs with wide pores as conduits for selective delivery of therapeutic drugs into cells (Binshtok et al., 2007; Li et al., 2011).

The hope for the future is that, in addition to the currently available closed and open state models for the P2XRs, a new model showing the putative pore dilated state will be resolved. As it is, there is already compelling evidence from crystallographic data for a similar trimeric family of ion channels, the ASIC receptors, giving credence to the idea that ion channels can occupy multiple conductance states of different diameter (Lingueglia et al., 1997; Baconguis and Gouaux, 2012). In addition, structural data suggests that the diameter of the open channel pore of the bacterial mechanosensitive channel, MscL, can change between 2 and 30Å depending on the degree to which the pore-forming transmembrane domains tilt relative to one another in the lipid bilayer (Doyle, 2004).

Separate downstream permeation pathways may contribute to some of the observed permeability changes

Although there is sound evidence supporting the ability for large ionic species to enter and permeate the channel of some P2XRs, this does not necessarily exclude the possibility that separate downstream permeation pathways also contribute to large ion transport. An initial piece of evidence favoring the view that the presence of other proteins, whether channel forming or not, might be necessary for the observed time-dependent changes in membrane permeability witnessed during prolonged ATP exposure was that the appearance of the phenomenon is far from consistent. Even in overexpression systems, the progressive increase in membrane permeability to large cations during prolonged ATP exposure often only occurs in a subset of the cells studied (Virginio et al., 1999a), and in other cases studies have failed to reproduce the phenomenon at all (Petrou et al., 1997; Klapperstuck et al., 2000; Pannicke et al., 2000). This may be due to the I_1 to I_2 transition being dependent on modification

of the P2XR channel by kinases or phosphatases, but may also be due to the need for some separate pore forming protein to be co-expressed in the same cells as the activated P2XRs. In some cases, a mismatch between the observed increase in large cation permeability of P2X7R mediated currents using electrophysiology, and observed uptake of cationic dyes in intact cell imaging experiments, suggests that at least some dye uptake might occur through a non-P2X7R-mediated pathway (Virginio et al., 1997; Jiang et al., 2005). Additionally, single channel studies in macrophages revealed that prolonged ATP exposure was associated with the openings of a large conductance (∼400 pS) pore permeable to large anions and cations (Faria et al., 2005), but these high conductance openings have not been observed in single channel studies of the recombinant P2X7R (Riedel et al., 2007). Furthermore, the increased permeability of macrophages to large ionic species during sustained ATP-exposure was found to be dependent on Phospholipase C-mediated elevations in $[Ca^{2+}]_i$ and MAP kinase, despite the fact that inhibition of neither of these pathways had any effect on P2X7R-mediated currents (Donnelly-Roberts et al., 2004; Faria et al., 2009).

A possible candidate for a putative secondary ion transport pathway was the gap junction-forming protein, Pannexin-1. A growing body of evidence suggests that there is a functionally significant interaction between P2X7R and Pannexin-1 (Pelegrin and Surprenant, 2006; Locovei et al., 2007; Gulbransen et al., 2012; Poornima et al., 2012; Xu et al., 2012), raising the compelling possibility that the latter is responsible for some of the observed ATP-dependent increase in membrane permeability to larger ionic species. Regardless, successive studies have shown that neither pharmacological blockade of Pannexin-1 nor inhibiting its expression affects the observed time-dependent change in permeability observed for P2X2R (Chaumont et al., 2008) P2X4R (Bernier et al., 2012), or P2X7R (Yan et al., 2008; Alberto et al., 2013). In addition, it appears that Pannexin-1 is directly inhibited by ATP within the relatively high concentration range required to activate native P2X7R receptors (Qiu and Dahl, 2009).

In cases in which prolonged ATP exposure correlates with an increase in membrane permeability to anionic species, the argument for a secondary permeation pathway independent of the P2XR pore itself is more compelling. Cankurtaran-Sayar et al. (2009) observed an ATP-mediated increase in permeability to large anions in HEK293 cells transfected with P2X7R, but found that this was due to a Ca^{2+}-dependent mechanism separate from that mediating permeability to large cations. Results from a study by Schachter et al. (2008) go as far as to suggests that, in some cases, the ATP-dependent increase in permeability to anions might occur irrespective of whether there are functional P2XRs present; a result that urges caution in studying P2XR pore dilation in cell models that likely contain other purinergic receptors coupled to second messenger cascades, which might be linked to these other permeabilizing pathways. Given that Pannexin-1 channels have been shown to be permeable to large anions (Ma et al., 2012; Poornima et al., 2012), it may yet transpire that these proteins have a role to play in ATP-mediated increases in membrane permeability to large ionic species in some settings.

CONCLUSION

The current understanding of ion permeation through the members of the P2X receptor family of cation permeable ion channels can be summarized as follows. Regardless of whether the P2X receptor is in the closed or open conformation, extracellular ions likely diffuse freely between the bulk solution and the extracellular vestibule of the channel by way of the three large intersubunit lateral portals. Upon ATP binding, signal transduction and channel gating, the TM domains withdraw from the central axis of the pore, iris-like, thus relieving the constriction formed by the TM2 domains in the closed channel state (**Figure 6**). Extracellular ions can then enter and conduct through the revealed transmembrane permeation pathway into the cell. Rather than a specific structural locus existing for ion selectivity, the data suggest that a number of sites within the open channel permeation pathway, some within the lateral portals and some deep within the narrow transmembrane pore, contribute to this essential ion channel property. Indeed, ion selection and conductance appear to be dynamic properties of P2XRs, with the ion permeable pore potentially inhabiting multiple open states with distinct permeation properties (Khakh and Lester, 1999; Virginio et al., 1999a; Samways et al., 2012).

Several questions remain, but one that is arguably of a particular pressing nature is precisely how these channels exhibit a preference for cations over anions. And extension of this question would include how some P2X receptors can become increasingly permeable to very large polyatomic cation species without apparently attenuating their ability to discriminate between small monovalent cations and anions. Another question is what role the intracellular domains play in ion permeation, whether direct by virtue of interacting with entering and exiting ions on the intracellular side of the membrane, or indirectly via effects on the arrangements of the TM domains in the open channel state.

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Heteromeric assembly of P2X subunits

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Transcripts and/or proteins of P2X receptor (P2XR) subunits have been found in virtually all mammalian tissues. Generally more than one of the seven known P2X subunits have been identified in a given cell type. Six of the seven cloned P2X subunits can efficiently form functional homotrimeric ion channels in recombinant expression systems. This is in contrast to other ligand-gated ion channel families, such as the Cys-loop or glutamate receptors, where homomeric assemblies seem to represent the exception rather than the rule. P2XR mediated responses recorded from native tissues rarely match exactly the biophysical and pharmacological properties of heterologously expressed homomeric P2XRs. Heterotrimerization of P2X subunits is likely to account for this observed diversity. While the existence of heterotrimeric P2X2/3Rs and their role in physiological processes is well established, the composition of most other P2XR heteromers and/or the interplay between distinct trimeric receptor complexes in native tissues is not clear. After a description of P2XR assembly and the structure of the intersubunit ATP-binding site, this review summarizes the distribution of P2XR subunits in selected mammalian cell types and the biochemically and/or functionally characterized heteromeric P2XRs that have been observed upon heterologous co-expression of P2XR subunits. We further provide examples where the postulated heteromeric P2XRs have been suggested to occur in native tissues and an overview of the currently available pharmacological tools that have been used to discriminate between homo- and heteromeric P2XRs.

Keywords: P2XR, subunit interface, homomer, heteromer, clustering, ligand binding site

ASSEMBLY OF P2XRs

TRIMERIC STRUCTURE OF P2XRs

Early electrophysiological measurements in bullfrog sensory neurons and single channel analysis of HEK cell-expressed P2X2Rs predicted that there are at least three ATP molecules needed to open a P2X channel (Bean, 1990; Ding and Sachs, 1999). Cross-linking studies and blue-native PAGE analysis of P2X1 and P2X3 receptors heterologously expressed in *Xenopus laevis* oocytes revealed the first biochemical evidence for a trimeric quaternary structure of P2XR channels (Nicke et al., 1998). This rather unexpected architecture was subsequently confirmed by atomic force microscopy (AFM) (Barrera et al., 2005), electron microscopy (EM), single particle analysis (Mio et al., 2005; Young et al., 2008) and finally the first crystal structure of a P2XR, the truncated zebrafish zP2X4R (Kawate et al., 2009), which constituted a major breakthrough in P2XR research. Unexpectedly, the crystal structure of the acid sensing ion channel (ASIC), a member of the ENaC/DEG (epithelial sodium channels/degenerin) superfamily, which shares the same topology and was published around the same time by the Gouaux group, also revealed a trimeric structure, although the two channels show no significant amino acid sequence relationships or similarities in the folding of their extracellular domains (Jasti et al., 2007; Gonzales et al., 2009; Kawate et al., 2009).

The overlapping expression patterns of various P2X subunits, the poor expression of functional $P2X_5$ and $P2X_6$ homomers in heterologous systems (see below), and the lack of correlation of the functional properties of heterologously expressed homomeric P2X3Rs with P2XRs found in dorsal root ganglions (Lewis et al., 1995), have early led to the assumption that P2XRs, like most ionic receptors, form heteromers. The existence of P2X heteromers is now firmly established but their specific composition and presence in native tissues remains in most cases enigmatic (see chapter Distribution of P2XR subunits). Likewise, their stoichiometry and determinants for subtype specific assembly are largely unclear. Biochemical and/or functional analysis of heterologously expressed P2X2/3 and P2X2/6 heteromers indicates a fixed stoichiometry of $P2X2(3)_2$ (Jiang et al., 2003) and $P2X(2)_26$ (Hausmann et al., 2012), respectively. In contrast, a variable, expression-level-dependent stoichiometry for P2X2/6 heteromers was observed in atomic force imaging experiments (Barrera et al., 2007). Co-purification experiments with the P2X1/2 heteromer suggest a $P2X1(2)_2$ stoichiometry (Aschrafi et al., 2004). So far, no evidence has been presented for the formation of complexes containing three different subunits.

ASSEMBLY DOMAINS AND MOLECULAR STRUCTURE OF THE P2XR

To investigate the role of the transmembrane domains (TMs) in subunit assembly, Torres and colleagues performed co-precipitation studies in HEK cells and found that the association of P2X2 subunits with either itself or P2X3 subunits was prevented if TM2 and the preceding 25 amino acids were deleted

(Torres et al., 1999b). To confirm the hypothesis that TM2 rather than the extracellular domain is critical for subunit assembly, these investigators made use of the finding that P2X6 subunits were able to co-immunoprecipitate with P2X1 but not P2X3 subunits (Torres et al., 1999a). Using chimeras in which the extracellular loops between P2X1 and P2X3 subunits were swapped they could demonstrate that only the chimera containing the P2X1 TMs was able to co-immunoprecipitate the P2X6 subunit. In a subsequent study on the hP2X5R splice variant that lacks the C-terminal end of the ectodomain and the outer half of the TM2, it was shown that tethering of the C-terminal end of the ectodomain by membrane insertion of TM2 and the intramembrane positioning of D355 are critical for homotrimeric assembly (Duckwitz et al., 2006). It was concluded that membrane insertion of TM2 restricts the conformational mobility of the ectodomain and thus enables correct positioning of assembly recognition sites located in the ectodomain, while D355 assists in the hydrogen bond-driven transmembrane helix-helix associations. In the zP2X4R crystal structure, however, it is seen that inter-subunit contacts are largely formed between the ectodomains (Kawate et al., 2009). The homotrimeric zP2X4R resembles a chalice, with the large extracellular domain raising ∼70 Å above the membrane plane and the six TM helices forming the shape of an hourglass. The single zP2X4 subunit structure has been compared with the shape of a jumping dolphin, in which the two TM helices and the largest part of the extracellular region form the fluke and the upper body, respectively. Attached to the large body domain, a flexible head domain, a dorsal fin, and right and left flippers have been defined. The body domain appears structurally rigid due to extensive β-sheet contacts within a β-sandwich motif. Three interfaces with close contact between adjacent subunits were defined; upper-body-to-upper-body, head-to-body and left-flipper-to-dorsal-fin. Thus, the contacts between neighboring subunits are restricted to the upper ectodomains. The lack of significant contacts between the lower bodies of the extracellular domains enables significant movements in these domains during ATP-induced channel opening (Kawate et al., 2009; Hattori and Gouaux, 2012). It was suggested that the more conserved body domains constitute a common assembly interface in all P2XRs, while the less conserved dorsal fin, head, and left flipper domains guide the subunit-specific assembly by the head-to-body and left-flipper-to-dorsal-fin contacts (Kawate et al., 2009). The low conservation of the latter domains has an important consequence: While homotrimeric P2XRs contain three identical subunit-subunit interfaces, heteromeric P2XRs form always three significantly different interfaces between the "head-to-tail" arranged subunits (see also "Specific characteristics of the ATP sites in heteromeric P2XRs").

THE INTERSUBUNIT ATP-BINDING SITE

Most of the conserved amino-acid residues involved in the interaction with ATP have been identified in mutagenesis-based studies (Ennion et al., 2000; Jiang et al., 2000; Roberts and Evans, 2004; Yan et al., 2005; Roberts and Evans, 2006; Wilkinson et al., 2006; Young et al., 2006; Fischer et al., 2007; Zemkova et al., 2007; Roberts et al., 2008; Donnelly-Roberts et al., 2009; Evans, 2009; Roberts et al., 2009; Browne et al., 2010; Evans, 2010; Bodnar et al., 2011). Based on disulfide cross-linking experiments, in which some of these residues were substituted by cysteine residues, it was concluded that the agonist binding site is located at the interface between two neighboring subunits (Marquez-Klaka et al., 2007). Crystallization of the zP2X4R showed that these residues surround a large intersubunit cavity, which was proposed to constitute the ATP binding site. This cavity is formed between two complementary half-shells contributed by the adjacent subunits A and B. When viewed from the side (i.e., in parallel to the membrane plane), the upper left and the lower right boundaries of each ATP-binding site are constituted by the upper body and the left flipper of subunit A and the lower body and dorsal fin of subunit B, respectively (Kawate et al., 2009). By labeling of engineered cysteines with thiol-reactive ATP-analogs (Jiang et al., 2011) and by voltage-clamp fluorometry studies (Lörinczi et al., 2012), it was confirmed that this cavity constitutes the ATP binding site and that the flexible head domain of subunit A that projects over the binding site moves substantially during ligand binding and/or channel gating (Hattori and Gouaux, 2012; Jiang et al., 2012; Lörinczi et al., 2012). The zP2X4 structure provided a basis for rational mutant design, *in silico* docking, and molecular dynamics simulations which were crucial to identify the three lateral ion-access-pathways, and to improve our understanding of the molecular mechanisms of ligand binding and channel gating (Jiang et al., 2010; Rokic et al., 2010; Allsopp et al., 2011; Bodnar et al., 2011; Jiang et al., 2011; Kawate et al., 2011; Samways et al., 2011; Wolf et al., 2011; Du et al., 2012a; El-Ajouz et al., 2012; Jiang et al., 2012; Lörinczi et al., 2012; Roberts et al., 2012; Hausmann et al., 2013). However, docking of ATP proved difficult due to the spatial diversity of the ATP-binding pockets of different P2X subtypes, the high flexibility of the critical lysine residues, and a multitude of possible binding-modes within the relatively large (compared to the ATP molecule) binding pocket. The precise mode of ATP-binding was only determined by crystallization of an ATP-bound zP2X4R (Hattori and Gouaux, 2012). The phosphate chain and the adenine ring of the ATP molecule are folded in an U-shaped configuration within the ATP-binding pocket. The phosphate oxygens of ATP are coordinated by the side chains of K70 and K72 within the lower body of subunit B and N296, R298, and K316 within the upper body of subunit A (zP2X4 numbering). In agreement with labeling experiments at the P2X2R using a thiol-reactive ATP-analog (Jiang et al., 2011), the adenine base of ATP is making hydrophobic interactions with L191 of the lower body and I232 of the dorsal fin (subunit B). It is further stabilized by hydrogen bonds with the side chain of T189 and the backbone of K70, both located within the lower body of subunit B. The ribose moiety is facing the solution (Lörinczi et al., 2012) and recognized solely by hydrophobic interactions with L217 within the dorsal fin of subunit B (Hattori and Gouaux, 2012).

SPECIFIC CHARACTERISTICS OF THE ATP-BINDING SITES IN HETEROMERIC P2XRs

Under the condition of similar conformations of the subunits and their uniform arrangement (Kawate et al., 2009; Hattori and Gouaux, 2012), three equivalent intersubunit ATP-binding sites can be assumed in one homomeric P2XR. Based on the zP2X4

structure, homology models of the ATP-binding sites of homomeric P2X1, P2X2, P2X3, P2X4, and P2X7 receptors have been generated so far (Keceli and Kubo, 2009; Roger et al., 2010; Allsopp et al., 2011; Bodnar et al., 2011; Jiang et al., 2011; Wolf et al., 2011; El-Ajouz et al., 2012; Jiang et al., 2012; Lörinczi et al., 2012; Roberts et al., 2012; Schwarz et al., 2012; Hausmann et al., 2013). As outlined above, three significantly different intersubunit binding sites are formed in the case of heteromeric P2X assemblies: one between two identical subunits and two at the heteromeric interfaces A/B and B/A. The structural differences of the latter two are mainly due to structural differences in the upper bodies and left flippers as exemplarily shown for P2X2 and P2X3 subunits (**Figure 1**) and lead to different interfaces and volumes of the hydrophilic cavities. In addition, the overlapping head domains differ significantly in their backbone conformation and side chain orientation. These domains are characterized by a highly conserved pattern of three disulfide bridges that constrain the rather non-conserved sequences between the cysteine residues in loops of different length and structure. Although these domains do not directly contribute to ATP binding, they appear to be important for channel gating and might have crucial influence on antagonist selectivities (Wolf et al., 2011; El-Ajouz et al., 2012; Hattori and Gouaux, 2012; Jiang et al., 2012; Lörinczi et al., 2012; Roberts et al., 2012). The side chains of residues responsible for the coordination of ATP, e.g., as illustrated for selected basic residues in **Figure 1**, are in different spatial orientation and in unequal distances relative to each other. Evidence for varying distances and/or orientation of residues critical for ATP binding was also obtained in cysteine cross-linking experiments at P2X1/P2X2 interfaces in the respective heteromer (Marquez-Klaka et al., 2009). Here, co-expression of K68C-P2X1 and F289C-P2X2 subunits resulted in spontaneous intersubunit cross-linking, while co-expression of F291C-P2X1 and K69C-P2X2 subunits did not produce significant amounts of SDS-resistant dimers. As a consequence of the spatial orientations, unequal binding sites are formed where ligands likely adopt substantially different binding modes and consequently different binding affinities and specificities. This is reflected in distinct pharmacological characteristics of heteromeric and homomeric P2XRs (Gever et al., 2006; Jarvis and Khakh, 2009; Coddou et al., 2011b) and may open the possibility for the development of ligands that are able to specifically block heteromeric P2XRs. However, so far it is not known, if a heteromeric P2XR can be blocked by specific targeting of one of the two heteromeric ligand binding sites or how many binding sites have to be occupied by antagonists to inhibit the channel.

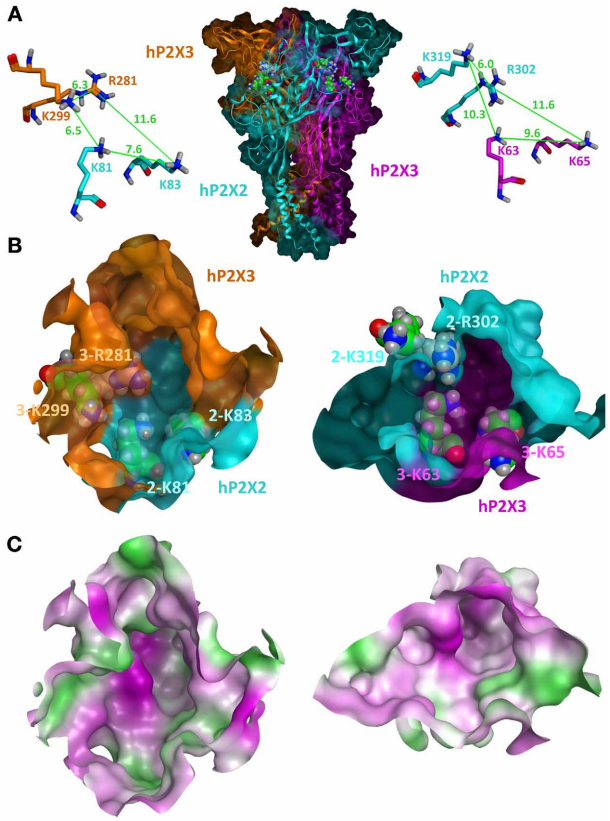

FIGURE 1 | Homology model of the closed state of the human P2X2/3R. (A) A side view of the heterotrimeric hP2X2(3)$_2$R in which both heteromeric interfaces are visible is shown in the middle. The P2X2 subunit is colored in cyan and the two P2X3 subunits are colored in orange and pink. Selected basic residues important for ATP binding are shown as spheres within the two intersubunit ATP-binding sites. For clarity, the same residues are shown as sticks in a close-up view with depiction of the distances between their side chains. **(B)** Frontal close-up views of the two heteromeric ATP-binding sites with a partial transparent surface to indicate the orientation of the residues. The coloring of the subunits is the same as in **(A)**. Differences in the three-dimensional structures and volumes of the hydrophilic cavities are clearly seen. **(C)** Surface representations of the same view as in **(B)** with gradual depiction of hydrophobic (green) or hydrophilic (pink) areas/residues. Neutral areas are shown gradually white. The hP2X2/3R homology model was generated and visualized by the molecular modeling program MOE2012.10 (Molecular Operating Environment 2012, CCG, Montreal, Canada) using the apo zP2X4 crystal structure (PDB entry 3H9V; (Kawate et al., 2009) as a template as previously described (Wolf et al., 2011; Hausmann et al., 2013).

COOPERATIVITY OF ATP BINDING IN P2XRs

Binding of agonist is supposed to induce channel opening by a closing movement of the head domain that is further propagated to the TM domains (Hattori and Gouaux, 2012; Jiang et al., 2012; Lörinczi et al., 2012). Dose response curves for ATP yield generally Hill-slopes greater than one (Bean, 1992; Brake et al., 1994) indicating that more than one agonist molecule binds before channel opening occurs. However, analyses of dose response curves are deceptively complex (Colquhoun, 1998) and the Hill-slope can only serve as a rough estimate of the minimum number of binding sites. The reduced Hill coefficients observed for agonist dose response curves at heteromeric P2XRs (Torres et al., 1998; Haines et al., 1999; Surprenant et al., 2000; Jiang et al., 2003) might suggest that the heteromers require fewer agonist molecules for opening but could also be explained by factors like altered cooperativity or desensitization. From electrophysiological analysis and mathematical modeling of the activation and deactivation kinetics of the P2X2, P2X3, and P2X7 receptors it was concluded that two ATP molecules are sufficient to open the channels while occupation of three binding sites enables dilation of the pore in P2X2 and P2X7 subtypes (Karoly et al.,

2008; Yan et al., 2010; Khadra et al., 2012). As detailed below, co-expression of wt (wild type) P2X6 subunits rescued ATP-elicited currents of P2X2 subunits with defective ATP-binding sites. Since receptors with one non-functional ATP-binding site can be activated, this supports the model in which binding of two ATP molecules is sufficient for channel gating (Wilkinson et al., 2006; Hausmann et al., 2012). Similar studies with concatenated subunits indicate that this is also valid for homomeric P2X2Rs (Stelmashenko et al., 2012). In contrast, photoaffinity labeling data at a homomeric non-desensitizing P2X2/1 chimera are better described by a model in which three BzATP molecules must bind to open the channel (Bhargava et al., 2012). In case that binding of two ATP molecules is sufficient to induce channel opening, ATP would need to bind and induce closure of the head domain in at least two of the three distinct ATP-binding sites in heteromeric P2XRs. For antagonists, the situation is likely different. Like in other ligand-gated ion channels, orthosteric antagonists of P2XR are generally larger molecules and supposed to block the closing movement or even induce an opening of the ATP-binding pocket (Du et al., 2012b), thereby preventing the initial gating step. Provided that simultaneous symmetric conformational changes of the three subunits should be favored, it might depend on the size of antagonist if targeting of one ATP binding sites is sufficient to prevent the ATP-induced gating of the whole trimeric channel. In case of the muscle-type nAChR for example, it appears, that occupation of only one agonist binding site by specific peptide antagonists is sufficient to inhibit channel opening, although it has to be considered that this channel has only two agonist binding sites (Groebe et al., 1995).

PROPERTIES OF HETEROLOGOUSLY EXPRESSED P2XRs

HOMOMERIC EXPRESSION

When heterologously expressed, most mammalian P2X subunits readily form homotrimeric complexes with functional and pharmacological properties described below. For P2X5Rs species-specific differences in expression efficiency and functional properties such as ion permeability were observed (Collo et al., 1996; Garcia-Guzman et al., 1996; Cox et al., 2001; Jensik et al., 2001; Ruppelt et al., 2001; Diaz-Hernandez et al., 2002; Wildman et al., 2002). This subunit occurs in humans predominantly as a non-functional splice variant (Le et al., 1997; Bo et al., 2003; Duckwitz et al., 2006) and expression of the mouse and rat isoforms is inefficient (Collo et al., 1996; Cox et al., 2001; Wildman et al., 2002). For P2X6Rs, heteromerization with other subunits appears to be obligatory for correct folding and trimeric assembly (see below) (Collo et al., 1996; Soto et al., 1996b; Nawa et al., 1998; King et al., 2000; Aschrafi et al., 2004; Barrera et al., 2005).

P2XRs are generally classified in rapidly desensitizing (P2X1 and P2X3), and slowly or non-desensitizing (P2X2, P2X4, P2X5, and P2X7) receptors although desensitization properties can change under cell-free conditions (Ding and Sachs, 2000). For P2X2, P2X4, and P2X7 receptors, additional permeability states have been described (Khakh et al., 1999; Virginio et al., 1999a,b; Khadra et al., 2012, 2013), for details see (Kaczmarek-Hajek et al., 2012), which are little understood on the molecular level.

AGONIST PHARMACOLOGY OF HOMOMERIC P2XRs

As a recent and detailed review of P2XR pharmacology is available (Coddou et al., 2011b), the following paragraphs summarize only some of the key functional properties of homomeric P2XRs with a focus on those characteristics that have been used to differentiate homomeric and heteromeric P2XRs.

The EC_{50} values for ATP range from submicromolar concentrations for P2X1, P2X3, and P2X5 to low micromolar concentrations for P2X2 and P2X4 receptors. At the P2X7R, EC_{50} values for ATP range from about 0.1 to 1 mM for the rat and mouse isoforms, respectively. BzATP is at least one order of magnitude more potent at this receptor (Rassendren et al., 1997) but has also considerable activity at other P2X isoforms (Anderson and Nedergaard, 2006). Agonist potency at P2X7Rs decreases if Ca^{2+} or Mg^{2+} are present in the recording solution. Ca^{2+} decreases the potency of orthosteric agonists independently of the free agonist concentration, thus acting as an allosteric inhibitor (Yan et al., 2011). In the case of Mg^{2+}, the inhibition seems to be due to both an inhibitory Mg^{2+} binding site and a lower or absent agonist activity of Mg^{2+}-bound ATP at the P2X7R (Virginio et al., 1997; Klapperstück et al., 2001; Acuna-Castillo et al., 2007). Differences in the sensitivity to free ATP and Mg^{2+}-bound ATP were also reported for P2X2 and P2X4 receptors but not seen with P2X1, P2X2/3, and P2X3 receptors (Li et al., 2013).

All P2XR agonists known so far are nucleotide analogs. ATPγS and αβ-meATP are metabolically more stable and widely used to investigate ATP-gated channels in native tissues. In addition, 2-MeSATP, diadenosine polyphosphates, and other nucleoside triphosphates are used as P2X agonists. Interestingly, fast desensitization and slow recovery from desensitization of P2X1R and P2X3R seems to be associated with the formation of high-affinity binding sites for ATP, αβ-meATP, and the antagonist trinitrophenyl-ATP (TNP-ATP). At homomeric P2X2Rs, αβ-meATP is an agonist with very low potency (EC_{50} >100 μM), while homomeric P2X4Rs are activated in a species-dependent manner with EC_{50} values ranging from 5 to 100 μM. P2X7Rs are not readily activated by αβ-meATP (EC_{50} >> 100 μM). In heteromeric assemblies, P2X1, P2X3, P2X5, or P2X6 subunits appear to confer αβ-meATP sensitivity. In contrast, the diadenosine tetraphosphate Ap_4A, is a full and potent agonist at the homomeric P2X2R (Pintor et al., 1996; Wildman et al., 1999a), but is inactive at the heteromeric P2X2/6R (King et al., 2000).

ANTAGONIST PHARMACOLOGY OF HOMOMERIC P2XRs

The large polysulfonated naphtylurea suramin and the pyridoxalphosphate derivative PPADS are among the earliest and still most widely used P2XR antagonists. Suramin also inhibits other purinergic receptors and G-proteins and more specific analogs ("NF compounds") have been developed. Of these, NF449 is highly selective for P2X1Rs and NF770 is moderately selective for P2X2Rs. PPADS is a more specific inhibitor for P2 receptors but acts in a non-competitive way. More potent analogs ("MRS compounds") with certain selectivity for P2X1 (MRS2220), P2X1 and P2X3 (MRS2257), and P2X1, P2X3, and P2X7 (MRS2159) have been developed (Coddou et al., 2011b).

The nucleotide analog TNP-ATP is a competitive antagonist with nanomolar affinity for recombinant P2X1, P2X3, and

P2X2/3 receptors, and micromolar affinity for recombinant P2X2 and P2X4 receptors (King et al., 1997; Virginio et al., 1998). Another nucleotide-based antagonist diinosine pentaphosphate (IP_5I) showed improved stability in native tissues and is a potent and selective P2X1R antagonist (King et al., 1999). Oxidized ATP is used as an irreversible P2X7 antagonist but requires extensive pre-incubation. New types of mostly non-nucleotidic and subtype-specific antagonist have been developed during the last two decades. These efforts focused mainly on the P2X3 and P2X7 subtypes that appeared to be the most relevant drug targets. Among others, the P2X3 and P2X2/3-selective antagonists RO-51, RO-3, TC-P262, AF353 (RO-4), A-317491 and the P2X7-selective antagonists A-438079, A-740003, A-804598, A-839977, AZ 11645373, AZ 10606120 are commercially available. In particular for P2X7Rs, however, species specificity has to be considered. The commonly used inhibitors KN-62 and BBG for example, are selective for human and rat P2X7 isoforms, respectively (Donnelly-Roberts et al., 2009).

At the P2X4R, which has been difficult to target, 5-BDBD has been shown to be a comparably potent blocker ($IC_{50} \sim 0.5\,\mu M$) (Donnelly-Roberts et al., 2008) and very recently, PSB-12054 was introduced as hP2X4R antagonist with submicromolar potency ($IC_{50} \sim 0.2\,\mu M$) (Hernandez-Olmos et al., 2012). In addition, more specific P2X2-antagonists, the anthrachinone derivatives PSB-10211 and PSB-1011, were introduced (Baqi et al., 2011).

ALLOSTERIC MODULATORS OF P2XRs

Acidification enhances responses of agonists and suramin at the rat P2X2R (King et al., 1997). Likewise, P2X2/3 (Stoop et al., 1997) and, with a distinct pattern, P2X2/6 (King et al., 2000) and P2X1/2 (Brown et al., 2002) receptors are modulated by protons (see below). Mutation of H319 to alanine removed the potentiating effect of acidification on rat P2X2Rs (Clyne et al., 2002). Its characteristic modulation by protons can help to distinguish P2X2Rs since all other homomeric P2XRs are inhibited by acidification [for details see (Coddou et al., 2011b)].

Mg^{2+} was recently shown to directly inhibit P2X1, P2X3, and P2X7 receptors, while P2X2 and P2X2/3 receptor responses were insensitive or less sensitive to Mg^{2+} when activated by supermaximal concentrations of free ATP (Li et al., 2013).

The trace metals Zn^{2+} and Cu^{2+} allosterically modulate P2XRs in complex subtype- and species-specific ways and via non-conserved binding sites [for details see (Coddou et al., 2011a)]. In summary, Zn^{2+} inhibits P2X1 and P2X7 receptor currents and modulates P2X2, P2X3, P2X4, and P2X5R currents in a biphasic way, i.e., it potentiates at low concentrations and inhibits at high concentrations. The Zn^{2+} binding site of the rat P2X2R has been localized at the interface between adjacent subunits (Nagaya et al., 2005). Like zinc, copper modulates the rat P2X2R in a biphasic way, while the human P2X2R and the P2X7R are only inhibited by zinc and copper (Tittle and Hume, 2008; Coddou et al., 2011a). A peculiarity of the P2X4R is that it is differentially modulated by Zn^{2+} and Cu^{2+}. While $Zn^{2+}$and Cu^{2+}both coordinate at the inhibitory allosteric site, Zn^{2+} can additionally bind to a positive allosteric site explaining the different modulatory effects of zinc and copper at the P2X4R (Coddou et al., 2003, 2007, 2011a).

The antiparasitic agent ivermectin (IVM) has agonist properties at invertebrate glutamate receptors (GluRs) and is also pharmacologically active at a number of other ligand-gated ion channels. At homomeric and heteromeric P2X4Rs it is an allosteric modulator (Khakh et al., 1999; Priel and Silberberg, 2004). In the absence of efficient P2X4R inhibitors, it has been widely used as a tool to dissect P2X4 subunit containing receptors. However, more recent studies show that IVM can also species-specifically potentiate P2X7R current amplitudes (Casas-Pruneda et al., 2009; Surprenant and North, 2009; Nörenberg et al., 2010).

PHARMACOLOGY OF HETEROMERIC RECEPTORS

In most functionally characterized heteromers (see below) the kinetic and ligand-binding properties of the constituting subunits are combined and the slowly desensitizing subunit generally dominates the kinetic, while the subunit with higher affinity for agonist and/or antagonist appears to increase the sensitivity of the heteromer for the respective ligand.

In agreement with this, the P2X2/3 heteromer appears to adopt largely the pharmacological profile (agonist potency, TNP-ATP sensitivity) of the P2X3 subunit, which is present twofold within the complex (Jiang et al., 2003). Although their stoichiometry is not known, the P2X1/4R and the P2X1/5R also show a pharmacological profile more similar to the P2X1R and kinetic properties that resemble the P2X4R or P2X5R, respectively (Coddou et al., 2011b). The often slightly lower potency of agonists at the heteromers is too subtle for a true discrimination from the P2X1 or P2X3 homomer and most likely reflects a lower affinity for ATP at the heteromeric ATP-binding sites. In case of the heteromeric P2X2/6 or P2X4/6 receptors the pharmacological properties are similar to that of the P2X2R or P2X4R, respectively (Coddou et al., 2011b). For the P2X2/6R a stoichiometry of two P2X2 and one P2X6 subunits was suggested (Hausmann et al., 2012). The heteromeric P2X1/2R shows a kinetic and pharmacological profile that resembles the P2X1R, although a $P2X1(2)_2$ stoichiometry has been suggested (Aschrafi et al., 2004). However, it adopted the pH sensitivity from the P2X2 subtype (Brown et al., 2002). The H^+ and Zn^{2+} sensitivity of the P2X2 subtype are also conferred to the P2X2/3 and P2X2/6 heteromers (Li et al., 1996; Stoop et al., 1997; King et al., 2000).

There are a few compounds that appear to be able to discriminate between homomeric and heteromeric P2XRs. The photoreactive [$\gamma-^{32}$P]8-Azido-ATP is an effective agonist at homomeric P2X3Rs but not at heteromeric P2X2/3Rs. It also efficiently labeled homomeric P2X3Rs, but was inefficient at homomeric P2X2Rs and heteromeric P2X2/3Rs (Koshimizu et al., 2002). Likewise, Ip_5I was shown to inhibit α,βme-ATP-induced responses of homomeric P2X1 and P2X3 receptors with low micromolar potency, but is virtually inactive at the heteromeric P2X2/3R and the homomeric P2X2R (King et al., 1999; Dunn et al., 2000). Also RO-85, an orally bioavailable drug-like P2X3R antagonist, is selective for the P2X3R over the P2X2/3R and other P2XR subtypes (Brotherton-Pleiss et al., 2010). Interestingly, all these substances appear to lose their affinity at the heteromeric receptor despite the fact that at least one P2X3-P2X3 interface is preserved in the heteromer. Possible explanations for this discrepancy would be, that the P2X3-P2X3 interface is markedly altered

by inclusion of the single P2X2 subunit in the complex and/or that occupation of one interface is insufficient and more than one ligand has to bind to produce an efficient channel block. So far, no compound has been identified, that is selective for any of the heteromeric receptors. Thus, it remains questionable, if the two requirements for selective heteromer targeting (1) selective binding to one (or both) heteromeric interfaces and (2) efficient blockade of the whole receptor by occupation of one (or both) heteromeric interface(s) can be fulfilled. In case of small antagonists, the specific recognition area (contributed by both subunits) might be too small and the critical lysine residues too flexible to allow high selectivity. Also, the small ligand volume and the comparably few interactions with both subunits might not provide sufficient steric hindrance to allosterically block the gating movements in all three subunits.

In this regard, it is also important to consider that P2XRs differ substantially from other ligand-gated ion channels in having three intersubunit ion access pathways that widen during channel opening (Kawate et al., 2011; Samways et al., 2011; Hattori and Gouaux, 2012; Roberts et al., 2012). In combination with the high flexibility of the lower ectodomains and their linkers to the TMs, this might enable P2XRs to tolerate or compensate also more or less pronounced antagonist induced conformational changes before a complete channel block occurs.

DISTRIBUTION OF P2XR SUBUNITS

Although transcripts for more than one P2X subunit are found in most cell types (**Table 1**), there is good evidence (in particular from studies on knockout mice) for homomeric P2X1, P2X2, P2X3, P2X4, and P2X7 receptors in at least some native tissues (e.g., Cockayne et al., 2005; Finger et al., 2005; Sim et al., 2007; Nicke, 2008). However, properties of heterologously expressed homomeric P2XRs more often do not match with those observed in native tissues. In addition to the heteromers described below and yet unidentified combinations of two or three different P2X subunits, splice variants and interacting proteins most likely contribute to the diversity of P2XR signaling.

HETEROOLIGOMERIZATION OF P2XR SUBUNITS

In a systematic biochemical analysis of pairwise co-expressed Flag- and HA-tagged rat P2X subunits in HEK cells (Torres et al., 1999a) it was found that (a) all subunits, except for P2X6 subunits, were able to homo-oligomerize (see also Aschrafi et al., 2004; Barrera et al., 2005), (b) P2X7 subunits did not hetero-oligomerize with other subunits (see also Nicke, 2008; Boumechache et al., 2009), and (c) the following pairs could be mutually co-purified: P2X1/2*, P2X1/3, P2X1/5*, P2X1/6, P2X2/3*, P2X2/5*, P2X2/6*, P2X3/5, P2X4/5, P2X4/6*, and P2X5/6. Heteromerization between P2X4 and P2X5 subunits was recently confirmed in an ELISA assay where a strong increase in the surface expression of a trafficking deficient P2X5 mutant by co-expression of the P2X4 subunit was observed (Compan et al., 2012). However, this heteromer has not been further characterized so far. In addition, P2X1/4* and P2X4/7* pairs were identified in co-purification studies (Nicke et al., 2005; Guo et al., 2007). Pairs marked with * have also been functionally investigated and partly confirmed in additional studies that are described below. It has to be noted, however, that the differentiation of a heterotrimeric assembly between two given subunits or association of their respective homotrimeric complexes can generally not be achieved by standard co-purification protocols. In particular, in case of the P2X4/P2X7 interaction, data from more detailed biochemical analysis are in favor of the latter (Nicke, 2008; Antonio et al., 2011).

P2X1/2 HETEROMERS

Biochemical evidence

Biochemical evidence for heteromeric P2X1/2Rs was further confirmed by studies in *Xenopus laevis* oocytes. In blue-native (BN)-PAGE and disulfide cross-linking studies, the metabolically labeled heterotrimeric P2X1/2R complexes were directly visualized and discriminated by their size from the respective homomers (Aschrafi et al., 2004; Marquez-Klaka et al., 2009). These data clearly excluded the possibility that the co-purification experiments were biased by artificial aggregation of the overexpressed protein or clustering of homotrimeric receptors (see below). Interestingly, no homotrimeric His-P2X1 complexes were detected in the BN-PAGE study and functional analysis of the receptors formed from substituted cysteine mutant P2X1 and P2X2 subunits revealed no current corresponding to homomeric P2X1Rs unless this subunit was injected in more than 6-fold excess. This suggests that in the presence of P2X2 subunits, the P2X1 subunit assembles preferentially as heteromer. However, discrepancies in the amount and speed in which the subunits are expressed could also account for this observation. Further analysis of the selectively radioiodinated heteromers in the plasma membrane revealed significantly more radioactivity in the band corresponding to the $P2X_2$ subunit than in the His-tagged $P2X_1$ subunit, suggesting a $P2X1(2)_2$ stoichiometry (Aschrafi et al., 2004).

Functional evidence

In a carefully conducted study by Brown et al. (2002) the subtle differences between the fast desensitizing homomeric P2X1R and the heteromeric P2X1/2R could be discriminated by their sensitivity to extracellular pH. In contrast to P2X1 homomers, which show decreased agonist potency at acidic pH and are not affected by alkaline pH, agonist efficacy at the P2X1/2 heteromer is increased under both alkaline and acidic pH (Brown et al., 2002). In contrast to the biochemical studies, hetero-oligomerization was found to be inefficient in this study and only one in six oocytes showed the pH-potentiated P2X1-like responses. The difficulty to resolve the P2X1/2 heteromer current could be partly overcome by specific disulfide cross-linking of oocyte-expressed P2X1 and P2X2 mutants resulting in non-functional P2X1/2Rs and their activation following reduction with dithiothreitol (Marquez-Klaka et al., 2009). However, the reduced agonist sensitivity in these mutants has to be considered. The fast desensitizing phenotype found for the P2X1/2 heteromer is somehow exceptional because in all other functionally described P2X heteromers the slowly desensitizing subunit determines the kinetic of the heteromer.

Table 1 | Summary of the distribution of P2XR subunits in selected mammalian cell types.

Cell line/type	Identified transcripts							Identified protein							Functional data similar to
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	
T-cells: Jurkat cell line[1]	(+)			+	+		(+)	+			+			+	Ca^{2+} imaging, siRNA: P2X1, P2X4, P2X7
T-cells: human primary CD4+[1]	(+)			+	+		+	+			+				Pharmacological inhibition, Ca^{2+} imaging: P2X1, P2X4
Mast cells: LAD2 and human lung mast cells[2]	+			+		(+)	+								Patch clamp: P2X1, P2X4, P2X7
Freshly isolated mouse peritoneal macrophages[3,4,46]	(+)			+			+	+			+				KO mouse: P2X1, P2X4
J774 cells[5]			+	+	(+)	+	+		+		+			+	Ca^{2+} imaging, patch clamp: P2X4, P2X7
Mouse spleen macrophages[5]			+	+	+	+	+	(+)	(+)	+	+	+	+	+	
Human B lymphocytes[6,7]	+			+			+	+	+		+			+	
HeLa cells[8]			(+)	+	+	+	+							+	Ca^{2+} imaging, changes in ATP-induced apoptosis: P2X7
Myocytes from renal artery[9]	+			+											Patch clamp: P2X1, P2X1/4
Endothelial cells[10,11,12]	+			+	(+)		+				+			+	Ca^{2+} imaging: P2X4; KO mouse: P2X4, P2X1; patch-clamp: P2X7
Dorsal root ganglia (DRG) neurons[13,14,15,16,17]		+ [a]	+	+					+	+		+			Patch clamp: P2X3, P2X2/3; KO mouse: P2X3, P2X2, P2X2/3
Nodose ganglia neurons[13,15]		+	+	+					+	+					Patch clamp, KO mouse: P2X2, P2X2/3
Superior cervical ganglion (SCG) neurons[13,18,19]	(+)	+	+	+	+	+	(+)	+	+	(+)	+		+		Patch clamp, Ca^{2+} imaging: predominantly P2X2; KO mouse: P2X1
Urinary bladder afferent neurons[15,20,21,22]									+	+					Bladder function, patch clamp: P2X3, P2X2/3; KO mouse: P2X2, P2X3, P2X2/3
P19 cells[23,24,25,26]															Ca^{2+} imaging: P2X2, P2X2/6, P2X4; RNAi: P2X2, P2X7
Undifferentiated		(+)	+	+	+	(+)	(+)		(+)	(+)	+		(+)		
Differentiated to progenitors	(+)	+	(+)	(+)	+	+	+	(+)	+	(+)	(+)		+	(+)	
Neuronally differentiated neural progenitors[27]		+	(+)	(+)	(+)	+									
Astrocytes[28,29,30,31]	+	(+)	+	+	+	+	+	+	+	+	+		+	+	Patch clamp: P2X1/5; YO-PRO uptake, RNAi: P2X7
Microglia[32,33,34,34,35,36,46]															Nerve injury, KO mouse: P2X4; patch clamp: P2X4, P2X7; YO-PRO uptake: P2X7
Freshly isolated	(+)			+			+								
Primary culture											+			+	
BV-2 cells				+		+	+				+				
C8-B4 cells			(+)	+			+				+			+	

(Continued)

Table 1 | Continued

Cell line/type	Identified transcripts							Identified protein							Functional data similar to
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	
Oligodendrocytes[38]														+	Ca^{2+} imaging: P2X7
Oligodendrocytes (progenitors)[37]								+	+	+	+			+	Ca^{2+} imaging, patch clamp: P2X7
Human salivary gland epithelial (HSG) cells[39]								+	+	+	+	+	+		
Human embryonic kidney (Hek) 293 cells[39]															
Normal conditions											(+)	(+)			
Grown past confluence								+		+	+	+	+		
Vascular smooth muscle cells[40,41,42]	+	+		+				+ [b]		+ [c]	+	+		+	Patch clamp: P2X1[b]
Non-cystic fibrosis epithelial cells (16HBE14o$^-$)[43]						+						+	+		
Cystic fibrosis cells (IB3-1)[43]				+	+	+					+	+	+		RNAi, Ca^{2+} imaging: P2X4, P2X6
Adrenal gland pheochromocytoma (PC12) cells[44,45]															Patch clamp, Ca^{2+} imaging: P2X2
Undifferentiated		+							+						
NGF-differentiated	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

[a] *Except primates,* [b] *except septal vessels,* [c] *except cerebral vessels.*

References in table: [1] *Woehrle et al., 2010a;* [2] *Wareham et al., 2009;* [3] *Sim et al., 2007;* [4] *Ulmann et al., 2010;* [5] *Coutinho-Silva et al., 2005;* [6] *Sluyter et al., 2001;* [7] *Wang et al., 2004;* [8] *Welter-Stahl et al., 2009;* [9] *Harhun et al., 2010;* [10] *Yamamoto et al., 2000;* [11] *Harrington et al., 2007;* [12] *Wilson et al., 2007;* [13] *Lewis et al., 1995;* [14] *Cockayne et al., 2000;* [15] *Cockayne et al., 2005;* [16] *Compan et al., 2012;* [17] *Serrano et al., 2012;* [18] *Xiang et al., 1998,* [19] *Calvert and Evans, 2004;* [20] *Vlaskovska et al., 2001;* [21] *Zhong et al., 2001;* [22] *Zhong et al., 2003;* [23] *Da Silva et al., 2007;* [24] *Resende et al., 2007;* [25] *Resende et al., 2008;* [26] *Yuahasi et al., 2012;* [27] *Schwindt et al., 2011;* [28] *Franke et al., 2001;* [29] *Kukley et al., 2001;* [30] *Lalo et al., 2008;* [31] *Yamamoto et al., 2013;* [32] *Tsuda et al., 2003;* [33] *Qureshi et al., 2007,* [34] *Raouf et al., 2007;* [35] *Ulmann et al., 2008;* [36] *Toulme et al., 2010;* [37] *Agresti et al., 2005;* [38] *Matute et al., 2007;* [39] *Worthington et al., 1999;* [40] *Nori et al., 1998;* [41] *Lewis and Evans, 2000;* [42] *Lewis and Evans, 2001;* [43] *Liang et al., 2005;* [44] *Michel et al., 1996;* [45] *Sun et al., 2007;* [46] *Hickman et al., 2013.*

Evidence from native systems

The solid biochemical evidence for P2X1/2 heteromers is in contrast to the very limited evidence for this heteromer in native tissues. P2X1 and P2X2 subunits are widely distributed and overlap in many tissues (Burnstock and Knight, 2004). Transcripts of both subunits were found for example in vascular smooth muscle (Nori et al., 1998). However, generally, expression levels differed greatly, suggesting at most a small contribution of the respective heteromer. Whole-cell patch clamp and calcium imaging studies in mouse sympathetic neurons revealed a dominant P2X2-like phenotype and an αβ-meATP-sensitive receptor population that was largely absent in P2X1 knockout mice (Calvert and Evans, 2004). This αβ-meATP-sensitive current was blocked by Ca^{2+} and alkaline pH and it was concluded that it corresponds to a P2X1/2 heteromer. However, involvement of a third subunit was suggested since the current was not potentiated at acidic pH and showed a relatively slow time course of response.

P2X1/4 HETEROMERS

Biochemical evidence

Biochemical evidence for heteromerization between co-expressed P2X1 and P2X4 subunits was excluded in two co-immunoprecipitation studies performed in HEK cells (Le et al., 1998; Torres et al., 1999a), but was later found by co-purification and subsequent BN-PAGE analysis of *Xenopus* oocyte-expressed P2X1 and P2X4 subunits (Nicke et al., 2005).

Functional evidence

Functional evidence for the formation of P2X1/4 heteromers came from the generation of a new phenotype upon co-expression of P2X1 and P2X4 subunits in *Xenopus* oocytes (Nicke et al., 2005). The proposed P2X1/4 heteromer shares the moderately desensitizing kinetics and fast recovery upon repeated activation with the P2X4R. In contrast to the P2X4 homomer and in common with the P2X1 homomer, however, it is activated by low micromolar concentrations of αβ-meATP and inhibited by suramin and TNP-ATP. Although these currents were reproducibly found upon co-expression of both subunits, they were comparably small, suggesting that previous attempts to detect this heteromer failed due to preferential homomerization of both subunits.

Evidence from native systems

While heterologously expressed P2X4Rs are efficiently expressed as homotrimers in the plasma membrane, only few native receptors have been identified that resemble exactly the

recombinant homomeric P2X4R, which is relatively insensitive to αβ-meATP (Coddou et al., 2011b). A possible explanation is that both P2X4R expression and its cycling between intracellular compartments and the plasma membrane are highly dynamic and a predominant localization of P2X4Rs in intracellular compartments has been described (e.g., Bobanovic et al., 2002; Qureshi et al., 2007; Stokes and Surprenant, 2009; Toulme et al., 2010). Thus, P2X4Rs appear to be expressed and/or translocated to the plasma membrane only under specific conditions (e.g., upon activation of microglial cells) and might require additional subunits to be stabilized at the plasma membrane. Receptors incorporating P2X4 subunits have been postulated in a wide variety of tissues but little evidence for a P2X1/4 heteromer in native tissues has been provided so far. The P2X4 subunit has the widest distribution pattern of all P2X subunits and overlap with P2X1 subunits is found in many tissues. For example, P2X1 and P2X4 subunits have been identified in different types of immune cells (Sim et al., 2007; Wareham et al., 2009; Woehrle et al., 2010b) and both subunits translocate to the immune synapse in stimulated T cells (Woehrle et al., 2010a). However, data from knockout mice (Sim et al., 2007) and pharmacological analysis (Wareham et al., 2009) indicate the presence of homomeric rather than heteromeric P2X1 and P2X4Rs in mouse macrophages and human mast cells, respectively. Co-expression of both subunits was also found by single cell RT-PCR in smooth muscle cells of renal resistance arteries (Harhun et al., 2010). Here, the characteristics of a current component that was insensitive to the P2X1 antagonist NF279 (Rettinger et al., 2000) were consistent with the properties of the P2X1/4 heteromer. A role of a P2X1/4 heteromer was also considered in neurogenic contractions in the guinea pig urinary bladder (Kennedy et al., 2007) and not excluded in coronary artery smooth muscle (Conant et al., 2008) and erythrocytes (Skals et al., 2009).

P2X1/5 HETEROMERS

Biochemical evidence

Biochemical evidence for the formation of heteromers consisting of P2X1 and P2X5 subunits was initially obtained from HEK293 cells transfected with epitope-tagged P2X1 and P2X5 subunits (Torres et al., 1998; Le et al., 1999). Here, the authors could demonstrate a direct association by co-purification. However, further biochemical evidence for P2X1 and P2X5 subunit interaction is still lacking in other *in vitro* models as well as in native tissues.

Several studies provide **functional evidence** for heteromerization of rat P2X1 and P2X5 subunits upon expression of both subunits in established expression systems like *Xenopus laevis* oocytes (Le et al., 1999; Rettinger et al., 2005) and cultured HEK293, CHO, and COS cells (Torres et al., 1998; Haines et al., 1999; Surprenant et al., 2000). The P2X1/5 heteromer shows distinct functional properties providing clear characteristics for its identification: (a) In contrast to the P2X1 homomer, its currents have a biphasic kinetic with a desensitizing peak and a non-desensitizing plateau current that are not desensitized by repeated agonist application. Depending on pH and ATP concentrations, the current response kinetics of the P2X1/5R differ (Surprenant et al., 2000). (b) It has a significant greater amplitude than the P2X5 homomer and, similar to the P2X1R, is activated by nanomolar ATP concentrations as well as by α,β-meATP (Torres et al., 1998; Haines et al., 1999; Le et al., 1999). (c) PPADS, suramin, and the specific P2X1R blocker NF449 antagonize with similar micromolar potencies the P2X1/5 heteromer and the P2X1 homomer (Haines et al., 1999; Rettinger et al., 2005), while the potency of TNP-ATP at the heteromer is markedly reduced in comparison to the P2X1 homomer and is more similar to that at the P2X5 homomer (Haines et al., 1999; Surprenant et al., 2000; Wildman et al., 2002). (d) A rebound current upon removal of agonist has been observed in some studies (Haines et al., 1999; Lalo et al., 2008). Peak currents of homomeric P2X5Rs are drastically decreased in a Ca^{2+} concentration dependent manner, whereas P2X1R responses are Ca^{2+} insensitive. Interestingly, increased extracellular Ca^{2+} significantly potentiated the steady-state currents of the P2X1/5 heteromer (Haines et al., 1999). Both acidification and alkalization have inhibitory effects on P2X1/5 heteromers while the homomeric receptors are only inhibited by an increased proton concentration (Surprenant et al., 2000).

Evidence from native systems

In contrast to the widely distributed expression of the P2X1 subunit, P2X5 subunit RNA has only been detected in a restricted number of tissues including the heart, sensory and motor neurons of the cervical spinal cord (Collo et al., 1996), vascular smooth muscle (Phillips and Hill, 1999), and astrocytes (Lalo et al., 2008). The presence of P2X1/5 heteromers was suggested in guinea-pig submucosal arterioles where an increase in spontaneous excitatory junction potentials and higher current amplitudes were measured following repetitive ATP application and increased Ca^{2+} concentration, respectively (Surprenant et al., 2000). The electrophysiological profile of this smooth muscle tissue supports rather the presence of P2X1/5 heteromers than P2X1 homomers that has been described in HEK293 cells expressing P2X1 and P2X5 subunits. In more recent studies, based on quantitative real time PCR and pharmacological characterization by whole cell voltage clamp experiments, evidence for P2X1/5Rs in acutely isolated cortical astrocytes was obtained (Lalo et al., 2008). Here, the heteromer appears to be involved in astrocytic excitability, which is driven by phosphoinositides and mediated through the lipid-binding domain of the P2X1 subunit (Ase et al., 2010).

P2X2/3 HETEROMERS

The P2X2/3 heteromer was the first one to be identified functionally and biochemically (Lewis et al., 1995; Radford et al., 1997). So far, it represents the best characterized P2X heteromer and solid evidence for its *in vivo* expression in sensory neurons and its functional role in sensory neurotransmission exists. As several valuable reviews regarding their presence in sensory and autonomic neurons and their physiological importance are available (North, 2002; Brederson and Jarvis, 2008; Burnstock, 2009a; Jarvis, 2010; Khakh and North, 2012), this chapter focuses only on some key findings and more recent studies.

Biochemical evidence and stoichiometry

Evidence for heteromerization of P2X2 and P2X3 subunits was first obtained from co-immunoprecipitation studies of P2X2 and P2X3 subunits heterologously expressed in Sf9 insect cells (Radford et al., 1997). Subsequent co-precipitation studies by Torres et al. (1999b) confirmed the interaction of P2X2 and P2X3 subunits in HEK cells and more recently, BN-PAGE analysis of oocyte-expressed affinity-tagged hP2X2 and hP2X3 subunits demonstrated that both subunits co-assemble in trimeric P2X2/3Rs, which are efficiently expressed at the plasma-membrane (Hausmann et al., 2012). By analysis of disulfide bond formations between engineered cysteine residues in P2X2 and P2X3 subunits a "head-to-tail" subunit arrangement was originally demonstrated (Jiang et al., 2003). Furthermore, this study revealed the presence of adjacent P2X3 subunits but not P2X2 subunits, indicating a $P2X2(3)_2$ stoichiometry of P2X2/3 channels. This stoichiometry was further confirmed by co-expression and functional analysis of P2X2 or P2X3 subunits containing single or double mutated ATP binding sites (Wilkinson et al., 2006). The $P2X2(3)_2$ stoichiometry is indirectly supported by the finding that the degree of potentiation by extracellular Zn^{2+} in the P2X2/3 heteromer was comparable to the limited Zn^{2+} effect seen with concatenated P2X2 trimers that contained only one wild type subunit with two Zn^{2+} binding site mutants (Nagaya et al., 2005).

Functional evidence

Functional evidence for the formation of P2X2/3 heteromers came initially from patch clamp analysis and comparison of rat nodose ganglia neurons and heterologously expressed P2X2 and P2X3 subunits (Lewis et al., 1995). This study revealed a non-desensitizing, αβ-meATP sensitive functional phenotype that could be unambiguously discriminated from the non-desensitizing α,β-meATP insensitive P2X2 and the fast desensitizing α,β-meATP sensitive P2X3 phenotypes. In addition, similar to the homomeric P2X2R, the heteromeric P2X2/3R is strongly potentiated at low pH values, while the homomeric P2X3R is much less pH sensitive and inhibited at low pH (King et al., 1996; Stoop et al., 1997; Wildman et al., 1999b). For a more detailed analysis of the pharmacologic and kinetic properties of heterologously expressed P2X2/3Rs see (Stoop et al., 1997; Virginio et al., 1998; Burgard et al., 2000; Liu et al., 2001; Spelta et al., 2002, 2003).

Evidence from native systems

Evidence from native systems led initially to the identification of heteromeric P2X2/3Rs; α,βme-ATP-elicited current responses in nodose ganglia neurons did not match those of any singly expressed P2X subunit but were reproduced by co-expression of P2X2 and P2X3 subunits (Lewis et al., 1995). Subsequently, convincing evidence for the existence of heteromeric P2X2/3 channels was also provided in sympathetic, trigeminal, and dorsal root ganglia neurons (Cook et al., 1997; Thomas et al., 1998; Burgard et al., 1999; Grubb and Evans, 1999; Ueno et al., 1999; Dunn et al., 2000, 2001; Lalo et al., 2001; Zhong et al., 2001; Petruska et al., 2002). Functional analysis of P2X2 and P2X2/P2X3 knockout mice further demonstrated/confirmed the presence of P2X2/3Rs in sensory and autonomic ganglia neurons and primary afferent nerve fibers in the urinary bladder (Cockayne et al., 2005) and defined the relative contribution of homomeric P2X2Rs and P2X3Rs and heteromeric P2X2/3Rs. This study revealed that ATP-induced currents in dorsal root ganglia (DRG) neurons are mediated by P2X3 and P2X2/3Rs, while those in nodose ganglion neurons are dominated by P2X2Rs and P2X2/3Rs. In sympathetic ganglion neurons, P2X3-containing receptors appear to be of minor functional importance and in the urinary bladder, P2X3Rs and P2X2/3Rs regulate urinary bladder reflexes. For more details about the role of these receptors in afferent sensory neurotransmission, mechanosensory transmission, and pain states see more recent reviews (Wirkner et al., 2007; Burnstock, 2009b; Jarvis, 2010; Burnstock et al., 2011; Khakh and North, 2012). Interestingly, a recent study by Serrano and colleagues showed that in contrast to rodent DRGs, P2X2 transcripts are virtually absent in human and monkey DRG neurons (Serrano et al., 2012). Hence, primate DRG neurons seem to be devoid of P2X2/3Rs and ATP-induced responses are mediated exclusively by P2X3Rs. This finding may significantly affect the translatability of rodent data to validate these receptors as targets for the treatment of pain. P2X2 and P2X3 subunits are also co-expressed in sensory nerve fibers in taste buds (Bo et al., 1999) and gustatory nerves and P2X2/P2X3 double-knockout mice exhibited abolished responses to taste stimuli (Finger et al., 2005; Eddy et al., 2009).

P2X2/5 HETEROMERS

Biochemical evidence

The rat P2X2/5 heteromer represents the most recent addition to the functionally characterized set of P2X subtypes. Apart from co-immunoprecipitation studies, biochemical evidence for its existence was provided by an ELISA assay that measured the increase in plasma membrane appearance of an HA-tagged trafficking-deficient P2X2 or P2X5 subunit by co-expression of the respective other subunit. In addition, their close spatial proximity was shown in bioluminescent resonance energy transfer studies and in bimolecular fluorescence complementation studies. In combination with cross-linking experiments using a membrane-impermeable cross-linker and native perfluorooctanoic acid (PFO)-PAGE analysis, these experiments indicated that P2X2/5 heterotrimers appear with both possible stoichiometries in the plasma membrane (Compan et al., 2012).

Functional evidence

Functional evidence for this heteromer was obtained upon co-expression of both subunits in *Xenopus* oocytes and HEK cells (Compan et al., 2012). Although the presence of homomeric P2X2Rs could not be prevented, these studies revealed a novel phenotype in oocytes with slightly reduced sensitivity to ATP, ATPγS, and BzATP. Interestingly, BzATP showed strongly reduced efficacy at this heteromer. Inhibition of the supposed heteromer by TNP-ATP was increased while αβ-meATP was ineffective at concentrations of 300 μM. Co-expression of both subunits in oocytes and/or HEK cells also produced ATP-activated time-dependent permeability changes for NMDG, YO-PRO-1, and

ethidium that showed a clearly different time course compared to homomeric P2X2Rs and higher absolute fluorescence values or even no saturation. Most remarkably, HEK cells co-expressing both subunits displayed plasma membrane blebbing and flipping of phosphatidylserine from the inside surface of the plasma membrane to the outside surface (PS flip), two hallmark properties of the P2X7R that were not seen in cells expressing P2X2 subunits alone.

Evidence from native systems

Based on co-immunoprecipitation data from total brain and brain stem as well as immunohistochemistry data from dorsal root ganglia, spinal cord and trigeminal mesencephalic nucleus neurons in the mid pons, the presence of P2X2/5 heteromers in these tissues was suggested (Compan et al., 2012). However, the P2X2/5 heteromer differs from a previously characterized P2XR in trigeminal mesencephalic nucleus in its insensitivity to α,β-meATP (Patel et al., 2001).

P2X2/6 HETEROMERS

Biochemical evidence and stoichiometry

Evidence for the interaction of P2X2 and P2X6 subunits was initially obtained by co-immunoprecipitation (Torres et al., 1999a) and more recently, heterotrimerization of these subunits was confirmed by BN-PAGE analysis of oocyte expressed affinity-tagged hP2X2 and hP2X6 subunits (Hausmann et al., 2012). In contrast, to the heterotrimeric hP2X2/6 complex that is efficiently expressed at the plasma membrane, hP2X6 subunits do not form trimeric complexes (Aschrafi et al., 2004; Hausmann et al., 2012) and are retained in the ER. In HEK cells, co-expression of wt P2X6 subunits rescued ATP-elicited currents of P2X2 subunits harboring mutations in ATP-binding residues (such as K69A) that lead to the formation of non-functional homomeric P2X2Rs. Co-expression of wt P2X2 subunits with mutant P2X6 subunits and *vice versa* revealed that functional P2X2/6 heteromers consist of two P2X2 subunits and one P2X6 subunit. The $P2X(2)_26$ stoichiometry is in contrast to an AFM study of antibody-tagged isolated receptors, in which a variable subunit stoichiometry that depended on the relative subunit expression levels was observed (Barrera et al., 2007). However, in this study receptors purified from a crude membrane fraction, which also contained intracellular membranes, were analyzed.

Functional evidence

Functional evidence for the existence of a P2X2/6 heteromer was initially shown in *Xenopus* oocytes (King et al., 2000). Expression of P2X6 subunits alone did not produce functional channels. Co-expression of both subunits resulted in ATP-induced currents that had fast activating and slowly desensitizing kinetics similar to homomeric P2X2Rs, but showed a biphasic current decay upon removal of ATP. In a subset of cells, an additional transient current component was present that was never seen in oocytes expressing exclusively the P2X2 subunit. Another remarkable difference is the loss of the agonist activity of Ap_4A, which is a full agonist at the P2X2R, but was almost inactive at the P2X2/6 heteromer (King et al., 2000). In contrast to homomeric P2X2Rs, which exhibit a current amplitude potentiation at high proton concentrations, heteromeric P2X2/6Rs showed a decreased current amplitude at acidic pH. In HEK cells, however, the co-expression of a four-fold excess of the P2X6 subunit with the P2X2 subunit revealed a current that was potentiated at decreasing pH values and that was not seen in HEK cells expressing exclusively P2X2 subunits (Hausmann et al., 2012). Thus, the cellular background might influence these properties.

Evidence from native systems

Co-expression of P2X2 and P2X6 transcripts has been described in some nuclei of the thalamus and hypothalamus and in specific laminae of the pineal gland (Collo et al., 1996). P2X2 and P2X6 subunits are also co-expressed in P19 embryonal carcinoma cells and neuronal stem cells (Resende et al., 2008; Schwindt et al., 2011). Ca^{2+}-imaging-based pharmacological analysis revealed that P2X4Rs or P2X4-containing heteromultimers mediate ATP-induced calcium-responses of undifferentiated P19 embryonal carcinoma cells, while P2X2Rs and possibly heteromeric P2X2/6Rs are the major mediators of calcium-responses in neuronally differentiated cells after retinoic acid treatment (Resende et al., 2008). However, it remained partially unresolved whether the ATP-induced calcium transients of neuronally differentiated P19 cells are mediated by homomeric P2X2 or heteromeric P2X2/6Rs (Resende et al., 2008), although an simultaneous upregulation of the P2X2 and P2X6 expression was shown during neuronal differentiation (Resende et al., 2007). Also, during neuronal differentiation of neuronal progenitor cells of neurospheres the expression of P2X2 and P2X6 subunits was upregulated (Schwindt et al., 2011). In summary, although P2X2 and P2X6 are co-expressed in several tissues and their simultaneous regulation during neuronal differentiation was shown, there is to our knowledge no clear and direct evidence for the presence of functional heterotrimeric P2X2/6 assemblies in native cells or tissues.

P2X4/6 HETEROMERS

Biochemical evidence

Biochemical evidence for the existence of P2X4/6 heteromers was initially demonstrated by co-purification of epitope-tagged P2X4 and P2X6 subunits upon expression in HEK293 cells (Le et al., 1998; Torres et al., 1999a). There is a controversy regarding the ability of the P2X6 subtype to form homotrimers, since its expression in *in vitro* models failed to generate a functional receptor (Soto et al., 1996b; Le et al., 1998; Khakh et al., 1999; Torres et al., 1999a; King et al., 2000; Aschrafi et al., 2004). It has been suggested that the P2X6 subtype needs to get posttranslationally glycosylated to form a functional homomeric receptor in HEK293 cells (Jones et al., 2004). However, endogenous P2X4Rs have been reported in HEK293 cells (Worthington et al., 1999) (own unpublished observations) and could promote P2X6 subunit assembly and trafficking in form of heteromers. Using AFM in combination with surface biotinylation and immunofluorescence analysis, it has been shown that P2X6 subunits expressed in NRK cells do not form homotrimers. Substitution of 14 uncharged N-terminal amino acid residues by charged residues increased glycosylation and plasma membrane insertion of P2X6 subunits, indicating that their ER release is inhibited by this uncharged N-terminal region (Ormond et al., 2006). Co-immunoprecipitation and

immunofluorescent studies in HEK293 cells showed a preferred assembly of P2X4 subunits into heterotrimeric P2X4/6Rs (Le et al., 1998; Bobanovic et al., 2002) and regulation of the P2X4/6 heteromer trafficking by the P2X4 subunit. The homomeric P2X4R showed an intracellular punctate distribution with sparse localization at the plasma membrane. In contrast, the GFP-tagged P2X6 subunit was diffusely distributed intracellularly and co-localized with the ER marker calreticulin, without any indication for the presence of the P2X6R at the cell surface. However, in the presence of the P2X4 subunit, the P2X6 subunit showed a similar punctate pattern as the homomeric P2X4R (Bobanovic et al., 2002).

Functional evidence

The functional differentiation between homomeric P2X4 and heteromeric P2X4/6 assemblies is difficult, since the biophysical and pharmacological differences are not that clear and controverse data exist regarding the functional expression and αβ-meATP sensitivity of the P2X6R (Collo et al., 1996; Jones et al., 2004). The sensitivity of homomeric P2X4Rs to the partial agonist αβ-meATP is relatively low and species-dependent with EC_{50} values of 7 and 19.2 μM for mouse and human receptors, respectively, whereas rat P2X4Rs showed only very weak responses (Jones et al., 2000). Upon co-expression of P2X4 and P2X6 subunits in *Xenopus* oocytes, receptors were observed that showed (1) a three to five fold higher sensitivity to the partial agonists αβ-meATP and 2MeSATP than the P2X4R, (2) an enhanced inhibition by PPADS, suramin, and RB-2, and (3) peak current amplitudes that differed from those obtained by expression of the P2X4 subunit alone. However, current kinetics and current potentiation by IVM, as well as the effects of modulators like protons and zinc ions were undistinguishable between P2X4 homomers and P2X4/6 heteromers (Le et al., 1998; Khakh et al., 1999).

Evidence from native systems

P2X4 and P2X6 RNAs are abundant and show a broad overlapping distribution throughout the central nervous system with significant amounts in the hippocampus and the cerebellum (Collo et al., 1996; Soto et al., 1996a,b). However, the function of homomeric P2X4 or heteromeric P2X4/6Rs in neurons remains unclear. Immunogold-labeling demonstrated the presence of P2X4 and P2X6 subunits in perikarya and dendritic spines of cerebellar Purkinje and hippocampal, pyramidal CA1 neurons, where they were suggested to form heteromeric assemblies (Rubio and Soto, 2001). Although P2X4 transcripts were found in hippocampal CA1 pyramidal neurons, αβ-meATP induced responses from most CA1 neurons remained unaltered upon IVM treatment, indicating that neither P2X4 homomers nor P2X4/6 heteromers are expressed in these cells (Khakh et al., 1999). However, a possible role of the P2X4 subunit in regulating synaptic plasticity was suggested based on studies with P2X4 knockout mice. The application of IVM on wt-mouse brain slices potentiated the EPSPs from CA1 neurons, whereas IVM had no effect in P2X4 knockout mice, which also showed an impaired long-term potentiation in the hippocampus (Sim et al., 2006). Early studies on rat hippocampal CA3 neurons revealed αβ-meATP-activated responses that were blocked by suramin, but were insensitive to PPADS (Ross et al., 1998), ruling out a contribution of P2X4Rs or P2X4/6Rs to neuronal excitation in these neurons. These findings were confirmed by later studies reporting that IVM had no effect on these cells (Mori et al., 2001; Kondratskaya et al., 2008). However, a significant contribution of P2X4 subunit-containing receptors to the generation of EPSPs was shown in neocortical neurons, where ATP- and αβ-meATP-mediated current responses were potentiated by IVM, but were not inhibited by PPADS (Lalo et al., 2007).

According to functional and biochemical analyses, P2X4 and P2X6 subunits are endogenously expressed in P19 murine embryonal carcinoma cells and show varying expression levels during the non-differentiated and neuronal progenitor states, suggesting a possible role for heteromeric P2X4/6Rs in regulation and induction of neurogenesis (Resende et al., 2007, 2008). P2X4 and P2X6 subtype expression was also found in human non-cystic and cystic fibrosis epithelial cells. The knockdown of the P2X6 subunit by siRNA in these cells resulted in a significant attenuation of Zn^{2+}-mediated Ca^{2+} influx, which was similar to the effect that occurred upon P2X4 subunit knockdown (Liang et al., 2005). Therefore, it was suggested that P2X4/6Rs play a role in the regulation of the Ca^{2+} influx that restores Cl^{-} secretion across the airway epithelia. Immunofluorescent stainings on human umbilicial endothelial cells revealed a colocalization of P2X4 and P2X6 subunits with VE-cadherin in cellular junctions, where they were suggested to be involved in the modulation of cell-cell adhesion processes via the mediation of Ca^{2+} signaling (Glass et al., 2002).

P2X4/7 HETEROMERS

P2X4R and P2X7R genes are located next to each other on human and rat chromosome 12 and murine chromosome 5. Both subunits share a high sequence similarity and show overlapping distribution in many tissues such as epithelia, endothelia, and immune cells.

Biochemical evidence

Biochemical evidence for an association between P2X4 and P2X7 subunits was originally provided by co-immunoprecipitation from transfected HEK cells and from bone marrow-derived macrophages (Guo et al., 2007). Several subsequent studies, however, failed to confirm an association between both subunits within heterotrimeric complexes: BN-PAGE analysis of P2X7 complexes from various native tissues (bone marrow, lymph node, salivary gland) that contained both subunits revealed exclusively complexes that corresponded in size to the homomeric P2X7 complex, which can be differentiated by its size from any heteromeric P2X7-containing complex (Nicke, 2008). Likewise, BN-PAGE analysis and cross-linking data from primary cultures of rat macrophages and mouse microglia revealed an interaction between homomeric P2X7 and P2X4 complexes rather than P2X4/7 heteromers (Boumechache et al., 2009). This study further showed that P2X4Rs were predominantly intracellular, whereas P2X7Rs were mainly localized to the plasma membrane. However, both complexes could be co-immunoprecipitated. Co-expression of P2X4 and P2X7 subunits in NRK cells increased the surface expression of P2X4 subunits about two-fold (Guo

et al., 2007). A pairing of P2X4 and P2X7 (and also P2X2 and P2X4) homotrimers was supported by cross-linking analysis and AFM imaging of HEK cell-expressed receptors (Antonio et al., 2011). In conclusion, there is currently no biochemical evidence for the presence of heterotrimeric P2X7Rs. It has to be noted, however, that P2X2 and P2X4 receptors have been found to be rather "sticky" proteins that appear to associate into higher aggregates more easily than other subunits (Aschrafi et al., 2004; Weinhold et al., 2010) (observation from the authors labs) and also P2X1 trimers appear to associate into dimers and higher complexes under certain conditions (Nicke, 2008). Whether this represents an expression-level and/or detergent-dependent artefact or is the result of a specific interaction is not clear at present. In the mouse alveolar epithelial E10 cell line, P2X4 and P2X7 receptors were shown to associate partly with lipid rafts and the P2X7R was found to interact with caveolin 1 (Barth et al., 2008). Co-immunoprecipitation, high resolution clear native PAGE, and BN-PAGE data suggest that all three proteins can be constituents of higher order protein complexes in these cells, but that caveolin 1 interacts only with P2X7Rs directly. In support of this, knock down by shRNAs demonstrated that downregulation of P2X7 subunits affects protein levels, localization, and complex organization of both caveolin 1 and P2X4. In contrast, P2X4 knockdown affected only P2X7 protein levels and localization but not caveolin 1 (Weinhold et al., 2010). In both cases, upregulation of the respective other P2X subunit was found. In the kidney, however, a mutual negative influence on subunit expression levels was found (Craigie et al., 2013). Using the respective knockout mice, it was shown that ablation of one subunit significantly reduced the mRNA levels of the respective other subunit.

Functional evidence for mutual interactions

Functional evidence for mutual interactions between P2X4 and P2X7 subunits and/or receptors has been found in HEK cells co-transfected with both subunits (Guo et al., 2007; Casas-Pruneda et al., 2009). Thus, co-expression of a dominant negative P2X4 subunit was found to reduce the P2X7R currents without reducing their number in the plasma membrane and to confer IVM and TNP-ATP sensitivity as well as some BBG resistance to the P2X7R (Guo et al., 2007). In another study, the ATP-activated current decay in TEA^+-containing solution was accelerated by co-expression of P2X4 subunits, which themselves showed no current under these ionic conditions. Furthermore, ethidium uptake was slowed down and decreased, a P2X fraction with lower ATP-sensitivity was observed, and a concentration-dependent lack of potentiation by IVM was seen in the presence of P2X4 subunits (Casas-Pruneda et al., 2009).

Evidence from native systems

Co-expression of mouse P2X4 and P2X7 subunits in HEK cells reproduced the properties of ATP-activated currents in parotid acinar cells better than expression of each subunit alone (Casas-Pruneda et al., 2009). In freshly isolated rabbit airway ciliated cells an ATP-gated cation channel has been characterized (Ma et al., 2006) that shares the following properties with the P2X7R but not the P2X4R: (a) low sensitivity to ATP, (b) modulation by external Na^+, (c) inhibition by extracellular divalent cations as well as (d) sensitivity to the P2X7 antagonists BBG and KN-62. However, in contrast to the P2X7R, this P2X(cilia) did not show pore dilation and, in agreement with P2X4R-associated properties, its current was augmented by Zn^{2+} and IVM. A mixture of both homomeric channels was excluded because the dose response curve of this channel could be described by a simple Hill equation and a P2X4/7R heteromer or a modified P2X4R were proposed as possible explanations. In support of a homomeric P2X7R, however, it was recently found that IVM also species-specifically potentiates P2X7R current amplitudes in a very similar manner as described for the P2X(cilia) (Casas-Pruneda et al., 2009; Nörenberg et al., 2010), see also Surprenant and North (2009). Adding to the complexity of P2X7R characterization, polymorphisms have been identified in human and mouse P2X7 isoforms that influence their ability to form dye permeable pores, at least when studied in native cell preparations (Gu et al., 2001; Adriouch et al., 2002; Le Stunff et al., 2004; Sorge et al., 2012). It has to be noted, however, that no obvious functional differences were found if the respective recombinant channels were studied (Boldt et al., 2003; Donnelly-Roberts et al., 2009; Schwarz et al., 2012; Xu et al., 2012), suggesting the involvement of cell-specific factors in pore formation and/or assay-dependent/methodological differences in these studies. Thus, species-specific differences in pharmacology as well as polymorphisms need to be considered when characterizing P2X7Rs in native tissues.

Functional interactions between both receptors were recently also shown in mouse macrophage RAW246.7 cells, where P2X4 knock down by shRNA suppressed ATP-induced cell death and release of HMGB1 and Il1β, and facilitated the production of reactive oxygen species. However, P2X4 subunit knock down did not affect P2X7-mediated pore formation and MAPK signaling (Kawano et al., 2012a,b). From similar experiments, it was suggested that the P2X4R positively modulates P2X7-dependent cytokine release from bone marrow-derived dendritic cells (Sakaki et al., 2013). Most recently, a supposedly P2X4-containing receptor was described in murine myenteric neurons. Compared to the homomeric P2X4R this receptor had lower ATP sensitivity but increased sensitivity to the antagonists PPADS and suramin and its current was potentiated by IVM. Since P2X2 and P2X7 subunits had also been reported to be expressed in these cells, the possibility of P2X2/4/7 heteromers was considered (Maria et al., 2013).

EVIDENCE FOR CLUSTERING OF TRIMERIC P2XRs

For heterologously expressed P2X2Rs it was observed that properties such as mean open times, open channel noise, potentiation by Zn^{2+} and pH, the EC_{50} value for ATP, the ability to form large pores, and the inward rectification depend on the P2X2 expression level or density (Ding and Sachs, 2002; Clyne et al., 2003; Fujiwara and Kubo, 2004), suggesting interactions between homotrimeric P2X2Rs. A physical interaction between P2X2Rs is supported by biochemical evidence for an increased tendency of this receptor to form higher order complexes (Aschrafi et al., 2004) and high densities of GFP-tagged P2X2Rs could be observed upon their activation-dependent clustering in embryonic hippocampal neurons (Khakh et al., 2001). In addition, physical interactions between homotrimeric P2X7 and

P2X4 receptors, and P2X2 and P2X4 receptors have been observed (Boumechache et al., 2009; Antonio et al., 2011). Whether these interactions are direct or via clustering molecules or within lipid domains and whether they have physiological relevance or represent overexpression artefacts remains to be determined. In this context, it is interesting that P2X4 and P2X7 were co-precipitated with the extracellular matrix component biglycan and soluble biglycan-induced clustering of P2X4 and P2X7 receptors with Toll-like receptor (TLR) 2/4 was found to underlie the activation of the inflammasome by this component (Babelova et al., 2009). Functional and physical interactions between P2XRs and other ion channels (e.g., members of the Cys-loop receptor and epithelial Na^+ channel families) have also been described [for a recent review see (Kaczmarek-Hajek et al., 2012)].

SUMMARY AND OUTLOOK

P2XRs contain three intersubunit binding sites for orthosteric ligands. In heteromeric receptors all three binding sites differ significantly and thus offer theoretically the possibility for subtype-specific targeting of heteromers. However, proof of this concept is still lacking.

For heterologously expressed receptors, a combination of pharmacology and current kinetics in most cases allowed the discrimination of heteromeric receptor responses from those of homomeric receptors. However, identification of matching responses in native cells proved difficult and was only convincingly achieved in case of the P2X1/5 and P2X2/3 heteromers. Thus, it is likely that additional factors such as receptor modifications (e.g., phosphorylation), P2X splice variants, interacting proteins, P2XR clustering, the physiological expression background, expression levels, and trafficking influence P2XR properties under native conditions.

Co-immunoprecipitation still represents one of the standard experiments to proof a physical interaction between proteins. However, several pitfalls need to be considered: First, the method does not allow to differentiate between association of trimeric receptors and heterotrimerization of subunits. Second, it is difficult to exclude that the observed interaction is due to artificial aggregation of the overexpressed protein or occurs artificially during the solubilization and purification process, which is generally optimized toward the detection of an interaction. Third, if performed in native tissues, this method also critically depends on the availability of reliable antibodies. Thus, methods for the direct visualization of complexes and biochemical and/or functional assays, which are able to detect interactions within the membrane, are needed and experiments should preferably be performed on native preparations or at physiological expression levels. With RNA knockdown technology, knockout animals, and transgenic animals expressing tagged P2XRs, suitable control experiments can be performed and in combination with the increasingly available subtype selective compounds, knockout animals provide valuable models to decipher the composition of heteromeric complexes by pharmacological means.

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Dynamic micro-organization of P2X7 receptors revealed by PALM based single particle tracking

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Adenosine triphosphate (ATP)-gated P2X7 receptors (P2X7Rs) are members of the purinergic receptor family that are expressed in several cell types including neurons. A high concentration of ATP is required for the channel opening of P2X7Rs compared to other members of this receptor family. Recent work suggests that ATP binding to members of the P2X receptor family determines the diffusion and localization of these receptors on the plasma membrane of neurons. Here, we employed single particle tracking photoactivated localization microscopy (sptPALM) to study the diffusion and ATP-dependence of rat P2X7Rs. Dendra2-tagged P2X7Rs were transfected in hippocampal neurons and imaged on proximal dendrites. Our results suggest the presence of two populations of P2X7Rs within the extra-synaptic membrane: a population composed of rapidly diffusing receptors and one stabilized within nanoclusters (∼100 nm diameter). P2X7R trajectories were rarely observed at synaptic sites. P2X7R mutations in the ATP-binding site (K64A) or the conserved phosphorylation site (K17A) resulted in faster- and slower-diffusing receptors, respectively. Furthermore, ATP differentially accelerated wild type and K17A-mutant receptors but not K64A-mutant receptors. Our results indicate that receptor conformation plays a critical role in regulating ATP-mediated changes in P2X7R diffusion and micro-organization.

Keywords: P2X7 receptors, P2X receptors, ATP, single particle tracking, PALM, diffusion

INTRODUCTION

ATP-gated purinergic P2X receptors (P2XRs) form homo- or hetero-trimeric receptors that can be composed of 7 different subunits, P2X1-P2X7. Slow-desensitizing P2X7Rs are unique members of the ATP-gated P2X receptor family with a characteristic long C-terminus (279 amino acids) and display unusually high EC_{50} for ATP (>100 μM) (Rassendren et al., 1997). P2X7Rs are expressed in both neuronal and non-neuronal cell types. However, the lack of specific antibodies and the existence of multiple splice variants of this receptor make it difficult to predict their expression and localization (Anderson and Nedergaard, 2006; Kaczmarek-Hájek et al., 2012). Recent development of P2X7-EGFP transgenic mice provides evidence of their expression in several brain regions and in both glial and neuronal cells (GENSAT, www.gensat.org). In the hippocampus of EGFP-P2X7R expressing mice, expression can be seen primarily in the dentate gyrus and CA3 region (*Cornu Ammonis*) suggesting a cell-type specific expression (GENSAT, http://www.gensat.org/).

The development of quantum dot based SPT (QD-SPT) studies has changed our understanding of activity-dependent dynamics of neurotransmitter receptors (Dahan et al., 2003; Groc et al., 2004; Ehlers et al., 2007; Lévi et al., 2008; Bannai et al., 2009). Recently, QD-SPT was successfully implemented to study ATP-dependent modulation of P2X receptor dynamics on the plasma membrane of spinal cord neurons (P2X2Rs, Shrivastava et al., 2011a), hippocampal neurons (P2X2Rs, Richler et al., 2011) and microglia (P2X4Rs, Toulme and Khakh, 2012). These studies suggested that calcium-influx through P2XRs along with the conformational changes induced by ATP binding, govern the diffusion of these receptors. A second important outcome of these studies in neurons suggested exclusion of P2X2R trajectories from synaptic areas despite their over-expression. However, a major limitation of QD usage is their large size (10–30 nm), which can restrict their accessibility to narrow spaces including the synaptic cleft. This shortcoming can be circumvented with single particle tracking photoactivated localization microscopy (sptPALM), an approach that makes use of genetically encoded fluorescent proteins.

PALM relies on the stochastic activation of photoactivatable or photoconvertible proteins to obtain super-resolution images. More precisely, using low intensity activation light, a small percentage of fluorescent proteins are activated at a given time-interval. Each fluorophore is then localized with high precision by fitting its fluorescence emission with a two-dimensional Gaussian function (Betzig et al., 2006; Hess et al., 2006). Since each photo-converted molecule remains visible for a short time period before photobleaching, it is possible to track their position allowing the generation of trajectories and the computation of diffusion coefficients. This approach of single particle tracking using PALM (sptPALM) has been recently successfully employed (Manley et al., 2008; Hoze et al., 2012). The stochastic activation of several well-separated molecules over a given time period provides a random sampling with hundreds of trajectories. In addition, rapid photobleaching allows the sampling of a large number of molecules (hundreds to thousands) with higher accuracy within a given field of view.

A high-level of ATP is released during tissue injury, inflammatory pain, and even in some neurodegenerative disorders (Orellana et al., 2011; Shrivastava et al., 2013, reviewed in Khakh and North, 2012). Therefore, it is important to understand ATP-dependent reorganization and clustering of P2X7Rs and identify factors defining their dynamics. More specifically, we aimed to study how ATP regulates the diffusion of P2X7Rs. We performed high-density mapping of P2X7Rs using the sptPALM approach. Even after over-expression and high-density imaging, P2X7Rs were rarely detected at synapses, suggesting a predominantly non-synaptic localization of these receptors and no synaptic enrichment. Consequently, we focused our study on extra-synaptic receptors. We found that non-synaptic P2X7Rs can be either freely diffusing or trapped within nanoclusters. Mutation disturbing the conserved N-terminus phosphorylation site (Boué-Grabot et al., 2000) revealed that the N-terminal conformation regulates P2X7R diffusion. In addition, perturbation of the ATP-binding pocket by point mutation (Wilkinson et al., 2006) or by ATP binding (Hattori and Gouaux, 2012) altered receptor diffusion. Altogether, our results suggest that the structural conformation of P2X7Rs determines their mobility and confinement on the plasma membrane.

MATERIALS AND METHODS

PLASMID AND ANTIBODIES

Rat P2X7R-EGFP plasmid was kindly provided by Francois Rassendran (Montpellier). The EGFP sequence was downstream of P2X7 coding region between *AgeI* and *NotI* restriction sites (pEGFP-N1 vector) and generated as described previously (Compan et al., 2012). Sequence coding for EGFP was replaced with Dendra2 sequence to generate P2X7-Dendra2 plasmid. Dendra2 is a monomeric fluorescent protein that undergoes irreversible photoconversion from green- to red-emitting state and has a high photo-conversion yield (Adam et al., 2009). Site-directed mutagenesis (Agilent) was performed on P2X7-Dendra2 plasmid to generate K17A and K64A mutant P2X7Rs. The following antibodies were used for immunocytochemistry: rabbit Tau (1:1000, Synaptic System), mouse MAP2 (1:1000, Millipore) and rabbit Synapsin (1:800, Synaptic System).

CELL CULTURE AND TRANSFECTION

Primary hippocampal cultures were prepared from 18-days-old Sprague–Dawley rat embryos. Neurons were plated at a density of 0.6×10^5 cells/well on 18 mm coverslips pre-coated with 80 μg/ml poly-D, L-ornithine (Sigma). Freshly dissociated cells were plated in neuronal attachment media consisting of 10% horse serum (PAA Labs), 1 mM sodium pyruvate (Life Technologies) and 2 mM glutamine (Life Technologies) in MEM (Life Technologies). Three hours after plating, media was replaced with neurobasal media containing 2 mM glutamine and 2% B27. Cells were maintained by replacing one-fourth of the medium with fresh culture medium every week. Transfection was performed using Lipofectamine-2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were transfected with 0.4 μg plasmid per coverslip at days *in vitro* (DIV) 12. Imaging was performed on DIV 14.

IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed following fixation of cells in 4% (w/v) paraformaldehyde (Serva Feinbiochemica, Germany) and permeabilization with 0.1% triton-X. Cells were incubated with primary antibodies against TAU (1:1000), MAP2 (1:1000) or Synapsin (1:800) for 1 h at room temperature. After washing, cells were incubated for 45 min at room temperature with appropriate secondary antibody and mounted on slides with Vectashield (Vector Labs).

PALM SETUP AND IMAGING

The PALM setup and imaging conditions used have recently been described in detail (Izeddin et al., 2011). PALM imaging was performed on an inverted Nikon Ti Eclipse microscope equipped with activation (405 nm) and excitation lasers (561 nm). Images were acquired using a 100× objective (N.A. 1.49). Before acquisition, any pre-converted P2X7-Dendra2 fluorescence was bleached using high-intensity excitation laser. Activation laser was maintained at low power to allow good separation of randomly converted molecules. Single-molecule Dendra2 signal was separated with a 561 nm dichroic (Di01-R561-25 × 36) and a 617 nm emission filter (FF01-617/73), expanded through a 1.5× lens in the tube-lens of the microscope. Images were acquired using an Andor iXon EMCCD camera (512 × 512 pixel with pixel size of 16 μm) at a frame rate of 20 ms. The z-position was kept stable using the perfect focus system (Nikon) integrated with the microscope.

For PALM imaging without tracking, cells were incubated with multi-colored beads (TetraSpeck, Invitrogen) to correct drift. Imaging was performed for 20,000 frames at 20 Hz. For sptPALM experiments, beads were not used and imaging was performed for 5000–6000 frames at 50 Hz. Imaging was performed in MEM medium without phenol red (Invitrogen) containing 2% B-27, 2 mM glutamine, 1 mM pyruvate, 33 mM glucose, and 20 mM HEPES.

IMAGE ANALYSIS

Multi-Trace Tracking (MTT) algorithm was used without the tracking feature (Serge et al., 2008; Izeddin et al., 2011) for detection of individual fluorophores. For visualization and generation of pointillist and rendered images, in-house written software for MATLAB was used and has been previously described (Izeddin et al., 2011). The point-spread function of each fluorophore was detected and fitted with a 2D Gaussian distribution. The resulting pointillist image consists of all the detections obtained during the period of acquisition. Rendering was performed on the super-resolution image by superimposing the position coordinates of the detected single molecules using a standard deviation σ that had been previously determined by the localization accuracy of single fluorophores (typically 10 nm).

Tracking of PALM traces and diffusion calculation was based on the mean square displacement (MSD) approach (Saxton and Jacobson, 1997), which has been previously used for QD-based studies (Bannai et al., 2009; Renner et al., 2010). The center of each fluorescent activation was determined by Gaussian fit based on the point spread function of the microscope with a spatial resolution of ∼20–50 nm. Activations in a given frame were

associated with the maximum likely trajectories estimated on previous frames of the image sequence. The MSD was calculated using $MSD(ndt) = (N–n)^{-1} \sum_{i=1}^{N-n}[(x_{i+n}–x_i)^2 + (y_{i+n}–y_i)^2]$, where x_i and y_i are the coordinates of an object on frame I, N is the total number of steps in the trajectory, dt is the time between two successive frames, and ndt is the time interval over which displacement is averaged (Saxton and Jacobson, 1997; Triller and Choquet, 2008). The diffusion coefficient D was calculated by fitting the first 2–5 points of the MSD plot vs. time with the equation $MSD(t) = 4D_{2-5}t + 4\sigma_x^2$, with σ_x as the spot localization accuracy in one direction. Area explored is defined as the total surface area covered by the trajectory divided by the number of steps in the trajectory.

Once trajectories were generated, they were localized on top of synapses or nanoclusters. Diffraction limited FM4-64 images were used to separate synaptic and non-synaptic trajectories. Super-resolved rendered images obtained using the MTT-algorithm, were used to differentiate between free trajectories and those trapped within nanocluster. As a first step, images were filtered by wavelet segmentation using an interface implemented in Metamorph (Racine et al., 2007) to generate background free masks. Trajectories overlapping the thresholded FM4-64 images were termed "synaptic" and those overlapping the thresholded nanoclusters were termed "trapped."

STATISTICS AND IMAGE PREPARATION

Experiments were performed on 3–4 independent cultures prepared from different animals on different days. Diffusion data presented shows the distribution of diffusion coefficients pooled from all independent experiments. Kolmogorov-Smirnov test was used to test the difference in distribution. Tracking, rendering, and image analysis were performed on Matlab. Images were prepared using Microsoft excel and Graph Pad Prism.

RESULTS

DENDRITIC EXPRESSION OF P2X7 RECEPTORS IN TRANSFECTED NEURONS

Photo-convertible Dendra2-tagged P2X7R plasmid was transfected in hippocampal neurons at DIV 12 (days *in vitro*) and experiments performed at DIV 14. To visualize the distribution and localization of P2X7-Dendra2 receptors, we performed immunocytochemistry to label axons (tau), dendrites (MAP2), and synapses (synapsin). P2X7-Dendra2 receptors showed a ubiquitous and uniformly diffused expression in most of the transfected neurons (**Figures 1Aa,Ba,Ca**). Tau staining revealed axons running at the periphery of processes expressing P2X7-Dendra2 receptors (**Figures 1Ab,Ac**), suggesting dendritic localization of these receptors. This dendritic localization is confirmed by overlap of P2X7-Dendra2 receptor expressing

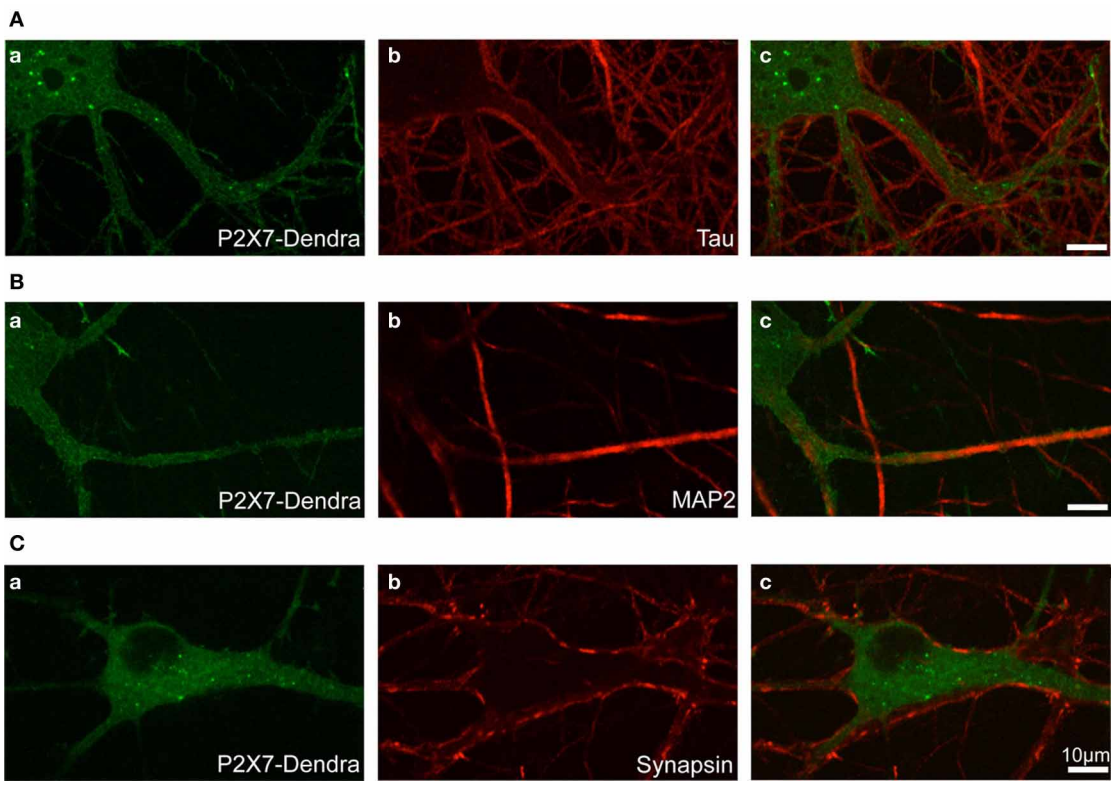


FIGURE 1 | Localization of transfected P2X7-Dendra2 receptors in dendrites. Dendra2-tagged P2X7Rs were transfected in hippocampal neurons at DIV 12. Forty-eight hours after transfection, immunocytochemistry was performed to determine the localization of P2X7Rs. **(Aa,Ba,Ca)** Diffused labeling of P2X7Rs with expression in cell body and neurites can be observed. **(Aa–Ac)** Labeling of axons with tau antibody shows axons running apposed to the processes expressing P2X7-Dendra2 receptors. **(Ba–Bc)** MAP2 labeling of dendrites show an overlap with P2X7-Dendra2 expressing neurites. **(Ca–Cc)** Synapse labeling with pre-synaptic synapsin protein shows pre-synaptic boutons are apposed to P2X7-Dendra2 expressing processes.

processes with MAP2 (**Figures 1Bb,Bc**). Labeling with the pre-synaptic marker synapsin further validated dendritic localization. Synapsin staining apposed P2X7-Dendra2 expressing processes (**Figures 1Cb,Cc**). These results suggest that transfected P2X7-Dendra2 receptors were targeted to proximal dendrites.

In agreement with previous reports (Díaz-Hernandez et al., 2008), we additionally observed the expression of P2X7Rs in growth cones and fine processes distant from the cell body (not shown), suggesting their localization in distal axons. We focused this work only on proximal dendrites to study post-synaptic P2X7R dynamics.

SUPER-RESOLUTION PALM IMAGING REVEALS NANOCLUSTERS OF P2X7Rs

Although fluorescence imaging has considerably accelerated the field of cell biology, the diffraction of light limits its optical resolution and the ability to accurately determine the size of a given structure and the localization of molecules of interest. Even more challenging is the localization of membrane receptors such as P2X7Rs that exhibit a predominantly diffused distribution (**Figure 1**). To overcome these obstacles, we employed PALM imaging to visualize the distribution of P2X7-Dendra2 receptors at much higher resolution (20–50 nm). PALM was performed on paraformaldehyde fixed (**Figure 2A**) and live (**Figure 2B**) neurons transfected with P2X7-Dendra2 receptors. **Figures 2Aa,Ba** show diffraction limited fluorescent images before PALM acquisition. While the distribution of P2X7Rs appeared unaltered, live cells exhibited much healthier morphology (**Figures 2Aa,Ba**). PALM was performed by green-to-red photoconversion of Dendra2 for a total of 20,000 frames at 20 Hz. PALM acquisitions are represented as pointillist (each point represents one detection, **Figures 2Ab,Bb**) or rendered (super-resolved, **Figures 2Ac,Bc**) images. Visual comparison of pointillist and rendered images of fixed cells (**Figures 2Ab,Ac**) showed a clustered distribution

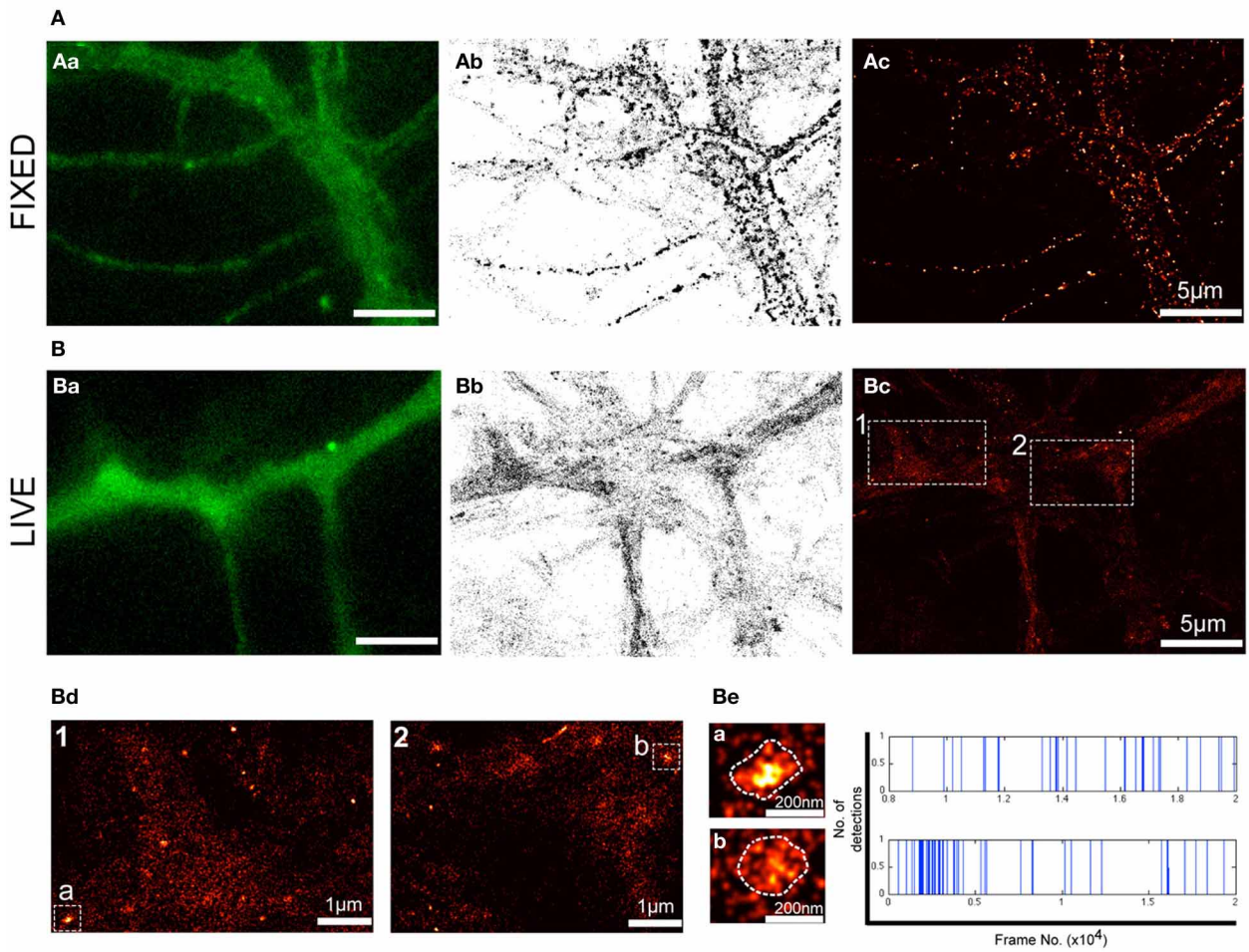


FIGURE 2 | PALM reveals nanoclusters of P2X7Rs. PALM imaging was performed on live **(A)** and fixed **(B)** neurons transfected with P2X7-Dendra2. **(Aa,Ba)** Representative fluorescent images of a section of dendrite before PALM imaging was performed. **(Ab,Bb)** Pointillist images displaying all detections obtained for P2X7-Dendra2. **(Ac,Bc)** Super-resolved rendered image generated with a pixel size of 5 nm. Note fixation-induced clustering in **(Ab,Ac)**. **(Bd)** Boxed-region 1 and 2 in **(Bc)** at higher magnification shows predominantly random, but also some nanoclusters of P2X7-Dendra2 receptors. **(Be)** Two random nanoclusters, "cluster a" from box 1 and "cluster b" from box 2, shown at higher magnification. Each detection within clusters "a" and "b" is represented as a blue vertical line and plotted as a function of time (x-axis).

compared to live cells (**Figures 2Bb,Bc**). Such large clusters of P2X7-Dendra2 receptors is believed to be a fixation artifact and therefore not suitable for further analysis. Hereafter, we only used live-cell PALM to study P2X7R dynamics.

Notably, in P2X7-Dendra2 transfected live neurons, we observed several sub-micron scaled clusters (hereafter referred to as "nanoclusters") (**Figure 2Bc**). Two representative regions (1 and 2) are shown at higher magnification (**Figure 2Bd**). Additionally, two representative nanoclusters, "a" and "b," from regions 1 and 2, respectively, are shown (**Figures 2Bd,Be**). The mean ± SEM (standard error of the mean) area of these nanoclusters was 8718 ± 442 nm^2. Considering a near circular shape of these nanoclusters, an average diameter of 105 ± 3 nm is approximated. To ascertain if these nanoclusters are real clusters and not generated due to multiple detections of a single fluorophore, we plotted all detections within these nanoclusters during the recording period. Representative traces for the two clusters are shown in **Figure 2Be** (right panel). Each blue vertical line represents the detection of a fluorophore at a particular period of acquisition (frame number). These traces indicate that detections within these nanoclusters were observed throughout the period of acquisition. Given that Dendra 2 is irreversibly photobleached following its photo-conversion, this suggests the presence of several fluorophores within the clusters.

Notably, the majority of P2X7-Dendra2 receptor detections were not clustered, suggesting a predominant presence as single molecules. The observed nanoclusters appear to be enriched in some dendrites. Altogether, these results suggest that transfected P2X7Rs exist in two populations, one as single molecules and a second stabilized in nanoclusters.

SINGLE PARTICLE TRACKING OF P2X7-DENDRA2 RECEPTORS USING PALM

Dendra2 undergoes a green-to-red shift in emission wavelength (photo-conversion) when exposed to UV light (405 nm laser). Following photo-conversion, Dendra2 remains switched on for a short time period before irreversibly photobleaching (Adam et al., 2009). This allows the detection and tracking of the fluorophore during the time-period it remains fluorescent. Fast acquisition at a rate of 50 Hz was performed for a short period of 2–3 min. Trajectories for each fluorophore were generated and diffusion properties analyzed. A representative fluorescent image showing a dendrite expressing P2X7-Dendra2 receptors (green) and synapses labeled with FM4-64 (red) is shown in **Figure 3Aa**. Synaptic labeling allowed us to monitor the mobility of P2X7-Dendra2 receptors within synapses. Threshold-images of nanoclusters obtained from rendered images were used to localize trajectories within these

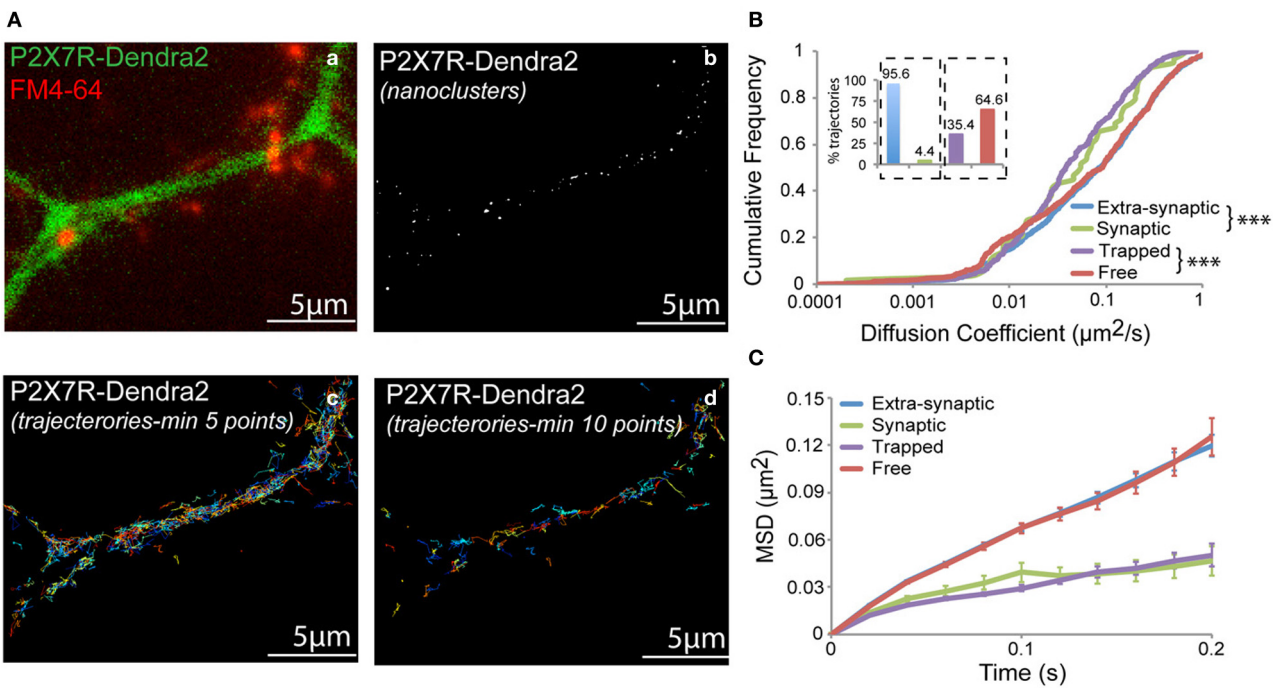


FIGURE 3 | sptPALM of P2X7-Dendra2 receptors. Live cell sptPALM was performed at 50 Hz on dendrites expressing P2X7-Dendra2. **(Aa)** Epi-fluorescence of a P2X7-Dendra2 (green) expressing dendrite and FM4-64 (red) labeled synapses. FM4-64 was used for identification of "synaptic" and "extra-synaptic" trajectories. **(Ab)** Representative threshold image obtained from a super-resolved rendered image shows nanoclusters used for localization of trajectories. Trajectories were localized as "trapped" or "free" within nanoclusters. **(Ac,Ad)** Example of trajectories obtained using the sptPALM approach. Minimum 10-point long trajectories were used for diffusion measurements in order to reduce calculation error. **(B)** "Synaptic" and "Trapped" trajectories show slower diffusion compared to "Extra-synaptic" and "Free" trajectories, respectively (Kolmogorov-Smirnov statistical test ***$p < 0.001$). Inset: Proportion of trajectories observed for each fraction (Synaptic + Extra-synaptic = 100% and Trapped + Free = 100%). **(C)** Mean square displacement (MSD) plot shows more confined P2X7Rs at synapses and within nanoclusters (trapped).

nanoclusters (**Figure 3Ab**). A distinct advantage of sptPALM is the increased number of trajectories that can be obtained within a short time interval (**Figures 3Ac,Ad**). Trajectories shown contain a minimum of 5- or 10-detection points. We performed MSD based diffusion analysis to estimate the diffusion coefficient of P2X7-Dendra2 receptors (Saxton and Jacobson, 1997; Triller and Choquet, 2008; Pinaud et al., 2010). To minimize computational error of diffusion coefficients, only trajectories consisting of at least 10-points were considered (**Figure 3B**). Trajectories of P2X7-Dendra2 receptors were grouped as extra-synaptic (blue) or synaptic (green) according to their localization over FM4-64, and as free (red) if present outside nanoclusters or trapped (purple) if inside nanoclusters. Only 4.4% of the total trajectories were observed at synapses (**Figure 3B**, inset, blue and green). A low probability of finding trajectories at synapses suggests a preferential extra-synaptic localization of P2X7Rs; thus the distinction between synaptic and extra-synaptic trajectories was not pursued in further experiments. Meanwhile, our data suggested that nearly 35% of the trajectories were localized on nanoclusters (**Figure 3B**, inset, purple and red). Distributions of diffusion coefficient (D) and MSD of P2X7Rs are plotted in **Figures 3B,C.** Whereas extra-synaptic (blue) and free (red) trajectories showed similar diffusion coefficients, P2X7-Dendra2 receptor trajectories showed slower diffusion within nanoclusters (purple) and synapses (green). The median diffusion coefficients ($\mu m^2/s$) are: extra-synaptic = 0.077 ($n = 1246$); synaptic = 0.054 ($n = 59$); free = 0.090 ($n = 597$); trapped = 0.038 ($n = 327$).

This difference in the diffusion coefficient is reflected in the shape of the MSD curve (**Figure 3C**) (Saxton and Jacobson, 1997). Extra-synaptic (blue) and free (red) trajectories showed anomalous diffusion (non-linearly dependent on time) whereas trapped (purple) and synaptic (green) trajectories exhibited relatively confined diffusion. The slow-diffusion and increased confinement within nanoclusters could be due to inter-molecular interactions between P2X7Rs and/or scaffolding molecules localized at these nanoclusters. Thus, sptPALM offers a useful tool to study P2X7 receptor diffusion in and out of nanoclusters formed by these receptors.

ALTERED DIFFUSION IN K17A AND K64A MUTANT P2X7Rs

We next generated two mutants to study the role of N-terminus phosphorylation and ATP-binding on P2X7R mobility. Lysine at positions 17 and 64 were substituted with alanine (K17A and K64A). Mutation of lysine at position 17 removes the putative protein kinase-C (PKC) phosphorylation site (15TXK17) without altering receptor gating (Boué-Grabot et al., 2000; Yan et al., 2008). Whereas mutation of lysine-64, found in the ATP-binding site, results in a non-functional P2X7R unable to conduct ions (Wilkinson et al., 2006). Analysis of the distribution of diffusion coefficients as measured by sptPALM revealed a slow diffusion of K17A mutant receptors and a fast diffusion of K64A receptors, relative to WT-P2X7Rs (**Figures 4A,B**). Notably, these differences in diffusion coefficients of WT and mutant receptors were observed for both free and trapped P2X7Rs. Similarly, MSD curves of the mutants display an opposite effect when compared to WT control. The downward shift of the MSD curve favors a more confined diffusion of K17A-mutant receptor relative to the WT P2X7Rs (red and blue, respectively, **Figures 4C–E**). Meanwhile, K64A-mutant showed a reduction in confinement as seen by an upward shift of the MSD curve (green and blue, **Figures 4C–E**). These results suggest that conformation of N-terminus phosphorylation site and ATP-binding site determines P2X7R diffusion behavior on the plasma membrane.

MODULATION OF P2X7R DIFFUSION BY ATP

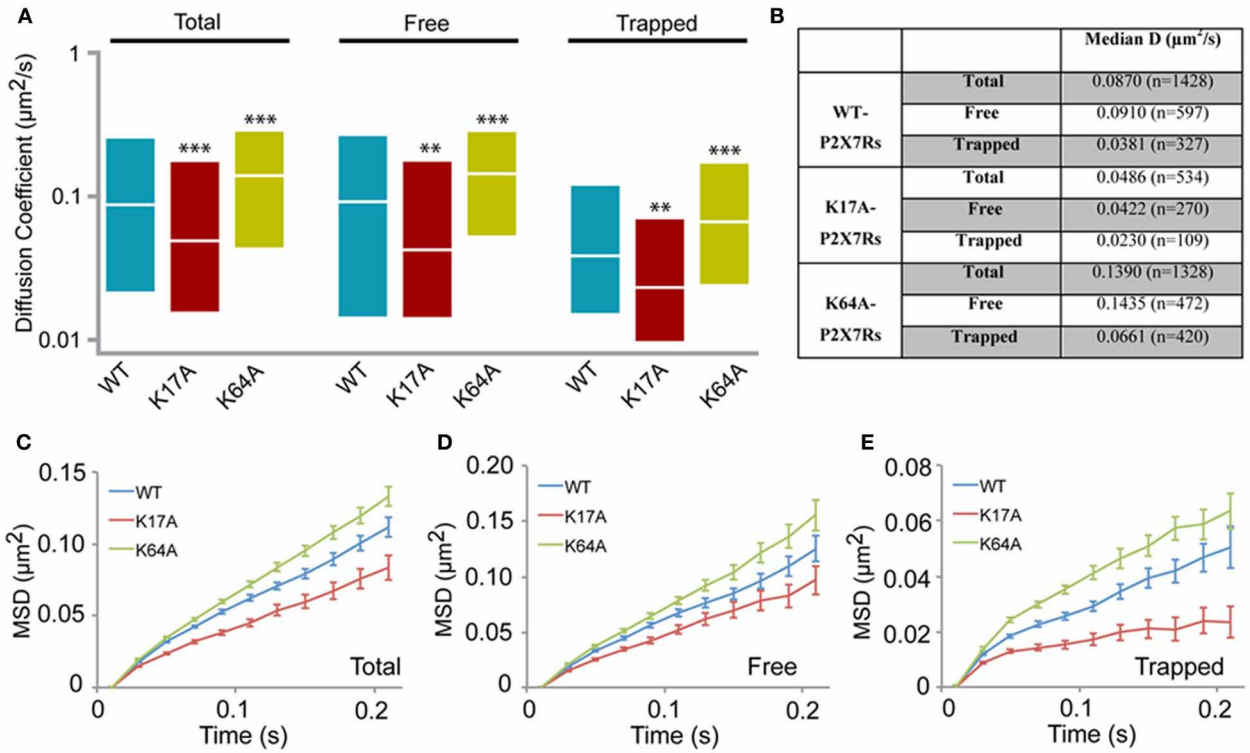
We next investigated whether ATP-binding alone could modify WT or mutated P2X7R mobility. One hundred micromolar (100 μM) ATP was added to the recording medium prior to imaging. Changes in diffusion coefficient were then measured for free and trapped populations. ATP-treated cells showed a small but significant increase in the diffusion coefficient of the free population of WT-P2X7Rs (**Figure 5A**, **Table 1**). While ATP-dependent acceleration was more pronounced for K17A-mutant P2X7Rs (**Figure 5B**, **Table 1**), ATP-binding site K64A mutant, showed no change in mobility for freely diffusing receptors (**Figure 5C**, **Table 1**). Notably, ATP had no effect on receptor mobility of trapped receptors (**Figures 5D–F**, **Table 1**). MSD plots also displayed no change in P2X7R confinement following ATP-treatment for both "free" and "trapped" receptors (Inset, **Figures 5A–F**).

Our inability to observe any change in diffusion coefficient and MSD of trapped P2X7Rs suggests that ATP does not affect this population of receptors. However, as the error in MSD calculation is higher for small length trajectories, we also looked at the surface area explored per unit length (length = number of time steps in the trajectory) of P2X7R trajectories that were trapped in nanoclusters. This parameter is independent of the length of the trajectories and gives a good estimate of receptor confinement. We observed a reduction in the area explored/length for both WT and mutant receptors (**Figure 6**). The median area/length (in pixels) was determined to be: $WT = 0.2175$ ($n = 2384$), $WT + ATP = 0.1902$ ($n = 2457$), K17A = 0.1305 ($n = 816$), K17A + ATP = 0.1104 ($n = 556$), K64A = 0.2387 ($n = 2911$), K64A + $ATP = 0.1923$ ($n = 2225$). The above data was obtained from trajectories with a minimum length composed of 5 detections. A similar reduction in area explored was observed with a minimum of 10-point trajectories. These observations suggest that receptors "trapped" within nanoclusters exhibit increased confinement in the presence of extracellular ATP. Notably, ATP had the highest effect on K64A-mutant receptors, which exhibit a disruption in the ATP-binding site (**Figure 6**). This suggests that direct binding of ATP has no role in mediating change in confinement within nanoclusters.

DISCUSSION

NON-SYNAPTIC NANOCLUSTERS OF P2X7Rs

Previous studies using QD based SPT suggested that members of the P2XR family are not enriched at synapses (Richler et al., 2011; Shrivastava et al., 2011a). Weak synaptic labeling of P2XRs could be due to weak expression of these receptors within the synapse or due to the inaccessibility of QD-antibody complexes to penetrate the synaptic cleft. To avoid size-bias of QD-labeling, we employed super-resolution PALM imaging to study the localization and dynamics of synaptic P2X7Rs. Following transfection



		Median D (μm²/s)
WT-P2X7Rs	Total	0.0870 (n=1428)
	Free	0.0910 (n=597)
	Trapped	0.0381 (n=327)
K17A-P2X7Rs	Total	0.0486 (n=534)
	Free	0.0422 (n=270)
	Trapped	0.0230 (n=109)
K64A-P2X7Rs	Total	0.1390 (n=1328)
	Free	0.1435 (n=472)
	Trapped	0.0661 (n=420)

FIGURE 4 | P2X7R mutants show altered diffusion. K17A and K64A mutants were generated by sight-directed mutagenesis from the wild-type (WT) P2X7-Dendra2 receptor plasmid. sptPALM was performed and diffusion analyzed. **(A)** Compared to WT-P2X7Rs, K17A-mutant showed slower diffusion, while K64A-mutant showed faster diffusion for total (100%), "free" (~65%) and "trapped" (~35%) population. Plotted data shows a distribution from 25 to 75th percentile and the median value (Kolmogorov-Smirnov statistical test $^{***}p < 0.001$, $^{**}p < 0.01$). **(B)** Median diffusion coefficient and number of trajectories for each population. **(C,D,E)** MSD plot shows an increased confined diffusion in K17A-mutant receptors and a decreased confined diffusion in K64A-mutant receptors with respect to wild-type P2X7Rs.

of P2X7R plasmid C-terminally tagged with photo-convertible Dendra2 protein, we could identify two different populations of P2X7Rs: freely diffusing and trapped as nanoclusters. However, neither of the two populations of P2X7Rs were observed at FM4-64 labeled synapses, supporting previous studies. Thus, it appears that members of the P2XR family are predominantly non-synaptic irrespective of cell type. Further experiments are needed to see if maturation of synapses and cell-types may contribute to the formation of nanoclusters.

A main drawback of PALM is the use of transfection, which may induce over-expression and modifications in the localization of proteins and receptors. Unfortunately, the absence of any specific antibodies suitable for single molecule imaging precludes the study on endogenous receptors. In any case, even tough we cannot completely rule out that nanoclusters of P2X7Rs could be due to over-expression, a number of observations point toward their existence. First, the difference in diffusion coefficient and confinement of WT and P2X7R mutants within nanoclusters suggests conformation-specific dynamics within these nanoclusters. Receptor aggregates are not expected to exhibit such behavior. Second, nanoclusters were rarely observed at synapses irrespective of synapse density, favoring non-random occurrence. Third, ATP modified the dynamics of molecules within the nanoclusters, suggesting an activity-dependent regulation within these nanodomains and indicating that there are signaling-related mechanisms that can modify the internal organization of nanoclusters. Therefore, such nanoclusters of P2X7Rs, possibly act as a signaling platform for P2X7Rs. This points toward the presence of P2X7R (or P2XR) interacting molecules, which may induce clustering at non-synaptic localizations. In fact, we observed non-synaptic clusters of P2X2R-subtype in spinal cord neurons supporting the existence of such receptor-stabilizing nanoclusters (Shrivastava et al., 2011a). In the same study, we found that ~20% of these non-synaptic P2X2Rs were associated with $GABA_A$Rs. Work by another group also observed that non-synaptic P2X4Rs were associated with $GABA_A$Rs (Jo et al., 2011). Such non-synaptic localization of members of P2XRs, along with their ability to interact with several cys-loop receptors, suggest that P2XRs may be stabilized by their interaction with cys-loop receptors (Khakh et al., 2000, 2005; Sokolova et al., 2001; Boué-Grabot et al., 2004a,b; Shrivastava et al., 2011a, reviewed in Shrivastava et al., 2011b). Notably, no cross talk involving P2X7Rs has been reported. In addition to neurotransmitter receptors (e.g., $GABA_A$Rs), P2XR-interacting proteins (reviewed in Kaczmarek-Hájek et al., 2012) and lipid rafts could also contribute to the clustering of P2X7Rs. Biochemical evidence suggests that P2X7Rs can be associated with lipid rafts (Garcia-Marcos et al., 2006; Barth et al., 2007; Gonnord et al.,

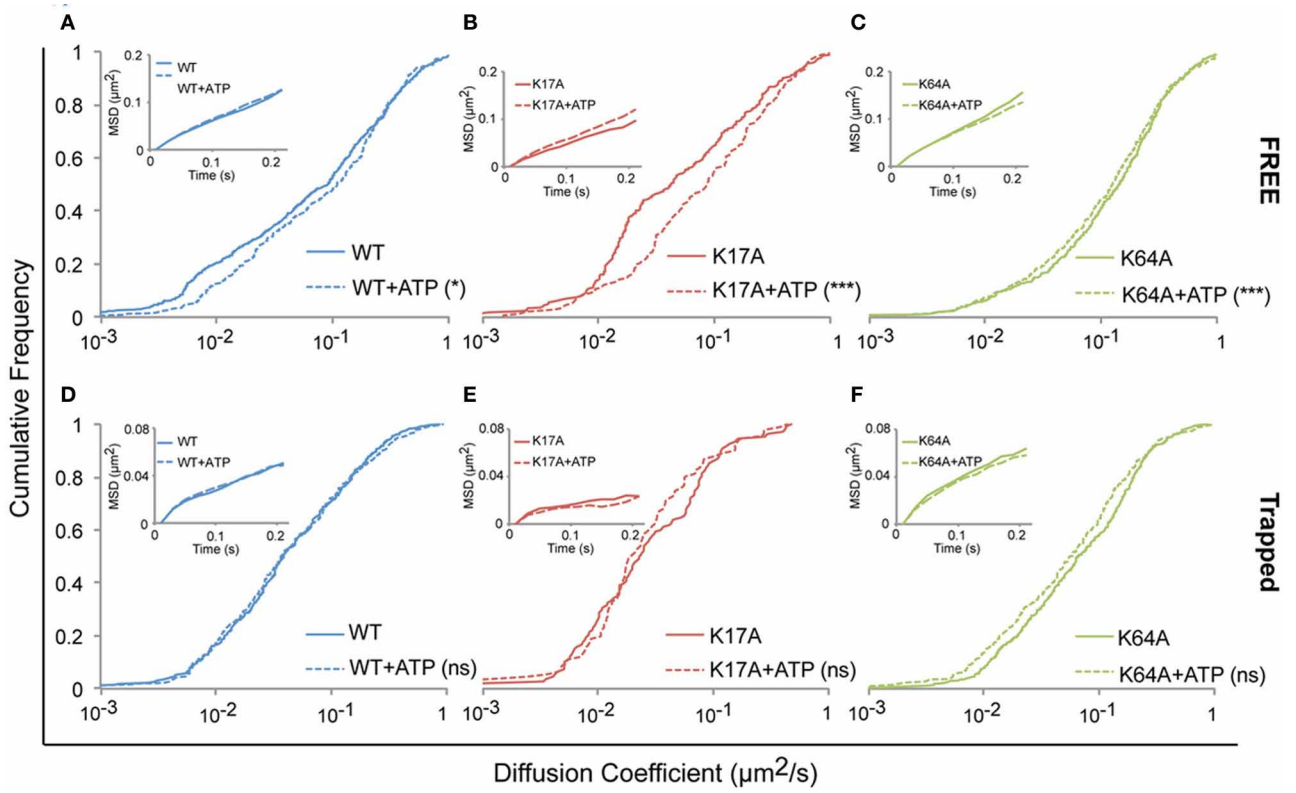


FIGURE 5 | ATP increased the mobility of P2X7Rs. Live cell sptPALM was performed either in the absence or presence of ATP (100 μM) in recording medium. (**A–C**, **Table 1**) ATP differentially increased the diffusion coefficient of "free" WT and K17A-mutant P2X7Rs but not of K64A-mutant receptors. (**D–F**, **Table 1**) No change in the diffusion coefficient of "trapped" receptors was observed for WT and mutant-P2X7Rs. Inset **(A–F)** shows no change in MSD under any condition. (Kolmogorov-Smirnov test. $^{***}p < 0.001$, $^{*}p < 0.05$). Median diffusion coefficient and number of trajectories are shown in **Table 1**.

Table 1 | (Supporting Figure 5) Median diffusion coefficient (μm²/s) and number of trajectories (>10 points) used for diffusion calculation.

		Control median *D* (μm²/s)	+ATP median *D* (μm²/s)
WT-P2X7Rs	Non-nanoclusters	0.0910 ($n = 597$)	0.1087 ($n = 403$)*
	Nanoclusters	0.0381 ($n = 327$)	0.0367 ($n = 365$)^{ns}
K17A-P2X7Rs	Non-nanoclusters	0.0422 ($n = 270$)	0.0855 ($n = 143$)***
	Nanoclusters	0.0230 ($n = 109$)	0.0192 ($n = 92$)^{ns}
K64A-P2X7Rs	Non-nanoclusters	0.1435 ($n = 472$)	0.1247 ($n = 504$)^{ns}
	Nanoclusters	0.0661 ($n = 420$)	0.0570 ($n = 327$)^{ns}

Kolmogorov-Smirnov test between Control and ATP treated cells.

2009) and could therefore provide a platform where P2X7Rs can cluster.

DYNAMICS OF P2X7Rs

The majority of P2X7Rs were observed as freely diffusing receptors within the extra-synaptic space. Even after overexpression and high-density sampling by sptPALM, we rarely observed P2X7R trajectories at synapses, suggesting their preference for extra-synaptic localization without any synaptic enrichment. Though nearly 65% of the trajectories were observed as free diffusing, we do not rule out that a small proportion of such free-trajectories could be due to saturation of the capacity of P2X7-clustering protein(s), resulting in over-spill of single P2X7Rs. Compared to WT-P2X7Rs, the diffusion of P2X7Rs lacking the putative PKC phosphorylation site (K17A-mutated) was more confined. Phosphorylation-dependent change in receptor conformation could be a possible explanation for the slower diffusion of K17A-mutant. However, recent work argues against this possibility. It has been reported that mutation of threonine within the conserved PKC site (15TXK17) resulted in a more sensitive agonist response. Contrarily, mutation of K-17 did not alter receptor gating, even though the phosphorylation site was removed (Yan et al., 2008, 2010). This suggests that phosphorylation itself is not a pre-requisite for P2X7R gating and it is the N-terminus structure that may shape P2X7R channel properties, including diffusion. Recent work from our laboratory showed that PKC-phosphorylation of glycine receptor reduced its binding affinity to the scaffold molecule gephyrin, thereby directly determining the amount of receptors at inhibitory synapses (Specht et al., 2011). Although unrelated to glycine receptors, a PKC phosphorylation-dependent interaction of P2XRs with scaffold

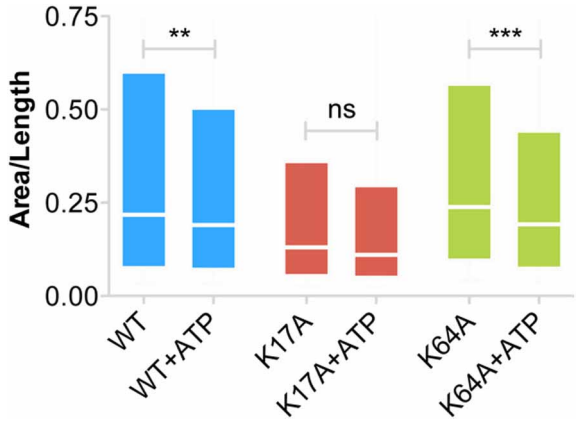


FIGURE 6 | Increased confinement of P2X7Rs trapped within nanoclusters. Area (pixels) per unit length of trajectory (number of steps) provides a good estimate of the area explored by trajectories and thus the size of the confinement domain. WT- and K64A-PX7Rs "trapped" within nanoclusters showed a reduction in the area explored or increased confinement in the presence of ATP. Graph shows a distribution from 25th percentile to 75th percentile. (Kolmogorov-Smirnov test. $^{***}p < 0.001$, $^{**}p < 0.01$, ns, no significant difference).

proteins or other receptors might contribute to their stability in the plasma membrane nanodomains. More work is needed to further understand how phosphorylation-state of P2XRs contributes to their diffusion, localization and interaction with other proteins.

Under physiological conditions, WT-P2X7Rs are composed of both "closed" and "open" receptors depending on the level of ATP (Yan et al., 2010). Previous studies based on the crystal structure of P2X4Rs showed significant structural differences between the ATP-bound (open) and unbound (closed) receptors (Kawate et al., 2009; Hattori and Gouaux, 2012). A mutation (K64A) within the ATP-binding pocket renders P2X7Rs non-functional and unresponsive to ATP (Jiang et al., 2000; Wilkinson et al., 2006). However, we found that the K64A-P2X7R mutant exhibited a faster diffusion compared to WT-P2X7Rs. Since the K64A-mutated receptors lack the ATP binding site, they are preferentially present in a "closed" conformation. Moreover as the sptPALM experiments were performed in an ATP-free medium, most WT P2X7Rs were also expected to be present in a closed conformation. Therefore, the faster diffusion of K64A compared to WT-P2X7Rs is likely due to differences in receptor conformation within the ATP-biding region caused by the point mutation.

In order to determine whether other perturbations (e.g., opening and closing) of the ATP-binding pocket also affect the diffusion coefficient of P2X7Rs, exogenous ATP was added to the imaging media. Following addition of ATP, an increase in the mobility was observed for WT- and K17A- but not for K64A-P2X7Rs. These results further support that perturbation of the ATP-binding pocket through either a point mutation (K64A) or through the opening of receptors after addition of ATP, causes a change in the diffusion coefficient of P2X7Rs. Consequently, ATP-dependent P2X7R conformation regulates its diffusion. It is likely that structural rearrangement within the extracellular ATP-binding pocket leads to changes in conformation of intracellular N- and/or C-terminus, thus modifying diffusion behavior. It should be emphasized that several other factors may contribute to the observed changes in diffusion such as basal phosphorylation state, interaction with scaffold, maturation of neurons, cell-type, network activity, etc. More work is needed to further understand the mechanism of such regulation.

ATP also had an effect on trapped receptors. Both WT and K64A-P2X7Rs trapped in nanoclusters exhibited a decrease in the area explored following ATP treatment. Thus ATP indirectly stabilizes P2X7Rs present within nanoclusters. Indeed, more work is needed to identify the mechanism, but involvement of other endogenous P2XR subtypes cannot be ruled out. ATP-dependent regulation of P2XR mobility and clustering has been demonstrated by several studies (Richler et al., 2011; Shrivastava et al., 2011a; Toulme and Khakh, 2012). We previously reported an ATP-dependent increase in the confinement of P2X2Rs in spinal cord neurons without any observable change in receptor diffusion coefficient (Shrivastava et al., 2011a). However, other studies reported an increase in diffusion coefficient along with reduced confinement following ATP-treatment of P2X2Rs in hippocampal neurons and P2X4Rs in microglia (Richler et al., 2011; Toulme and Khakh, 2012). While the latter work investigated an immediate (∼30 s) effect on P2XR diffusion following ATP-application, our work on P2X2Rs in spinal cord neurons looked at the response within 30 min of ATP application. In fact, under similar experimental conditions as those used in our previous work, here we observed only a marginal acceleration of freely diffusing P2X7Rs but an increased confinement of nanocluster-trapped P2X7Rs. It cannot be excluded that these minor variations in the diffusion behavior of P2XR-subtypes could result from intrinsic properties of the receptors and/or depend on cell-types and local calcium concentration.

Altogether, these results suggest a conserved mechanism for the control of P2XR diffusion dynamic, where the binding of ATP on P2XRs initially results in an increased mobility that is eventually followed by slow-down and/or clustering. This hypothesis is strengthened by two additional observations: first, a competitive antagonist, TNP-ATP (2′, 3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate) that binds the same site as ATP was found to increase clustering of P2X2Rs (Shrivastava et al., 2011a). Second, ATP-induced the formation of hot spots of P2X2-EGFP receptors in transfected neurons (Khakh et al., 2001). Thus, ATP-dependent conformation of P2XRs determines their mobility and clustering on the plasma membrane. An increased level of ATP has been observed in neuro-inflammatory conditions such as neuropathic pain, as well as in neurodegenerative disorders including Alzheimer's disease (Orellana et al., 2011; reviewed in Khakh and North, 2012; Shrivastava et al., 2013). We recently observed that ATP-dependent activation of P2XRs indirectly contributes to the pathogenicity in Alzheimer's disease (Shrivastava et al., 2013). Rapid reorganization of P2XRs following ATP-sensing may create P2XR-clusters that can contribute to neuronal dysfunction by enhancing local calcium-influx in cells. Further work on P2XR dynamics and their nano-organization will shed new light on the involvement of ATP-receptors in physiopathology.

AUTHOR CONTRIBIUTION

Amulya N. Shrivastava, Pamela C. Rodriguez, Marianne Renner, and Antoine Triller designed the experiments and wrote the manuscript. Amulya N. Shrivastava and Pamela C. Rodriguez performed the experiments. Marianne Renner developed the tools for analysis of sptPALM data.

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P2X4 receptors and neuropathic pain

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Neuropathic pain, a debilitating pain condition, is a common consequence of damage to the nervous system. Neuropathic pain is often resistant to currently available analgesics. A growing body of evidence indicates that spinal microglia react and undergo a series of changes that directly influence the establishment of neuropathic pain states. After nerve injury, P2X4 receptors (P2X4Rs) are upregulated in spinal microglia by several factors at the transcriptional and translational levels. Those include the CC chemokine CCL21 derived from damaged neurons, the extracellular matrix protein fibronectin in the spinal cord, and the transcription factor interferon regulatory factor 8 (IRF8) expressed in microglia. P2X4R expression in microglia is also regulated at the post-translational level by signaling from other cell-surface receptors such as CC chemokine receptor (CCR2). Importantly, inhibiting the function or expression of P2X4Rs and P2X4R-regulating molecules suppresses the aberrant excitability of dorsal horn neurons and neuropathic pain. These findings indicate that P2X4R-positive microglia are a central player in mechanisms for neuropathic pain. Thus, microglial P2X4Rs are a potential target for treating the chronic pain state.

Keywords: P2X4 receptor, microglia, neuropathic pain, spinal cord, fibronectin, CCL21, IRF8, BDNF

INTRODUCTION

Injury to the nervous system arising from bone compression in cancer, diabetes mellitus, infection, autoimmune diseases, or traumatic injury results in debilitating chronic pain states (so-called neuropathic pain). Characteristic symptoms of neuropathic pain include spontaneous pain, hyperalgesia (increased pain perception of noxious stimuli), and tactile allodynia (pain hypersensitivity to normally innocuous stimuli). Neuropathic pain is refractory to currently available treatments, such as non-steroidal anti-inflammatory drugs and opioids (Costigan et al., 2009). Neuropathic pain is now considered not just a symptom of disease but also the consequence of disordered functioning of the nervous system (Costigan et al., 2009; Beggs et al., 2012).

Extensive lines of evidence from basic pain research using diverse animal models of neuropathic pain indicated that neuropathic pain is a reflection of the aberrant excitability of dorsal horn neurons evoked by peripheral sensory inputs (Woolf and Salter, 2000; Costigan et al., 2009). While neurons have long been considered the only cell type involved in neuropathic pain, recent studies have shown that pathologically altered neurotransmission requires communication with glial cells, in particular microglia, activated in the spinal cord in response to peripheral nerve injury (PNI; Watkins et al., 2001; Tsuda et al., 2005; McMahon and Malcangio, 2009; Ren and Dubner, 2010; Tsuda et al., 2013).

Microglial cells are resident macrophages in the central nervous system (CNS), which derive from primitive macrophages in the yolk sac (Ginhoux et al., 2010). In the adult, microglia are ubiquitously distributed throughout the brain and spinal cord and have small cell body bearing branched and motile processes, which monitor the local environment in the CNS (Davalos et al., 2005; Nimmerjahn et al., 2005). Microglia show a stereotypical long-term response to a wide range of stimuli that threaten physiological homeostasis, including PNI. In response to PNI, microglia activation in the spinal cord progresses through hypertrophic morphology, an increase in cell number, and altered gene expression (Tsuda et al., 2005; Suter et al., 2007; Tsuda et al., 2009b). Activated microglia induce or enhance expression of various genes including neurotransmitter receptors such as purinergic P2 receptors (Pocock and Kettenmann, 2007). By responding to extracellular stimuli such as ATP, activated glia evoke various cellular responses such as production and release of bioactive factors including cytokines and neurotrophic factors (Inoue, 2006), which in turn leads to hyperexcitability of dorsal horn neurons and neuropathic pain.

Among purinergic P2 receptors [ionotropic receptors (P2XRs) and metabotropic receptors (P2YRs)], activated microglia express several subtypes of P2XRs and P2YRs, and these receptors play a key role in establishing and maintaining neuropathic pain states (Tsuda et al., 2012, 2013). In this article, we highlight recent advances that further increase our understanding of the mechanisms underlying neuropathic pain, with a specific focus on P2X4 receptor (P2X4R) in spinal microglia after PNI.

P2X4R IN SPINAL MICROGLIA AND NEUROPATHIC PAIN

The first observation that demonstrated the important role of P2X4R in neuropathic pain was that established tactile allodynia after PNI was reversed by pharmacological blockade of P2X4Rs in the spinal cord (Tsuda et al., 2003). Immunohistochemical studies revealed that expression of P2X4Rs in the spinal cord was

upregulated exclusively in microglia. These results indicated that PNI-induced pain hypersensitivity depended on ongoing signaling via microglial P2X4Rs. Furthermore, animals with P2X4R knock-down or knock-out in the spinal cord were resistant to PNI-induced tactile allodynia (Tsuda et al., 2003; Ulmann et al., 2008; Tsuda et al., 2009a), indicating a necessity for P2X4Rs. The impact of microglial P2X4R stimulation in transforming tactile information to pain was demonstrated by an *in vivo* microglia transfer approach (Tsuda et al., 2003). It was found that intrathecal delivery of P2X4R-stimulated microglia caused normal rats to develop allodynia and indicated that microglial P2X4R stimulation is sufficient (Tsuda et al., 2003, 2005). Furthermore, it was demonstrated that activation of microglial P2X4Rs stimulated the synthesis and release of brain-derived neurotrophic factor (BDNF; Ulmann et al., 2008; Trang et al., 2009) and that BDNF then causes an altered transmembrane anion gradient in a subpopulation of dorsal horn lamina I neurons presumably through the downregulation of the neuronal chloride transporter KCC2, which in turn renders GABA and glycine effects depolarizing, rather than hyperpolarizing, in these neurons (Coull et al., 2005; **Figure 1**). Thus, P2X4R-stimulated microglia release BDNF as a crucial factor to signal lamina I neurons, causing an aberrant nociceptive output that contributes to neuropathic pain (Beggs et al., 2012). Therefore, microglial P2X4Rs are central players in the pathogenesis of neuropathic pain.

REGULATION OF P2X4R EXPRESSION IN MICROGLIA: TRANSCRIPTIONAL, TRANSLATIONAL, AND POST-TRANSLATIONAL LEVELS

Upregulation of P2X4R expression in microglia is a key process in the pathogenesis of neuropathic pain. Several studies have identified molecules involved in the upregulation of P2X4R expression in spinal microglia after PNI.

A clue to identifying an inducer of P2X4R expression in microglia was that microglial P2X4R upregulation was observed following PNI but not peripheral tissue inflammation (Tsuda et al., 2003), raising the possibility that a factor derived from injured primary afferent sensory neurons might be involved. Recently, it was shown that the chemokine CCL21 [chemokine (C-C motif) ligand 21] was induced in injured dorsal root ganglion (DRG) neurons and transported to the central terminals of the dorsal horn (Biber et al., 2011). Mice treated with CCL21-neutralizing antibody and mice deficient for CCL21 showed attenuation of tactile allodynia and microglial P2X4R upregulation. CCL21 treatment increased the expression of P2X4R in cultured microglia, indicating a direct action of CCL21 on microglia. Intrathecal supply of CCL21 in CCL21-deficient mice rescued PNI-induced tactile allodynia in those mice. Thus, CCL21 derived from injured DRG neurons directly contributes to microglial P2X4R expression and neuropathic pain (Biber et al., 2011; **Figure 1**).

Because blood–spinal cord barrier functions collapse after PNI occurs (Beggs et al., 2010; Echeverry et al., 2011), proteins leaking from the blood might change P2X4R expression in microglia. The extracellular matrix protein fibronectin might be such a protein. The level of fibronectin protein was elevated in the dorsal horn after PNI (Nasu-Tada et al., 2006; Echeverry et al., 2011). Fibronectin stimulation induces upregulation of P2X4R mRNA and protein in primary cultured microglial cells (Nasu-Tada et al., 2006). Using integrin blockers *in vitro* and *in vivo*, it was shown that fibronectin/integrin signaling was crucial for augmentation of P2X4R expression and PNI-induced tactile allodynia (Tsuda et al., 2008a). Furthermore, intrathecal injection of fibronectin to naïve animals produced tactile allodynia, a behavior not observed in P2X4R-deficient mice administered fibronectin (Tsuda et al., 2008a). Regarding intracellular signaling mechanisms underlying P2X4R upregulation by fibronectin, microglial Lyn tyrosine kinase, a member of Src-family kinases (SFKs) that belong to the non-receptor protein tyrosine kinase family, is an important molecule as upregulation of P2X4R gene expression in response to fibronectin is not observed in microglial cells lacking Lyn (Tsuda et al., 2008b). In spinal cord microglial cells, Lyn was the predominant SFK (Tsuda et al., 2008b) amongst the five members (Src, Fyn, Lck, Yes, and Lyn) that are known to be expressed in the CNS (Salter and Kalia, 2004). Lyn expression in the spinal cord *in vivo* is highly restricted to microglia, and following PNI the level of Lyn is increased (Tsuda et al., 2008b), which is interferon-γ signaling dependent (Tsuda et al., 2009b). Mice lacking Lyn suppress PNI-induced tactile allodynia and the upregulation of spinal P2X4R expression after PNI (Tsuda et al., 2008b). Following Lyn tyrosine kinase activation, two intracellular signaling cascades are distinctly activated: one is a pathway through phosphatidylinositol 3-kinase (PI3K)-Akt and the other is through mitogen-activated protein kinase kinase (MAPK kinase, MEK)-extracellular signal-regulated kinase (ERK; Tsuda et al., 2009c). Signaling through the PI3K-Akt pathway induced degradation of p53 via mouse double minute 2 in a proteasome-dependent manner. The consequence of an attenuated repressive effect of p53 may be associated with enhanced P2X4 gene expression. However, activated MEK-ERK signaling in microglia exposed to fibronectin enhanced eukaryotic translation initiation factor 4E (eIF4E) phosphorylation status via activated MAPK-interacting protein kinase-1, which may play a role in regulating P2X4 expression at translational levels (**Figure 1**). The Lyn-ERK signaling pathway seems likely to be active in spinal microglia after PNI as pharmacological inhibition of SFK effectively suppressed ERK activity in spinal microglia (Katsura et al., 2006). Thus, Lyn might be a key kinase in the molecular machinery mediating the upregulation of P2X4R in microglia (**Figure 1**).

To detect extracellular ATP, P2X4Rs are expressed on the cell surface of microglia. However, a large amount of P2X4R protein within microglia (and macrophages) localizes predominantly to intracellular lysosomal compartments (Qureshi et al., 2007). Interestingly, P2X4R protein remains stable within the proteolytic environment of lysosomes. How P2X4R protein is recruited to the cell surface of microglia remains elusive, but recent studies have shown that trafficking of P2X4R protein to the cell surface occurs when microglia are stimulated by a Toll-like receptor (TLR)4 agonist lipopolysaccharide (Boumechache et al., 2009; Toulme et al., 2010) or the Ca^{2+} ionophore, ionomycin (Qureshi et al., 2007). Furthermore, the chemokine CCL2 increased P2X4R protein levels on the cell surface (without changing total cellular expression) via CC chemokine receptor (CCR2) (Toyomitsu et al., 2012). Notably, CCL2 changed the distribution

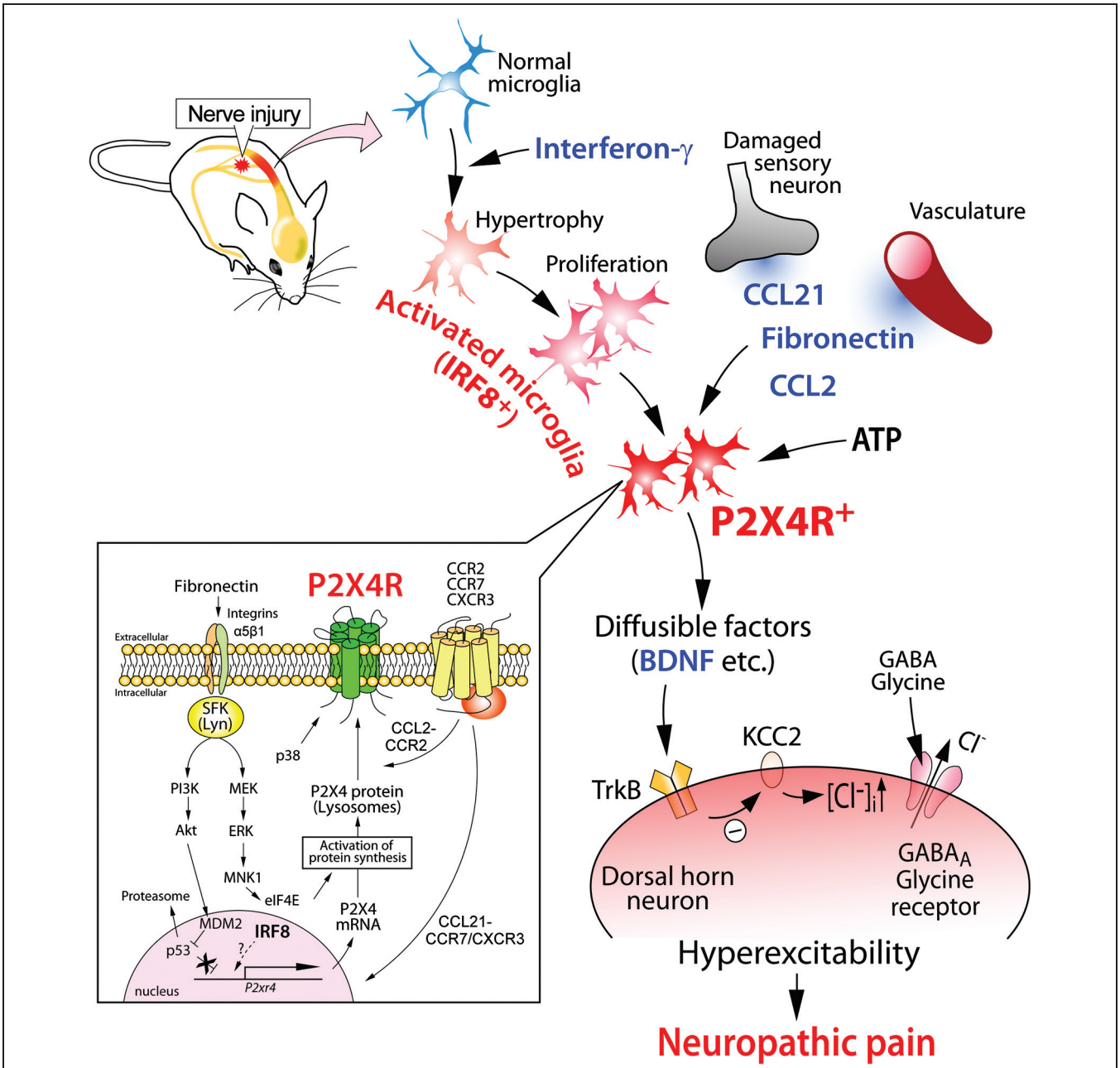

FIGURE 1 | Schematic illustration of the potential mechanisms by which P2X4R in activated microglia modulate pain signaling in the dorsal horn after PNI. Nerve injury activates microglia in the dorsal horn of the spinal cord. Activated microglia show increased expression of P2X4R. The upregulation of microglial P2X4R expression involves signaling by fibronectin and chemokine (C-C motif) ligand 21 (CCL21). CCL2 signaling promotes P2X4R trafficking to cell surface of microglia. P2X4R is activated by ATP and, in turn, release bioactive diffusible factors, such as BDNF. BDNF downregulates the potassium-chloride transporter KCC2 via TrkB, causes an increase in intracellular [Cl^-], and leads to the collapse of the transmembrane anion gradient in dorsal horn neurons which in turn induces depolarization of these neurons following stimulation by GABA and glycine. The resultant hyperexcitability in the dorsal horn pain network induced by factors from activated microglia may be responsible for neuropathic pain.

of lysosomes with P2X4R protein within microglial cells and induced the release of a lysosomal enzyme (Toyomitsu et al., 2012). Thus, CCL2 might promote the expression of P2X4R protein on the cell surface of microglia through exocytosis of P2X4R-containing lysosomes (**Figure 1**). A recent study using single-molecule imaging to track P2X4Rs in the processes of microglia showed that lateral mobility of P2X4Rs is enhanced in activated microglia by the p38 MAPK pathway that selectively regulates slowly mobile P2X4Rs (Toulme and Khakh, 2012). These results indicated that microglial P2X4Rs are dynamically

regulated on the cell surface (**Figure 1**). Thus, post-translational regulation to enhance P2X4R expression and mobility on the cell surfaces of microglia might render cells hyper-responsive to extracellular ATP, which may be important in neuropathic pain.

TRANSCRIPTIONAL FACTOR FOR REACTIVE STATES OF MICROGLIA

PNI activates microglia and converts them to reactive phenotypes through the activation of gene transcription. For example, as described above, activated microglia with high levels of P2X4R expression are essential for PNI-induced neuropathic pain. Appropriate changes in gene expression patterns required for transformation into reactive phenotypes might be tightly regulated by cell type-specific transcription factors. Recent studies identified interferon regulatory factor 8 (IRF8) as a transcription factor in microglia (Horiuchi et al., 2012; Masuda et al., 2012; Minten et al., 2012; Kierdorf et al., 2013) that was critical for their activation and neuropathic pain (Masuda et al., 2012). IRF8 is a member of the IRF family (IRF1–9), and is expressed in immune cells such as lymphocytes and dendritic cells (Tamura et al., 2008). Within the spinal cord, IRF8 expression was markedly upregulated in microglia, but not in neurons or astrocytes, after PNI (Masuda et al., 2012). The microglia-specific upregulation of IRF8 occurs as early as postoperative day 1, peaks on day 3, and persists for at least several weeks. IRF8-deficient mice showed a reduction of PNI-induced tactile allodynia with no change in basal mechanical sensitivity. Notably, suppressing the upregulated expression of spinal IRF8 after PNI by intrathecal administration of a small interfering RNA (siRNA) targeting IRF8 in wild-type mice with allodynia caused a significant recovery of tactile allodynia. This indicated an ongoing activation of IRF8 in spinal microglia. *In vitro* and *in vivo* studies demonstrated that IRF8 promoted the transcription of P2X4R and other molecules associated with reactive phenotypes [innate immune response (TLR2), chemotaxis (P2Y12R and the chemokine receptor CX3CR1), and inflammatory components (interleukin-1β, cathepsin S, and BDNF)]. Nevertheless, nerve injury-induced proliferation of spinal microglia was not affected by IRF8 deficiency, indicating IRF8 deficiency does not impair all reactive processes of microglia. Rather the transcription factor contributes to determining the reactive phenotypes of microglia by changing the expression of a set of genes including P2X4R (**Figure 1**). However, several important issues remain. How is IRF8 expression induced in microglia after PNI? To determine whether IRF8 directly binds to promoter regions of these genes is also important. Answers to these issues will provide new insights into the molecular mechanisms underlying microglial activation and neuropathic pain.

MARKERS OF ACTIVATED MICROGLIA *IN VIVO*

A widely used index for predicting the status of microglia activity *in vivo* is the expression levels of microglial markers [complement receptor 3 (CR3, recognized by OX-42) and Iba1]. Increased immunolabeling of these markers is a principal feature of a variety of PNI models (Tsuda et al., 2005; Suter et al., 2007), but it remains unclear whether their expression can be linked to tactile allodynia following PNI as in some cases CR3 expression is not well correlated with the degree of allodynia (Colburn et al., 1997; Winkelstein and DeLeo, 2002; Tsuda et al., 2005; Suter et al., 2007). In addition, OX-42 immunofluorescence was still observed in P2X4R-knockdown animals that had attenuated PNI-induced allodynia (Tsuda et al., 2003). In contrast, as described above, microglia-specific molecules including P2X4R and IRF8 are necessary for the pathology of neuropathic pain, in both its development and maintenance phases. Therefore, alterations in the expression or phosphorylation of these molecules are likely to be more useful markers to assess reactive states of microglia *in vivo*.

CONCLUDING REMARKS

We have primarily focused on the role of P2X4R expressed in spinal microglia in neuropathic pain (**Figure 1**). Pharmacological, molecular, and genetic studies on P2X4Rs described above provide compelling evidence that P2X4R-positive microglia are a central player in the mechanisms of neuropathic pain and might be promising targets for treating neuropathic pain. Furthermore, the upregulation of microglial P2X4R expression has also been reported in animal models of stroke (Cavaliere et al., 2003), brain tumor (Guo et al., 2004), traumatic brain injury (Guo and Schluesener, 2005; Zhang et al., 2007), spinal cord injury (Schwab et al., 2005), epilepsy (Ulmann et al., 2013), and in human acute inflammatory demyelinating polyradiculoneuropathy (Zhang et al., 2008). Recently, it was demonstrated that P2X4R-positive microglia are essential for morphine-induced hyperalgesia via a P2X4R-BDNF-KCC2 disinhibition cascade between microglia and dorsal horn neurons (Ferrini et al., 2013). It is expected that an increased understanding of the phenotype of P2X4R-positive microglia will provide us with exciting insights into pain mechanisms and clues to aid the development of new therapeutic agents for the management of neuropathic pain and other CNS diseases.

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