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INVITED REVIEW

Joel R. Gever · Debra A. Cockayne · Michael P. Dillon · Geoffrey Burnstock · Anthony P. D. W. Ford

Pharmacology of P2X channels

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Abstract Significant progress in understanding the pharmacological characteristics and physiological importance of homomeric and heteromeric P2X channels has been achieved in recent years. P2X channels, gated by ATP and most likely trimerically assembled from seven known P2X subunits, are present in a broad distribution of tissues and are thought to play an important role in a variety of physiological functions, including peripheral and central neuronal transmission, smooth muscle contraction, and inflammation. The known homomeric and heteromeric P2X channels can be distinguished from each other on the basis of pharmacological differences when expressed recombinantly in cell lines, but whether this pharmacological classification holds true in native cells and in vivo is less well-established. Nevertheless, several potent and selective P2X antagonists have been discovered in recent years and shown to be efficacious in various animal models including those for visceral organ function, chronic inflammatory and neuropathic pain, and inflammation. The recent advancement of drug candidates targeting P2X channels into human trials, confirms the medicinal exploitability of this novel target family and provides hope that safe and effective medicines for the treatment of disorders involving P2X channels may be identified in the near future.

Keywords P2X · Purinergic · ATP · Ion channel · Antagonist

J. R. Gever (✉) · A. P. D. W. Ford
Department of Biochemical Pharmacology,
Roche Palo Alto,
3431 Hillview Avenue,
Palo Alto, CA 94304, USA
e-mail: joel.gever@roche.com

D. A. Cockayne · A. P. D. W. Ford
Department of Neuroscience, Roche Palo Alto,
3431 Hillview Avenue,
Palo Alto, CA 94304, USA

M. P. Dillon
Department of Medicinal Chemistry,
Roche Palo Alto,
3431 Hillview Avenue,
Palo Alto, CA 94304, USA

G. Burnstock
Autonomic Neuroscience Centre,
Royal Free and University College Medical School,
Rowland Hill Street,
London, NW3 2PF, UK

Introduction

Receptors activated by adenosine 5′-triphosphate (ATP), and related di- and tri-phosphate nucleotides, were originally named P2 receptors to differentiate them from P1 receptors, activated most potently by adenosine [35]. In 1985, Burnstock and Kennedy further proposed dividing P2 receptors into P2X and P2Y receptor families, initially on the basis of differences in agonist and antagonist potencies and, later, on the basis of differences in receptor structure and signal transduction mechanism [1, 41]. Accordingly, it is now widely accepted that the terms P2X and P2Y describe ligand-gated ion channels and G protein-coupled receptors, respectively [91, 247].

Our understanding of P2X channels emerged gradually at first from pharmacological investigations of native excitable tissues, and then exploded with great interest after their molecular cloning and characterization in the mid-1990s (Fig. 1). Seven P2X receptor subunits have been identified that share less than 50% identity and range in length from 379 to 595 amino acids. P2X receptor subunits share a similar structural topology consisting of two transmembrane domains connected by a large extracellular loop containing the putative ATP binding site, and intracellular N and C termini of various lengths [24, 78, 154, 165, 226, 254, 290, 300, 305]. In the last decade, the subunit composition of functional P2X channels has been elucidated, especially in recombinant systems, along with an understanding of their biophysical characteristics, such as ion selectivity, permeability, and kinetics of activation and inactivation. Data from a variety of experimental

techniques, including chemical cross-linking followed by native polyacrylamide gel electrophoresis (PAGE), mutagenesis, and atomic force and electron microscopy, support the idea that P2X channels exist as homomeric and heteromeric trimers [4, 10, 151, 215, 227]. These channels are selectively permeable to cations (p_{Ca2+} approximately twofold to fivefold greater than p_{Na+} and p_{K+}) [30, 81, 192, 300], and different trimers display unique pharmacological properties [28, 172, 188, 192, 228, 245, 292]. Significant progress has also been made in ascribing functions to various mammalian P2X subtypes in both physiological and pathological settings, in virtually every cell type and organ system [42].

Despite these advances, progress has been less impressive in certain regards. First, in many tissues and cells it remains to be established which homomeric or heteromeric form(s) of P2X channels transmit ionotropic responses to ATP, a discrepancy that may be attributable to the failure of recombinant expression systems to fully elaborate the characteristics of native P2X channels. Secondly, there remains a paucity of potent and selective pharmacological tools. Agonists that can selectively activate distinct members of this family have not been found, and with the exception of two notable family members, progress has been slower than perhaps anticipated in identifying selective inhibitors. Thus, exploration of therapeutic potential remains still very superficial.

The focus of this review is on the pharmacology of P2X receptors, with the aim of reviewing each *reasonably* established channel trimer, and a goal of capturing a) pharmacological characteristics that reflect the greatest distinctiveness and b) properties that have been identified more recently (over the last 3–5 years). The reader should be aware that many recognized properties of P2X receptors are based on data from recombinant channels, expressed heterologously in either oocytes or mammalian cells, and the degree to which these properties deviate from the functional characteristics of native channels is not entirely clear. A second caveat is that as a general guiding rule, robust pharmacological classification depends heavily on the determination of 'constants' that are derived under conditions closely approximating thermodynamic equilibrium. However, the nature of P2X channels, especially varying rates of desensitization, makes it very difficult (if not impossible) to ensure thermodynamic equilibrium has been established. Accordingly, a review of the literature will reveal many "dependent" variables—EC_{50} and IC_{50} estimates—dependent on the experimental conditions employed. In many cases, because of the difficulty or impossibility in attaining steady-state conditions (e.g., in standard electrophysiological or calcium flux studies), or in clearly establishing "simple, reversible competition", one essentially cannot estimate equilibrium dissociation constants. This means that a clear fingerprint cannot yet be established for many of the P2X channels, and until truly selective antagonists are developed, it will probably remain a challenge. The arrival of novel antagonists will provide a greater opportunity to study channels under conditions that more closely approximate true equilibrium—for example, using radioligand binding approaches. Until then, one must remain cautious when claiming unequivocal characterizations based on agonist EC_{50} or antagonist IC_{50} estimates.

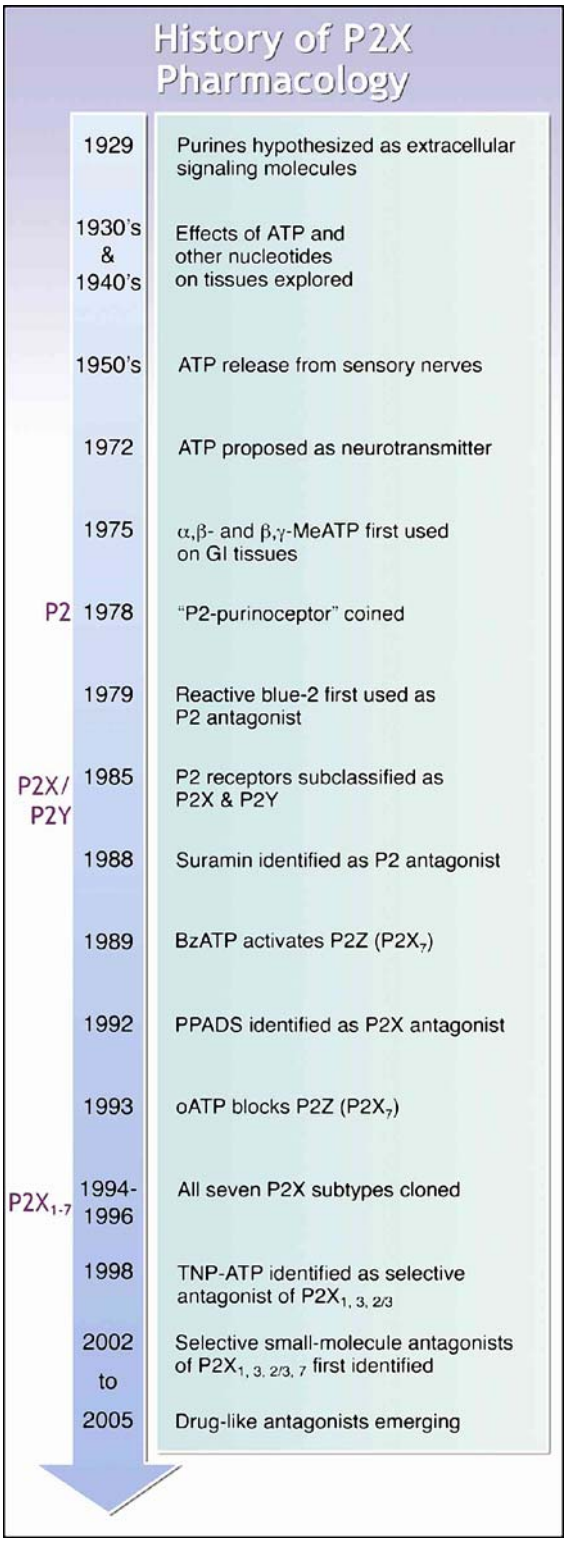

Fig. 1 Timeline of the discovery of P2 receptors and the highlights of their pharmacological characterization. References used to construct timeline: [2, 11, 20, 24, 34, 35, 41, 51, 63, 73, 75, 88, 96, 106, 126, 144, 146, 164, 184, 192, 209, 218, 264, 285, 300, 309]

Homomeric $P2X_1$ channels

Key messages

1. $P2X_1$ channels are predominantly expressed in smooth muscle and platelets where they regulate smooth muscle contractility and various prothrombotic functions.
2. Pharmacologically, $P2X_1$ is almost identical to $P2X_3$ in terms of agonist and kinetic properties. However, β,γ-MeATP has a higher potency for $P2X_1$ versus $P2X_3$.
3. Many $P2X_1$ selective antagonists are available but drug-likeness is low. The only non-acidic small molecule $P2X_1$ antagonist is RO-1 (see Recent advances).

Localization and function

The gene encoding the $P2X_1$ protein subunit was first cloned from rat vas deferens [300], and although $P2X_1$ messenger ribonucleic acid (mRNA) and protein have a fairly broad tissue distribution, most notable is its dense localization within the smooth muscle lining a variety of hollow organs including the urinary bladder, intestines, arteries, and vas deferens [42, 63, 217, 300, 301]. A role for $P2X_1$ in smooth muscle contractility emerged from early studies demonstrating that ATP was the neurotransmitter involved in atropine-resistant, nonadrenergic, noncholinergic contractions of the guinea pig detrusor smooth muscle [34]. These neurogenic contractions could be mimicked by ATP and suppressed by desensitization after exposure to the hydrolytically stable ATP analog, alpha,beta-methylene ATP (α,β-MeATP) [39, 40, 162]. Electrophysiological recordings also showed that ATP and α,β-MeATP elicited dose-dependent membrane depolarization and inward currents in isolated detrusor smooth muscle cells that showed rapid desensitization [93, 139, 140]. It is now well-established that $P2X_1$ channels mediate the purinergic component of sympathetic and parasympathetic nerve-mediated smooth muscle contraction in a variety of tissues including urinary bladder [132, 235, 303], vas deferens [184, 217, 294], saphenous vein [311], and the renal microvasculature [141]. Consistent with this, $P2X_1$-mediated inward currents are abolished in the detrusor smooth muscle, vas deferens, and mesenteric arteries of mice lacking the gene encoding $P2X_1$ protein subunits [217, 303, 304]. Nerve-mediated vasoconstriction and contraction of the urinary bladder and vas deferens are also reduced by ~50–70% in these mice [217, 303, 304].

$P2X_1$ is also present in blood platelets [203], and ATP activation of $P2X_1$ receptors has been implicated in the regulation of various platelet functions including shape change [256] and aggregation under increased sheer stress conditions [79, 122]. Platelets from $P2X_1$-deficient mice have deficits in aggregation, secretion, adhesion, and thrombus growth under certain in vitro conditions [122]. $P2X_1$-deficient mice also have reduced mortality and thrombus formation in models of systemic thromboembolism and laser-induced vessel wall injury, respectively [122]. Conversely, transgenic mice overexpressing human $P2X_1$ protein subunits in the megakaryocytic cell lineage exhibit hypersensitive platelet responses in vitro, and increased mortality in a model of systemic thromboembolism [234]. Taken together, these data suggest that $P2X_1$ channels may play an important role in platelet physiology and hemostasis.

Activation

Two defining characteristics of the homomeric $P2X_1$ channel are its rapid desensitization kinetics and its sensitivity to activation by α,β-MeATP [80, 300]. In cells expressing recombinant rat or human $P2X_1$, α,β-MeATP is generally less potent than ATP and 2-(methylthio) ATP (2-MeSATP) (pEC_{50}≈6–7), and somewhat more potent than adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S) (pEC_{50}≈5.5) [14, 80, 292, 300, 301]. These characteristics are shared by the homomeric $P2X_3$ channel, and, therefore, cannot be used to uniquely define $P2X_1$. However, beta, gamma-methylene ATP (β,γ-MeATP) is reported to be equipotent to α,β-MeATP at $P2X_1$, but approximately 30- to 50-fold less potent at $P2X_3$, and >100-fold less potent at $P2X_{2,4,5,7}$ [30, 80, 96, 97, 173, 285]. Consequently, β,γ-MeATP has been used as a selective agonist in some studies investigating $P2X_1$-mediated smooth muscle contraction (e.g., urinary bladder, vas deferens, saphenous veins) [178, 216, 217, 232, 288, 294, 311]. Adenosine 5′-diphosphate (ADP) was originally reported to be an agonist at $P2X_1$ with moderate potency (pEC_{50}=4.1–5) [14, 80]; however, it has been shown that this activity was imparted by impurities. Indeed, purified ADP at concentrations as high as 1 mM fail to elicit currents in oocytes expressing human $P2X_1$ [204]. One report further suggests that 3′-*O*-(4-benzoyl)benzoyl ATP (BzATP) may be the most potent agonist at $P2X_1$ with a reported pEC_{50} of 8.74, approximately 100-fold more potent than α,β-MeATP [14]. Recently, a recombinant chimeric rat $P2X_2/P2X_1$ receptor, incorporating the N terminus and first transmembrane domain of $P2X_2$ (conferring non-desensitizing kinetics) with the extracellular loop, second transmembrane domain and C terminus of $P2X_1$ (retaining $P2X_1$ pharmacology), was used to unmask nanomolar potency of ATP (pEC_{50}=8.5) and other nucleotide agonists [252]. The deactivation rate of currents (τ) through the rat $P2X_2/P2X_1$ chimera after washout of agonist was inversely related to potency (e.g., for ATP, τ =63 s and pEC_{50}=8.5, while for α,β-MeATP, τ =2.5 s and pEC_{50}=7.2), leading the authors to conclude that the rate-limiting step in the recovery from desensitization was the rate of agonist unbinding. A similar finding has recently been reported for the rapidly desensitizing $P2X_3$ channel (see $P2X_3$ section below) [243, 252].

Diadenosine polyphosphates are also known to be agonists at $P2X_1$, with potencies similar to ATP, and selectivity for rat $P2X_1$ over rat $P2X_2$, $P2X_3$ and $P2X_4$. Only Ap_6A is a full agonist (pEC_{50}=6.1 at $P2X_1$, 5.8 at $P2X_3$, <<4 at $P2X_2$ and $P2X_4$), whereas, Ap_5A (pEC_{50}=6.0

at $P2X_1$, ≈5.9 at $P2X_3$, <<4 at $P2X_2$ and $P2X_4$) and Ap_4A (pEC_{50}=7.4 at $P2X_1$, 6.4 at $P2X_4$, 6.1 at $P2X_3$, 4.8 at $P2X_2$) are partial agonists, with Ap_4A being at least tenfold selective for $P2X_1$ over the other P2X channels tested [320]. Conversely, diinosine polyphosphates (synthesized through the deamination of Ap_nAs by the AMP-deaminase of *Aspergillus* sp.) are potent $P2X_1$ antagonists (see below) [171].

Inhibition

The first antagonists shown to block $P2X_1$ channels were the non-selective P2 antagonist, suramin [75], and the non-selective P2X antagonist, pyridoxal-5′-phosphate-6-azo-phenyl-2,4-disulfonate (PPADS) [184, 300]. Subsequently, several analogs of both suramin and PPADS were synthesized that had increased $P2X_1$ potency and selectivity [143, 253, 341]. NF023 is a suramin analog that was first identified as a P2X selective antagonist based on inhibition of α,β-MeATP-evoked vasoconstriction in pithed rats [299]. After a thorough pharmacological characterization using two electrode voltage-clamp recordings in oocytes expressing recombinant P2X channels, NF023 was shown to be a $P2X_1$ antagonist (pIC_{50}=6.6) with selectivity over $P2X_3$ and $P2X_{2/3}$ (~35- to 100-fold) and $P2X_2$ and $P2X_4$ (~400-fold or greater) [277]. Even greater potency was achieved with the discovery of another suramin analog, NF279, which has a pIC_{50} of 7.7 and increased selectivity over rat $P2X_3$ (85-fold) and human $P2X_4$ (>15,000-fold) [253]. Unlike NF023, NF279 is a reasonably potent rat $P2X_2$ antagonist with a pIC_{50} of 6.1 (40-fold less potent than at rat $P2X_1$). The mechanism of antagonism of NF279 and NF023 was further investigated using non-desensitizing $P2X_2$-containing channels ($P2X_2$ for NF279 and the chimeric $P2X_2/P2X_1$ for NF023) to avoid the agonist–antagonist hemi-equilibrium conditions present in rapidly desensitizing channels. Incubation with either NF023 or NF279 resulted in parallel, surmountable shifts in the concentration–response curves to ATP, consistent with competitive antagonism [252, 253].

PPADS analogs with increased potency and selectivity have also emerged. MRS2220 was the first PPADS analog identified with modest selectivity for rat $P2X_1$ (pIC_{50}=5) over rat $P2X_3$ (pIC_{50}=4.2) and $P2X_2$, $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ (inactive up to 100 μM) [143]. Pyridoxal-5′-phosphate-6-azo-naphthyl-5-nitro-3,7-disulfonate (PPNDS), another PPADS analog, inhibited α,β-MeATP-induced isometric contractions of rat vas deferens with a pK_B=7.43 (vs 6.59 for PPADS), and inward currents of rat $P2X_1$-expressing oocytes with pIC_{50}=7.84 (vs 7.06 for PPADS). PPNDS also blocked guinea pig ileum smooth muscle contractions evoked by adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS) with a pA_2=6.13 (vs 6.2 for PPADS) [185].

Certain nucleotides have also been shown to be potent and selective $P2X_1$ antagonists. 2′,3′-O-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) and other related trinitrophenyl ATP analogs (e.g., TNP-ADP, TNP-AMP, and TNP-GTP) are 300- to 4,000-fold selective for $P2X_1$ (pIC_{50}=8.22), $P2X_3$ (IC_{50}=8.5–9.0) and $P2X_{2/3}$ (IC_{50}=7.4–8.2) over $P2X_2$, $P2X_4$ and $P2X_7$ (pIC_{50}≤5.9) [309]. As mentioned previously, diinosine polyphosphates are also potent $P2X_1$ antagonists, possibly acting via stabilization of the desensitized state of the channel (see $P2X_3$ section below). Ip_5I is the most potent and selective for rat $P2X_1$ (pIC_{50}=8.5), being 900-fold selective over $P2X_3$ (pIC_{50}=5.6) and >1000-fold selective over $P2X_2$ (inactive up to 30 μM) [171].

As is the case with all P2X receptors, agonist-evoked currents through $P2X_1$ are altered by extracellular pH, being reduced at pH 6.3 but unaffected at pH 8.3 [81, 117]. Although extracellular calcium has been shown to reduce currents through most P2X channels, $P2X_1$ is unaffected up to concentrations as high as 100 mM [281].

Homomeric $P2X_2$ channels

Key messages

1. $P2X_2$ channels are widely distributed throughout the peripheral and central nervous system, and on many non-neuronal cell types, where they play a role in sensory transmission and modulation of synaptic function.
2. $P2X_2$ channels exhibit agonist activity and slow desensitization kinetics similar to $P2X_4$ and $P2X_5$.
3. $P2X_2$ channels are the only homomeric P2X subtype potentiated by acidic conditions; they are also potentiated by Zn^{2+}, but inhibited by other divalent cations at high concentrations.

Localization and function

The gene encoding the $P2X_2$ subunit was first cloned from neuronally derived rat pheochromocytoma PC12 cells [24], and subsequent localization studies have demonstrated a broad tissue distribution. $P2X_2$ is expressed within the peripheral and central nervous systems (CNS), where it plays a role in ATP-mediated fast synaptic transmission at both nerve terminals and at interneuronal synapses. Within the CNS, $P2X_2$ receptors are localized within the cortex, cerebellum, hypothalamus, striatum, hippocampus, nucleus of the solitary tract, as well as in the dorsal horn of the spinal cord [42, 63, 160, 161, 168, 169, 237, 265, 270, 312, 313, 325]. Accordingly, $P2X_2$ channels may have wide-ranging functions in the regulation of many CNS processes including memory and learning, motor function, autonomic coordination, and sensory integration. Several studies have proposed a role for homomeric $P2X_2$, and possibly heteromeric $P2X_2$-containing channels, in ATP-mediated facilitation of inhibitory γ-amino butyric acid-mediated (GABAergic) synaptic transmission in the hippocampus and dorsal horn [8, 22, 133, 167]. $P2X_2$ is also heavily expressed in the peripheral nervous system on both sensory

and autonomic ganglion neurons [45, 59, 63, 201, 202, 270, 313, 330, 336–339], signifying roles in afferent and efferent signaling pathways, and in the enteric nervous system where homomeric $P2X_2$ channels are thought to mediate fast synaptic excitation on S-type myenteric neurons [48, 94, 233, 250, 340]. Numerous recent studies have implicated both peripheral and central $P2X_2$ channels in chemosensory transduction in a variety of physiological systems, including the regulation of respiratory control in response to hypoxia and hypercapnia (via sensory neurons within neuroepithelial bodies and the carotid body and the ventrolateral medulla) [107, 121, 205, 242, 258, 335], and in the detection of chemical stimuli, such as odorants (via trigeminal neurons in the nasal epithelium) [279], and taste (by gustatory nerves) [86].

$P2X_2$ protein subunits are also expressed on many non-neuronal cell types including cells of the anterior pituitary [312] and adrenal medulla [312], endothelial and epithelial cells [15, 120, 175], epithelial and other support cells within the cochlea [131, 145, 175], skeletal, cardiac and smooth muscle [118, 119, 155, 191, 263], interstitial cells of Cajal [43, 44], and lymphocytes [69]. A role for $P2X_2$ in many of these tissues has yet to be defined, but may involve functions of ATP, such as autocrine/paracrine regulation of hormone release, exocytosis/endocytosis, regulation of sound transduction, smooth muscle contractility, and pacemaker activity.

$P2X_2$ is unique among other P2X receptor subunits in that multiple splice variants of the human, rat and guinea pig $P2X_2$ mRNA have been identified that are capable of producing channels with different functional properties (see below) [25, 50, 130, 180, 200, 270]. Given the ability of full-length $P2X_2$ protein subunits to form heteromeric assemblies with truncated $P2X_2$ splice variants or other P2X subunits (e.g., $P2X_3$ or $P2X_6$, see corresponding sections below), $P2X_2$-containing channels in whole tissues or animal studies may function in a manner not entirely predicted by in vitro studies utilizing recombinant full-length $P2X_2$ subunits expressed in cell lines.

Activation

On the basis of a similar rank order of agonist potencies and slow desensitization kinetics after activation, $P2X_2$ can be grouped with $P2X_4$ and $P2X_5$. ATP, ATP-γ-S, and 2-MeSATP are the most potent agonists, with similar pEC_{50}s that are commonly reported as ranging from 5.1 to 6.3 [14, 80, 173, 174, 200, 225]. Bz-ATP has been reported to be a less potent partial agonist [80, 212], and α, β-MeATP, β,γ-MeATP, ADP, and uridine 5′-triphosphate (UTP) are inactive up to 100–300 μM [14, 24, 80, 173, 174]. The only diadenosine phosphate capable of gating $P2X_2$ channels is Ap_4A (pEC_{50}=4.8); Ap_2A, Ap_3A, Ap_5A, and Ap_6A are all inactive up to 100 μM [240, 320].

One property that differentiates $P2X_2$ from all other homomeric P2X channels is the ability of acidic pH to potentiate ATP-evoked currents [174, 281]. ATP concentration–response curves at recombinant rat $P2X_2$ channels expressed in oocytes are facilitated by protons, with a maximal potentiation at pH 6.5 (pK_a=7.05 for potentiation), producing a shift of the pEC_{50} from 5.3 (pH=7.4) to 5.9 (pH=6.5) and no change in the maximal response [173]. Conversely, ATP-evoked currents are reduced under basic conditions (pEC_{50}=4.5 at pH 8.0) [173]. The ATP binding site of the $P2X_2$ channel is likely to include a histidine residue within the extracellular loop, and mutation of this residue to an alanine (H319A) significantly reduces the pH sensitivity of $P2X_2$ expressed in oocytes [58]. Extracellular histidine residues (His^{120} and His^{213}) may also be important in mediating the potentiation of currents through $P2X_2$ by Zn^{2+} (1–10 μM) [24, 57, 58, 224, 322]. It has been hypothesized, based on results from mutational studies, that the Zn^{2+} binding site resides at the interface between $P2X_2$ subunits on homomeric channels [221]. To date, this is the only evidence for a regulatory intersubunit binding site for any factor on a P2X channel, although intersubunit binding sites have been demonstrated to be present in other ion channels including $GABA_A$, glycine, and nicotinic receptors (for discussion, see [47, 113, 268]).

$P2X_2$ channels are known to dilate after prolonged agonist activation, a characteristic shared by homomeric $P2X_4$, $P2X_5$, and $P2X_7$ channels [166, 307]. Recent studies using fluorescence resonance energy transfer (FRET) have shown that the increased permeability of $P2X_2$ channels after pore dilation is due to the movement of subunit cytosolic domains, resulting in a transition from a state of high to lower ionic selectivity (measured as permeability to *N*-methyl-D-glucamine; NMDG) over the course of ~13 s [87]. Although in recent years certain evidence has suggested that the ATP-evoked cellular uptake of some large molecular weight fluorescent dyes, such as quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(triethylammonio)propyl]-diiodide (YO-PRO-1) may not pass exclusively through a pore intrinsic to P2X channels, the FRET work with $P2X_2$ [87], and other evidence from experiments with $P2X_7$ channels, suggests that NMDG most likely does pass directly through a dilated P2X channel (see $P2X_7$ section below) [153].

Inhibition

There are no known selective or highly potent $P2X_2$ antagonists. PPADS, TNP-ATP, and reactive blue-2 are approximately equipotent inhibitors of ATP-evoked currents through human or rat $P2X_2$ channels (pIC_{50}s range from 5.4 to 6.4), clearly less potent than at the homomeric $P2X_1$ or $P2X_3$ channels [14, 173, 200, 309]. Suramin has been reported to be of similar potency (pIC_{50}=5.4–6.0) as PPADS and TNP-ATP at $P2X_2$ [80, 214, 322] in some studies, while others have reported suramin as having a threefold to tenfold lower potency (pIC_{50}=4.5 to 5.0) than these antagonists [14, 173].

As stated above, currents through $P2X_2$ channels are potentiated by Zn^{2+}, whereas, other divalent cations (e.g., Mn^{2+}, Mg^{2+}, Ca^{2+} and Ba^{2+}), at extracellular concentrations of 1–7 mM, have all been shown to reduce ATP-

evoked currents through rat $P2X_2$ channels expressed in oocytes. It is speculated that this inhibition may occur through open channel blockade [71, 173].

Several splice variants of the wild-type rat $P2X_2$ channel ($rP2X_{2a}$) have been identified, but only one variant (rat $P2X_{2b}$; containing a 69-amino-acid deletion in the C terminus) has been shown to form functional channels [25, 270]. The key difference is that the homomeric rat $P2X_{2b}$ channel expressed in oocytes has a more rapid desensitization ($P2X_{2b}$, τ =12–27.5 s; $P2X_{2a}$, τ =56–115 s), and reduced sensitivity to antagonists such as PPADS and suramin [25, 200, 270]. A human splice variant ($hP2X_{2b}$) with a similar amino acid deletion in the C terminus has also been isolated from pituitary tissue, but had identical desensitization characteristics and sensitivity to agonists and antagonists as the wild-type human $P2X_{2a}$ channel [200]. Thus, regions in the C terminus thought to be important in controlling the desensitization kinetics of the rat $P2X_2$ channel (e.g., Val^{370}, Pro^{373}–Pro^{376}) apparently do not regulate the kinetics of the human $P2X_2$ channel in the same manner [179, 273].

Homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ channels

Key messages

1. $P2X_3$ and $P2X_{2/3}$ channels are predominantly localized on peripheral and central terminals of unmyelinated C-fiber and thinly myelined Aδ sensory afferents, where they mediate sensory neurotransmission.
2. $P2X_3$ and $P2X_{2/3}$ channels are pharmacologically similar, and like $P2X_1$, are selectively gated by α, β-MeATP. These channels differ, however, in their desensitization kinetics and in their sensitivity to extracellular ions.
3. Non-acidic, "drug-like" $P2X_3$/$P2X_{2/3}$ antagonists have been identified (see Recent advances).

Localization and function

Homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ channels have become increasingly recognized as playing a major role in mediating the primary sensory effects of ATP [36–38, 88, 149, 231]. The gene encoding the $P2X_3$ protein subunit was originally cloned from dorsal root ganglion (DRG) sensory neurons [51, 192] and, in the adult, $P2X_3$ and $P2X_{2/3}$ channels are predominantly localized on small-to-medium diameter C-fiber and Aδ sensory neurons within the dorsal root, trigeminal, and nodose sensory ganglia [23, 42, 77, 313]. Electrophysiological studies on sensory neurons from $P2X_2$- and $P2X_3$-deficient mice have confirmed that $P2X_3$ and $P2X_{2/3}$ channels account for nearly all ATP responses in DRG sensory neurons [33, 59, 60, 246], while $P2X_2$ and $P2X_{2/3}$ channels are predominant in nodose sensory neurons [59, 289, 309]. $P2X_3$ and $P2X_{2/3}$ channels are present on both the peripheral and central terminals of primary sensory afferents projecting to a number of somatosensory and visceral organs including the skin, joint, bone, lung, urinary bladder, ureter, and gastrointestinal tract [26, 27, 60, 104, 135, 142, 176, 191, 257, 313, 314, 328, 329, 333, 334]. Accordingly, central $P2X_3$ and $P2X_{2/3}$ channels are present within the dorsal horn of the spinal cord and within the nucleus tractus solitarius (NTS), where they appear to play a role in the presynaptic modulation of glutamate release [114, 156, 222, 223]. $P2X_3$ and $P2X_{2/3}$ channels are also present within the enteric nervous system, where they are thought to mediate excitation of AH-type intrinsic sensory neurons [13, 94, 241, 302]. Recent studies have demonstrated that epithelial tissues, including the bladder uroepithelium, airway epithelial cells, and pulmonary neuroepithelial bodies, express $P2X_3$ and $P2X_{2/3}$ channels, where they may modulate certain mechanosensory or chemosensory responses [92, 316].

Several studies have shown that $P2X_3$ is expressed during development in various regions of the brain and in regions of the spinal cord outside of the dorsal horn; however, a role for $P2X_3$ during development of the nervous system has not been clearly established [55, 56, 170, 282].

$P2X_3$ and $P2X_{2/3}$ channels have been characterized as fulfilling a role in nociceptive transmission and mechanosensory transduction within visceral hollow organs [88, 94, 149]. Studies using pharmacological agents, such as the $P2X_1$, $P2X_3$, and $P2X_{2/3}$ selective antagonist TNP-ATP [128, 148, 296–298], and the $P2X_3$, $P2X_{2/3}$ selective antagonist A-317491 [146, 207, 327] (see below), have shown that peripheral and spinal $P2X_3$ and $P2X_{2/3}$ channels are involved in transmitting persistent, chronic inflammatory and neuropathic pain. $P2X_3$-deficient mice [60, 278], and animals treated with $P2X_3$-selective antisense [7, 127, 137] or small interfering RNA (siRNA) [72] have revealed similar findings.

$P2X_3$ receptors also play a role in visceral mechanosensory transduction where according to the "tubes and sacs" hypothesis proposed by Burnstock, ATP released from the epithelial lining of visceral hollow organs can activate $P2X_3$ and/or $P2X_{2/3}$ channels on adjacent primary sensory afferents [36]. Within the urinary bladder [84, 284, 310] and ureter [177] for example, ATP is released from the urothelium upon distension. Distension leads to increased afferent nerve activity that is mimicked by ATP and α,β-MeATP, and attenuated in $P2X_3$-deficient mice [259, 310]. ATP and α,β-MeATP can directly stimulate the micturition reflex in conscious rats, and this is inhibited by TNP-ATP [236]. Moreover, $P2X_3$-and $P2X_2$-deficient mice have reduced urinary bladder reflexes [59, 60]. A similar role has been postulated in gastrointesintal tissues where α,β-MeATP excites extrinsic [176, 329] and intrinsic [12, 13, 37] afferents, and $P2X_3$-deficient mice have impaired peristalsis [13].

Activation

Like $P2X_1$, native and recombinantly expressed homomeric $P2X_3$ channels respond to α,β-MeATP with a rapidly desensitizing inward current (typically described as bi-exponential decay with a fast component of τ_{d1}≈30–100 ms and a slow component of τ_{d2}≈250–1,000 ms) at concentrations (pEC_{50}=5.7–6.3) approximately 100-fold lower than those required to activate other homomeric P2X channels [14, 33, 51, 89, 97, 112, 192, 225, 255, 308]. When tested side-by-side in the same assay systems, ATP and 2-MeSATP (pEC_{50}=6.1–6.9) have been consistently shown to be slightly more potent than α,β-MeATP [14, 97, 225, 255]. Most studies have determined that ATP-γ-S is of similar potency as α,β-MeATP (pEC_{50}=6.2–6.3) [14, 225], although it was originally reported to be less potent [51]. Again, as at $P2X_1$, BzATP is the most potent agonist at homomeric $P2X_3$ channels, with the concentration required to elicit half-maximal responses (pEC_{50}=7.1–7.5) being approximately fivefold lower than that required for ATP or 2-MeSATP [14, 225]. Overall, the distinguishing pharmacological features between $P2X_3$ and $P2X_1$ include lower sensitivity of $P2X_3$ to L-β,γ-MeATP (pEC_{50}<4 at $P2X_3$; pEC_{50}~5.5 at $P2X_1$) [51, 80, 97, 246] and Ap_4A (pEC_{50}=6.1–6.3 at $P2X_3$; pEC_{50}=7.4 at $P2X_1$) [14, 320]. Conversely, Ap_3A appeared to be a $P2X_3$-selective agonist in one report (pEC_{50}=6.0 at rat $P2X_3$; pEC_{50}<4 at $P2X_1$, $P2X_2$ and $P2X_4$) [320], but it has since been reported to be a significantly weaker partial agonist at human $P2X_3$ (pEC_{50}=4.7, 53% of ATP-evoked maximal response) and inactive at rat $P2X_3$ (pEC_{50}<4) [14]; this finding remains controversial. It has also been recently suggested that desensitized $P2X_3$ channels bind some agonists (e.g., ATP) with very high affinity (<1 nM), and that the subsequent rate of recovery from desensitization is primarily dependent on the rate of agonist unbinding [243].

The heteromeric $P2X_{2/3}$ channel shares many of the activation characteristics of homomeric $P2X_3$ including selective gating by α,β-MeATP and a similar rank order of agonist potencies [14, 192, 199]. However, the key difference is that α,β-MeATP-evoked inward currents through recombinant or natively expressed (nodose ganglion neurons) $P2X_{2/3}$ channels are slowly desensitizing [33, 192]. In fact, the relatively sustained agonist-evoked cation influx through $P2X_{2/3}$ channels has enabled the use of mechanism of action studies requiring agonist–antagonist equilibrium (i.e., Schild-style curve shift experiments) to better understand putative antagonist binding sites (see below).

Another fundamental way in which $P2X_{2/3}$ channels differ from $P2X_3$ is in their opposite response to changes in pH. Like $P2X_2$, inward currents through $P2X_{2/3}$ channels (recombinantly expressed in oocytes or natively expressed in rat nodose ganglion neurons) are strongly increased under acidic conditions by as much as 250% at pH 6.3, and strongly decreased under basic conditions by about 75% at pH 8.0. In contrast, currents through $P2X_3$ channels are much less sensitive to variations in pH, being unaffected at modestly basic (pH 8.0) or acidic (pH 6.5) conditions, and only significantly reduced in a much more acidic environment (pH 5.5) [195, 196, 281, 323]. In fact, the agonist-evoked response of $P2X_{2/3}$ channels is extremely sensitive to small changes in extracellular pH (pK_a=7.1–7.2) [195, 196], a factor that must be taken into consideration when comparing the potency estimates of competitive antagonists from studies conducted under different assay conditions (e.g., TNP-ATP and A-317491; see below).

Channels containing $P2X_3$ subunits appear to be sensitive to positive allosteric modulation by agents such as cibacron blue, ethanol, and Zn^{2+}. Cibacron blue elicited a threefold to sevenfold increase in the maximal ATP-evoked Ca^{2+} influx through recombinant homomeric human $P2X_3$ channels (but not $P2X_1$, $P2X_2$, or $P2X_7$) expressed in 1321N1 astrocytoma cells (pEC_{50} for potentiation=5.9), and pre-incubation with 3 μM cibacron blue increased the pEC_{50} of ATP from 6.4 to 7.3 [3]. Because the actions of cibacron blue were independent of ATP concentration, and mediated both a leftward shift of the agonist concentration–effect curve and a rightward shift of the concentration–effect curve of a non-competitive antagonist (PPADS), it was concluded that cibacron blue positively modulates ATP activation of $P2X_3$-mediated inward currents via an allosteric binding site [3]. ATP-evoked currents through $P2X_3$ channels are also potentiated by high concentrations of ethanol (5–200 mM), but, unlike cibacron blue, ethanol produces only a modest increase in ATP potency (from pEC_{50}=5.6 to 6.0 in the presence of 100 mM ethanol) with no change in the maximal response [66]. Neither ethanol nor cibacron blue has been tested on $P2X_{2/3}$ channels, so it is unknown if the heteromer retains the sensitivity to these agents exhibited by the homomeric $P2X_3$ channel. Agonist-evoked inward currents through both homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ channels are sensitive to positive modulation by Zn^{2+} (pEC_{50} for potentiation=4.9–5.0) [194, 196, 323]. For example, in oocytes expressing recombinant rat $P2X_3$ channels, 100 μM Zn^{2+} increased the potency of ATP from pEC_{50}=5.3 to 6.1, with no change in the maximal response [66]. Furthermore, because the potentiation of $P2X_3$-mediated inward currents in oocytes by ethanol and Zn^{2+} were synergistic, not additive, and the maximal potentiation by Zn^{2+} was increased in the presence of ethanol, the authors concluded that ethanol and Zn^{2+} are acting on different sites or by different mechanisms [66].

Inhibition

As with $P2X_1$, the activation of $P2X_3$ and $P2X_{2/3}$ channels by α,β-MeATP is sensitive to inhibition by TNP-ATP. Nanomolar concentrations of TNP-ATP can inhibit α,β-MeATP-evoked inward currents and Ca^{2+} influx in cell lines expressing recombinant rat $P2X_3$ (pIC_{50}=9.0) and $P2X_{2/3}$ (pIC_{50}=8.3–8.5) channels [32, 309], representing an approximately 1,000-fold or greater selectivity over other homomeric P2X channels. Similarly, α,β-MeATP-evoked currents through natively expressed rat $P2X_3$ (DRG neurons) and $P2X_{2/3}$ (nodose ganglion neurons) channels

are also inhibited by TNP-ATP with pIC_{50}s of 9.1–9.5 and 7.7, respectively [76, 112]. Not surprisingly, based on the structural similarity to ATP, TNP-ATP is thought to be a competive antagonist of ATP-mediated responses at $P2X_3$ and $P2X_{2/3}$ channels. In a manner consistent with competitive antagonism, pre-incubation with increasing concentrations of TNP-ATP produced parallel and surmountable rightward shifts (slope of Schild plot ≈1) of α,β-MeATP concentration-effect curves in 1321N1 cells expressing the heteromeric $P2X_{2/3}$ channel, or a $P2X_{2\text{-}3}$ chimeric channel composed of subunits incorporating the N terminus and first transmembrane domain of $P2X_2$ (conferring non-desensitizing kinetics) with the extracellular loop, second transmembrane domain and C terminus of $P2X_3$ (retaining $P2X_3$ pharmacology). In these experiments, the affinity estimates (pA_2) of TNP-ATP were 8.7 (human $P2X_{2\text{-}3}$), 8.2 (rat $P2X_{2/3}$) and 8.7 (human $P2X_{2/3}$) [32, 225]. A similar affinity estimate (K_D≈2 nM) was determined in experiments measuring the on- and off-rates of TNP-ATP on rat $P2X_{2/3}$ channels, where it was illustrated that the high affinity of TNP-ATP derives primarily from fast binding (k_{+1}≈100 μM^{-1} s^{-1}) and not slow unbinding (k_{-1}≈0.3 s^{-1}) [280]. Further evidence that TNP-ATP acts at the ATP binding site is the observation that pre-incubation of rat DRG neurons (natively expressing homomeric $P2X_3$ channels) with approximately pIC_{80} concentrations of TNP-ATP (10 nM) significantly reduced the rate of desensitization of α,β-MeATP-evoked currents, as would be expected of a competitive antagonist [89].

In addition to TNP-ATP, both suramin and PPADS are antagonists of rat $P2X_3$- and $P2X_{2/3}$-mediated responses. Antagonism occurs at concentrations (pIC_{50}=5.4–6.5) similar to those required to block activation of $P2X_1$ and $P2X_5$ channels, and lower than those required to block $P2X_2$, $P2X_4$, and $P2X_7$ channels [14, 112], although the human $P2X_3$ channel has been reported to be somewhat less sensitive to suramin (pIC_{50}≤4.8) than the rat $P2X_3$ channel (pIC_{50}=6.1) [14, 97]. As previously discussed in the $P2X_1$ section, Ip_5I is a $P2X_1$-selective antagonist that has moderate potency as an antagonist of inward currents through native or recombinantly expressed $P2X_3$ channels (pIC_{50}=5.6–6.9) [76, 171]. Recently, it was observed that Ip_5I inhibited $P2X_3$-mediated inward currents in rat DRG neurons only when pre-exposed to desensitized receptors, suggesting that this antagonist inhibits $P2X_3$ (and presumably $P2X_1$) activity through stabilization of the desensitized state of the channel [89].

High extracellular concentrations of calcium inhibit α,β-MeATP-evoked currents through rat $P2X_3$ (pEC_{50}=1.1) and $P2X_{2/3}$ (pEC_{50}=1.8) channels [308]. Additionally, increasing the extracellular but not the intracellular concentration of Ca^{2+} from 1 to 10 mM has been shown to speed the recovery of $P2X_3$ channels from the desensitized state, and this was true even if the increase was reversed several minutes before activating the channels. These data suggest that Ca^{2+} (and other polyvalent cations like Gd^{3+} and Ba^{2+}) bind to an extracellular site to alter channel recovery [65].

Homomeric $P2X_4$ channels

Key messages

1. $P2X_4$ subunits are widely distributed within neuronal and non-neuronal tissues.
2. $P2X_4$ channels localized on activated microglia have been implicated in chronic inflammatory and neuropathic pain.
3. Species differences exist in the responses of $P2X_4$ channels to α,β-MeATP and PPADS.
4. $P2X_4$ channels can be differentiated from $P2X_2$ and $P2X_5$ channels by differing activation sensitivity to pH and Zn^{2+}.

Localization

The gene encoding the $P2X_4$ protein subunit was originally cloned from rat brain [20], and $P2X_4$ may be the most widely distributed of the P2X channels. mRNA and protein localization studies indicate that the $P2X_4$ subunit is expressed in several regions of the rat brain (particularly cerebellar Purkinje cells) and spinal cord [18, 20, 30, 42, 63, 95, 260, 276, 315], autonomic and sensory ganglia [18, 30, 330], arterial smooth muscle [18, 105, 193, 230], osteoclasts [125, 219], parotid acinar cells [63, 287], kidney [18, 95, 206], lung [18, 30, 276], heart [18, 95, 276], liver [18, 95], pancreas [18], and human B lymphocytes [272]. The functional role of $P2X_4$ in most of these tissues is still unclear. However, several recent studies have demonstrated that $P2X_4$ receptor expression is increased on activated spinal cord microglia after spinal nerve injury, spinal cord injury, or formalin-induced inflammatory pain [116, 138, 266, 295]. Moreover, intraspinal administration of $P2X_4$ antisense oligonucleotides decreased the induction of $P2X_4$ receptors on spinal microglia, and suppressed the development of tactile allodynia after spinal nerve injury [295]. Intraspinal administration of TNP-ATP and PPADS also suppressed tactile allodynia in this study; however, these antagonists are not selective for $P2X_4$ channels and may mediate reversal of chronic pain through other P2X channels. These findings suggest that ATP and $P2X_4$ may be important in the modulation of chronic inflammatory and neuropathic pain by spinal cord microglia, a topic that has received considerable recent attention [318].

Activation

Homomeric $P2X_4$ channels generally produce a slowly-desensitizing inward current in response to ATP [20, 30, 95]. $P2X_4$ channels are activated most potently by ATP, with pEC_{50}s in recombinant systems ranging from 4.7 to 5.5 for rat [20, 30, 168, 214, 267, 276] and 5.1 to 6.3 for human [14, 95, 157]. $P2X_4$ can also be activated by

2-MeSATP and CTP, but, in most cases these compounds were observed to be ≥ tenfold less potent partial agonists [95, 267, 276]. There may be species differences regarding the sensitivity of $P2X_4$ channels to activation by α,β-MeATP. α,β-MeATP is a weak partial agonist at recombinant mouse and human $P2X_4$ expressed in human embryonic kidney (HEK293) cells or oocytes, [14, 95, 157] whereas, at rat $P2X_4$, it has been shown to behave as a moderately potent antagonist of ATP-evoked inward currents (pIC_{50}=5.3) [157]. β,γ-MeATP has consistently failed to activate rat or human $P2X_4$ channels at concentrations up to 300 μM [30, 95]. To summarize, $P2X_4$ channels respond to ATP and 2-MeSATP with slowly desensitizing currents at ~tenfold or higher concentrations than is required to activate $P2X_1$ and $P2X_3$. $P2X_4$ channels are also generally insensitive to activation by methylene-substituted ATP analogs, a pattern of agonist activity shared by $P2X_2$ and $P2X_5$.

As is the case for $P2X_2$, ATP-evoked currents through $P2X_4$ channels can also be positively modulated by Zn^{2+}, with up to a threefold increase in the potency of ATP and no change in the maximal response at physiologically relevant concentrations (0.1–10 μM) [64, 95, 323]. However, unlike $P2X_2$, ATP-evoked currents through rat $P2X_4$ are also potentiated by ivermectin, as has been previously shown for $GABA_A$ and α_7 nicotinic channels [67, 181, 182]. In oocytes expressing recombinant rat $P2X_4$, ivermectin increased the potency of ATP tenfold, and increased the maximal response by 50–300% with a pEC_{50} for potentiation of 6.6, but had no effect on $P2X_2$, $P2X_3$, $P2X_{2/3}$, or $P2X_7$ [168]. Recently, single-channel recordings of ATP-evoked currents through human $P2X_4$ expressed in HEK293 cells suggested that ivermectin increases maximal channel currents after binding to a high affinity site (pEC_{50}=6.6), and may also bind to a low affinity site (pEC_{50}=5.7) to increase the affinity of ATP by stabilizing the open-channel conformation [244].

Inhibition

An unusual property of the rat $P2X_4$ receptor that differentiates it from other P2X channels is its relative insensitivity to classic, non-selective P2X antagonists, such as suramin and PPADS, at concentrations as high as 100–500 μM [30, 157, 276]. Indeed, there have even been reports that suramin, PPADS, and cibacron blue at some concentrations can potentiate ATP-evoked currents in rat and mouse $P2X_4$ [20, 214, 293]. However, the *rat* $P2X_4$ may be uniquely insensitive as moderate sensitivity of the human $P2X_4$ has been reported for several antagonists, including PPADS (human $P2X_4$ pIC_{50}=4.6–5.0; rat $P2X_4$ pIC_{50}<3.3), suramin (human $P2X_4$ pIC_{50}=3.7; rat $P2X_4$ pIC_{50}< 3.3), bromphenol blue (human $P2X_4$ pIC_{50}=4.1; rat $P2X_4$ pIC_{50}<3.5), and cibacron blue (human $P2X_4$ pIC_{50}=4.4; rat $P2X_4$ pIC_{50}=3.9) and the mouse $P2X_4$ has also been reported to be inhibited by PPADS (pIC_{50}=5.0) with potency similar to that seen at the human $P2X_4$ [95, 157]. It has been hypothesized that PPADS acts in part by forming a Schiff base with a lysine residue in $P2X_1$ and $P2X_2$ which in $P2X_4$ is replaced by a glutamate at the analogous position (Glu^{249}); indeed, when this residue is replaced by a lysine, the resultant $P2X_4$ mutant is sensitive to inhibition by PPADS [30]. However, the human $P2X_4$ has only one lysine (Lys127) not present in the rat $P2X_4$ in the region of the ectodomain (between residues 81 and 183) shown to confer sensitivity to PPADS and mutation of this residue to a lysine in the rat $P2X_4$ (N127K) did not produce a PPADS-sensitive channel [95]. Consequently, the increased sensitivity of the human $P2X_4$ to inhibition by PPADS cannot be simply explained by a difference in the ability of PPADS to form a Schiff base via lysine residues.

As with $P2X_3$ channels, acidic conditions (pH 6.3-6.5) decrease currents through $P2X_4$ but basic conditions (pH 8.0-8.3) have little or no effect [281, 323]. This is another key difference from $P2X_2$ where ATP-evoked inward currents are increased at low pH and decreased at high pH [173, 174]. ATP-evoked currents through rat $P2X_4$ can also be inhibited by high concentrations of ethanol (5-500 mM) and mutant studies have suggested that histidine 241 in the extracellular loop is probably involved [66, 332].

Homomeric $P2X_5$ and heteromeric $P2X_{1/5}$ channels

Key messages

1. Expression of $P2X_5$ has been most closely linked with differentiating cells, particularly in skeletal muscle and skin.
2. Recombinantly expressed $P2X_5$ channels from some species (human, chick, bullfrog) respond to activation with robust currents, whereas, others (rat, zebrafish) respond much more weakly.
3. $P2X_5$ channels have unusually high chloride permeability and unusually slow recovery from desensitization.
4. Unlike $P2X_5$, $P2X_{1/5}$ can be activated by α,β-MeATP and blocked by TNP-ATP with reasonable potency.

Localization and function

$P2X_5$ mRNA and immunoreactivity are found in a variety of tissues including brain, spinal cord, heart, and eye [19, 42, 63, 96, 150, 261]; moreover, it has become apparent in recent years that $P2X_5$ expression is most evident in differentiating tissues, including skeletal muscle [61, 210, 262] and epithelial cells of the nasal mucosa [102], gut [111], bladder and ureter [191], and skin [109, 111, 136]. It has been shown that activation of $P2X_5$-containing channels by ATP inhibits proliferation and increases differentiation of rat skeletal muscle satellite cells through phosphorylation of a mitogen-activated protein kinase (MAPK) signaling pathway [262]. Additionally, $P2X_5$ protein subunits are expressed in squamous cell

carcinomas of the skin and prostate and may play a regulatory role in the proliferation and differentiation of certain types of cancer cells [46, 110].

In human, mRNA expression has been reported to be low in many of the tissues mentioned previously, and instead appears to be expressed at the highest levels in tissues related to the immune system, such as thymus, spleen, lymph node, leukocytes, appendix, and bone marrow [190]. Additionally, both mRNA and immunohistochemical localization studies indicate that $P2X_5$ is present in cultured human epidermal keratinocytes [109, 136]. However, due to the scarcity of published data involving human tissues, the expression and function of $P2X_5$ channels in humans is still unclear.

Activation

The initial pharmacological characterization of the homomeric rat $P2X_5$ channel was impaired by the inability to detect a robust functional response when expressed in recombinant cell lines [63, 96]; however, subsequent work has highlighted some potentially important interspecies differences. For example, recombinant chick, bullfrog, and human $P2X_5$ channels respond to ATP with large, rapidly activating, slowly desensitizing inward currents, whereas, recombinant rat and zebrafish $P2X_5$ respond very poorly to ATP [17, 19, 63, 70, 96, 150, 190]. ATP and 2-MeSATP are typically full agonists with similar pEC_{50}s ranging from 4.8 to 5.7 in all species tested [17, 63, 96, 261]. In most species, methylene-substituted ATP analogs (i.e. α,β-MeATP and β,γ-MeATP) are weak or inactive agonists [96, 150], although in one recent study using rat $P2X_5$ expressed in oocytes, α,β-MeATP was a partial agonist (pEC_{50}=6.0, ~50% of maximal ATP-evoked current) with a potency comparable to ATP (pEC_{50}=6.4) [321]. Only the chick $P2X_5$ channel appears to be consistently sensitive to activation by α,β-MeATP, with currents as large as 80% of the maximum evoked by ATP [19, 261].

Additionally, both the chick and human $P2X_5$ channels have been reported to have relatively high chloride permeability ($p_{Cl^-}/p_{Na^+}=p_{Cl^-}/p_{Cs^+}$=0.5), an unusual property for P2X channels and one of the few traits differentiating $P2X_5$ from $P2X_2$ [17, 261]. Although ATP-evoked currents through $P2X_5$ channels are slowly desensitizing, recovery from desensitization is also very slow requiring 20–60 min to recover to 30–65% [19, 150, 261, 321]. Human, chick, and bullfrog $P2X_5$ have also been reported to dilate to a large pore upon prolonged exposure to ATP in a fashion classically seen with $P2X_7$, although also seen with $P2X_2$ and $P2X_4$ [17, 19, 150].

The $P2X_{1/5}$ channel, as with other heteromeric P2X channels (e.g., $P2X_{2/3}$), uniquely combines some of the pharmacological and biophysical characteristics observed for the individual homomeric channels constructed from the constituent subunits. For example, whereas $P2X_1$ channels respond to ATP with a rapidly desensitizing current, and $P2X_5$ channels respond with a relatively slowly desensitizing current, $P2X_{1/5}$ channels have a characteristic biphasic response to ATP consisting of a transient peak current followed by a sustained plateau current [117, 189, 292]. In addition, a rebound inward current after the plateau current has been observed when large inward peak currents are elicited [117], possibly suggesting passage from the desensitized state to a closed state through an intermediate open state [231]. The calcium permeability of $P2X_{1/5}$ ($p_{Ca^{2+}}/p_{Na^+}$=1.1) more closely resembles $P2X_5$ ($p_{Ca^{2+}}/p_{Na^+}$=1.5) than $P2X_1$ ($p_{Ca^{2+}}/p_{Na^+}$= 3.9–5.0), but, unlike the $P2X_5$ receptor, there is no evidence that the $P2X_{1/5}$ receptor can dilate to a large pore upon prolonged exposure to ATP [17, 81, 286, 300].

Pharmacologically, $P2X_{1/5}$ channels more closely resemble $P2X_1$ than $P2X_5$. The rank order of agonist potencies acting on recombinant rat $P2X_{1/5}$ channels has been reported as ATP ≥ 2-MeSATP > ATP-γ-S ≥ α,β-MeATP ≥ β,γ-MeATP > ADP, a rank order similar to the homomeric $P2X_1$ channel, although only ATP and 2-MeSATP were reported to be full agonists, while ATP-γ-S, α,β-MeATP, β,γ-MeATP and ADP were partial agonists [80, 117, 286, 300]. In studies where recombinant rat $P2X_1$ and $P2X_{1/5}$ channels expressed in HEK293 cells or oocytes were tested side by side, ATP and α,β-MeATP were approximately equipotent at $P2X_{1/5}$ (pEC_{50}=6.2–6.4 for ATP; pEC_{50}=5.3–6.0 for α,β-MeATP) and $P2X_1$ (pEC_{50}=6.2 for ATP; pEC_{50}=5.6–5.8 for α,β-MeATP) [117, 189, 292].

The magnitude of ATP-evoked inward currents through homomeric rat $P2X_5$ channels is approximately doubled by moderate concentrations of Zn^{2+} (1–100 μM), but high concentrations (1 mM) block currents [321]; the effect of Zn^{2+} on $P2X_{1/5}$ channels has not been published. With regard to positive modulation of agonist activity, $P2X_{1/5}$ is unlike either homomeric $P2X_1$ or $P2X_5$ channels. Thus, whereas high concentrations of extracellular calcium inhibits ATP-evoked currents through rat $P2X_5$ and have no effect on rat $P2X_1$, a potentiation of currents through rat $P2X_{1/5}$ is reported with a maximal increase of 40–60% at 50 mM Ca^{2+} [117, 286, 321].

Inhibition

Like $P2X_2$, but unlike $P2X_4$, PPADS and suramin are effective antagonists of ATP-evoked currents through $P2X_5$ channels at concentrations as low as 1 μM [19, 63, 96, 150]. In HEK293 cells expressing human $P2X_5$, PPADS (pIC_{50}=6.7) and suramin (pIC_{50}=5.5) are moderately potent antagonists but TNP-ATP is barely effective (1 μM producing 11% inhibition) [17]. $P2X_5$-mediated inward currents are also reduced in an acidic extracellular environment (pH ≤6.5) but basic conditions have no effect [321]. The only ion shown to inhibit currents through $P2X_5$ channels is calcium, which exhibits a half-maximal effect at an extracellular concentration of 6.7 mM [117].

No selective antagonists of the $P2X_{1/5}$ channel have been described, so it is difficult to distinguish this channel from other P2X channels on the basis of antagonist potencies. PPADS and suramin block ATP-evoked currents

through recombinant rat $P2X_{1/5}$ channels with potencies (pIC_{50}=6.2 and 5.8, respectively) similar to those seen using recombinant rat homomeric $P2X_1$ and $P2X_5$ channels [14, 117]. However, the potency of TNP-ATP (pIC_{50}s range from 6.1 to 7.2) is intermediate between $P2X_1$ (pIC_{50}=8.2) and $P2X_5$ (pIC_{50}<5) [17, 117, 189, 286, 309]. In fact, TNP-ATP may be a weak partial agonist at the rat $P2X_{1/5}$ channel [286]. Also, unlike either $P2X_1$ or $P2X_5$ (and in common only with the homomeric $P2X_7$ channel), both low (6.3) and high (8.3) pH reduce ATP-evoked currents through $P2X_{1/5}$ channels, whereas, only low pH has been reported to inhibit currents through $P2X_1$ or $P2X_5$ homomers [281, 286, 321].

In summary, homomeric $P2X_5$ channels can be distinguished from the other rapidly activating, slowly desensitizing, α,β-MeATP-insensitive P2X channels (e.g., $P2X_2$ and $P2X_4$) primarily on the basis of differential modulation by pH and sensitivity to potentiation by ivermectin ($P2X_4$ only). Recombinant heteromeric $P2X_{1/5}$ channels behave in some respects like $P2X_1$ (agonist activity and lack of pore dilation) and, in other respects, like $P2X_5$ (calcium permeability and presence of sustained current), but in many essential ways they are unique (sensitivity to TNP-ATP and pH and kinetic response). The physiological relevance of the heteromeric $P2X_{1/5}$ channel is unknown. However, it has been hypothesized that $P2X_{1/5}$ may mediate excitatory junction potentials at arterial neuroeffector junctions in guinea pig [286]. In light of the relatively small currents through homomeric $P2X_5$ channels and the fairly widespread distribution of mRNA and immunoreactivity for $P2X_5$ (see above), it seems reasonable that $P2X_5$ may function in some tissues in heteromeric form.

Homomeric $P2X_6$ and heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels

Key messages

1. $P2X_6$ is present throughout the CNS where it often colocalizes with $P2X_2$ and/or $P2X_4$.
2. $P2X_6$ does not form functional homomeric channels without extensive glycosylation, at which point they can be activated by α,β-MeATP and blocked by TNP-ATP.
3. Heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels retain many characteristics of homomeric $P2X_2$ and $P2X_4$, respectively, and it is difficult to distinguish between these channels.
4. Homomeric $P2X_6$ channels differ from heteromeric channels containing $P2X_6$ subunits on the basis of sensitivity to α,β-MeATP, pH, ivermectin and/or antagonists such as TNP-ATP, PPADS, and suramin.

Localization and function

$P2X_6$ mRNA expression and immunoreactivity are expressed throughout the CNS, particularly in portions of the cerebellum (Purkinje cells) and hippocampus (pyramidal cells) [21, 42, 63, 229, 260, 331]. Additionally, expression of $P2X_6$ has been reported in sensory ganglia [330], thymus [105], skeletal muscle [210, 263], gland cells of the uterus, granulose cells of the ovary, bronchial epithelia [63], and human salivary gland epithelial cells [326]. Recently, $P2X_6$ was shown to be the only P2X subtype to be upregulated in human heart tissue (cardiac fibroblasts and in a cardiomyocyte-enriched cell population) from patients with congestive heart failure (CHF) compared to normal human hearts [5]. As $P2X_6$ does not form functional homomeric channels under most circumstances, it has been hypothesized that $P2X_6$ functions in vivo primarily as a heteromeric channel in combination with other P2X subunits known to be expressed in the same regions (e.g., $P2X_2$ and $P2X_4$).

Activation

Until recently, $P2X_6$ was thought to be largely incapable of forming functional homomeric channels when expressed in either oocytes or HEK293 cells [63, 168, 188], primarily due to a failure to even form homo-oligomers [10, 291]. One study found that $P2X_6$ was retained in the endoplasmic reticulum of oocytes as tetramers and high molecular mass aggregates, and failed to be exported to the membrane surface [4]. However, recent data suggests that nonfunctional $P2X_6$ channels can be expressed on the plasma membrane of HEK293 cells if they are partially glycosylated, and that further glycosylation leads to a functional homomeric $P2X_6$ channel [158]. In this case, the rat $P2X_6$ channel can be differentiated from $P2X_2$ or $P2X_4$ by an increased sensitivity to activation by ATP (pEC_{50}=6.3 at $P2X_6$; 5.3 and 4.5 at $P2X_4$ and $P2X_2$, respectively) and α,β-MeATP (pEC_{50}=6.2 at $P2X_6$; <4.5 at $P2X_4$ and $P2X_2$) [24, 80, 157, 158]. ATP induced rapid inward currents through rat $P2X_6$ channels, but the rate of current decay after agonist was removed was significantly slower than the current decay through $P2X_{2/3}$ channels expressed in the same HEK293 cell line [158].

When co-expressed with $P2X_2$ or $P2X_4$ in oocytes, $P2X_6$ can also form heteromeric $P2X_{2/6}$ or $P2X_{4/6}$ channels, respectively [172, 188]. The heteromeric $P2X_{4/6}$ channel is pharmacologically similar to the homomeric $P2X_4$ channel, and may differ only slightly in the potencies of 2-MeSATP (pEC_{50}=5.1 at rat $P2X_{4/6}$; pEC_{50}=4.6 at rat $P2X_4$) and α,β-MeATP (pEC_{50}=4.9 at rat $P2X_{4/6}$; pEC_{50}=4.3 at rat $P2X_4$), but not ATP (pEC_{50}=5.4 at rat $P2X_{4/6}$; pEC_{50}=5.2 at rat $P2X_4$) [188]. ATP-evoked currents in oocytes expressing $P2X_{4/6}$ or $P2X_4$ channels behave virtually identically in the presence of 10 μM Zn^{2+}, where currents are potentiated by a factor of 1.8, or under basic conditions, where at pH 8.0 currents are slightly increased to 121 and 106% of pH 7.5 control responses for $P2X_{4/6}$ and $P2X_4$, respectively. As with the homomeric $P2X_4$ channel, ivermectin marginally potentiates agonist-evoked currents in oocytes expressing $P2X_{4/6}$ channels, shifting the pEC_{50} of α,β-MeATP from 4.6 to 4.8 in the presence of 3 μM ivermectin

[168]. Similarly, the heteromeric $P2X_{2/6}$ and homomeric $P2X_2$ channels are also virtually identical in their rank order of agonist activation (ATP=ATP-γ-S=2-Me-SATP>>BzATP, α,β-MeATP, β,γ-MeATP, ADP, Ap_nA), and when expressed in oocytes they were similarly responsive to ATP (pEC_{50}=4.7 and 4.5 for $P2X_2$ and $P2X_{2/6}$, respectively) [172]. Both heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels differ from the homomeric $P2X_6$ channel primarily by their significantly lower sensitivity to α,β-MeATP, and by the greater sensitivity to pH ($P2X_{2/6}$) or ivermectin ($P2X_{4/6}$) imparted by the other P2X subunits comprising the heteromeric channel.

Inhibition

ATP-evoked currents through the functional glycosylated homomeric $P2X_6$ channel can be blocked by TNP-ATP (pIC_{50}=6.1) and PPADS (pIC_{50}=6.1), but not suramin (27% reduction at 100 μM) [158]. The sensitivity to inhibition by TNP-ATP and PPADS is in marked contrast to the heteromeric $P2X_{4/6}$ channels which, like the homomeric $P2X_4$ channel, is relatively insensitive to inhibition by 10 μM PPADS (38% inhibition), suramin (41% inhibition) or reactive blue-2 (26% inhibition but >45% potentiation in rat $P2X_4$) [188]. The heteromeric $P2X_{2/6}$ channel is similarly sensitive to inhibition by suramin (pIC_{50}=5.2) as the homomeric $P2X_2$ channel (pIC_{50}=5.0), but more sensitive than the homomeric $P2X_6$ channel (see above) [172]. However, $P2X_2$ and $P2X_{2/6}$ channels can be distinguished on the basis of their differing responses to activation under acidic conditions. Under moderately acidic conditions (pH 6.5), the potency of ATP at both $P2X_2$ and $P2X_{2/6}$ channels increases relative to responses evoked at pH 7.5 (from pEC_{50}=4.8 to 5.9 at $P2X_2$; from 4.5 to 5.1 at $P2X_{2/6}$). Under more strongly acidic conditions (pH 5.5) the potency of ATP at $P2X_2$ increases further (to pEC_{50}=6.3) with no change in the maximal response, whereas at $P2X_{2/6}$ the maximal ATP-evoked response is dramatically decreased (76% reduction) [172].

To summarize, the homomeric $P2X_6$ channel differs from the heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels primarily on the basis of their relative sensitivities to α,β-MeATP, pH and/or ivermectin, and additionally by their differing sensitivity to inhibition by TNP-ATP, PPADS, and suramin. The differences are more subtle between the heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels and the homomeric $P2X_2$ and $P2X_4$ channels, respectively, but a potential way to distinguish them is on the basis of different responses to pH (at pH 5.5, maximal response to ATP unaffected at $P2X_2$ but reduced at $P2X_{2/6}$) or reactive blue-2 (potentiates $P2X_4$ but slightly inhibits $P2X_{4/6}$).

Homomeric $P2X_7$ channels

Key messages

1. $P2X_7$ channels are predominantly localized on immune cells and glia, where they mediate proinflammatory cytokine release, cell proliferation, and apoptosis.
2. $P2X_7$ protein subunits form only homomeric channels, and activation requires unusually high concentrations of agonist.
3. $P2X_7$ channels allow passage of larger molecular weight molecules upon prolonged agonist exposure.
4. Potent and selective antagonists, some with drug-like properties, have been identified in recent years.

Localization and function

The $P2X_7$ receptor, formerly known as the cytolytic P2Z receptor [9, 16, 82], is predominantly expressed on cells of the immune system, such as macrophages/monocytes, dendritic cells, lymphocytes, and mast cells, as well as on various types of glia within the peripheral and central nervous system, including microglia, astrocytes, oligodendrocytes, and Schwann cells [29, 31, 42, 52, 62, 69, 90, 249]. $P2X_7$ protein subunits are also expressed on epithelial cells, fibroblasts, osteoblasts, and some neuronal populations [68, 101, 111, 269, 274].

Activation of the $P2X_7$ channel has been associated with multiple cellular functions [231, 319]. However, it is best characterized for its role in mediating the processing and release of mature, biologically active interleukin-1β (IL-1β) and interleukin-18 (IL-18) from immune cells and glia [49, 85, 108, 208, 239, 248]. Macrophages and microglia pretreated with the $P2X_7$ receptor antagonists KN-62 or periodate-oxidized ATP (oATP) (see below), or from $P2X_7$-deficient mice, fail to release IL-1β when challenged with ATP or BzATP [108, 208, 248, 275]. Consistent with this, $P2X_7$-deficient mice have a decreased incidence and severity of disease in a model of monoclonal anti-collagen antibody-induced arthritis [183], and deficits in models of chronic inflammatory and neuropathic pain [52]. A role for $P2X_7$ in neurodegeneration and CNS inflammation has also been postulated based on its role in cytokine, reactive oxygen species, and neurotransmitter release from microglia and astrocytes, induction of cell death, and its upregulation around β-amyloid plaques in a transgenic mouse model of Alzheimer's disease [49, 74, 186, 238]. Priming of macrophages or microglia with β-amyloid peptide is a potent co-stimulus for $P2X_7$-mediated cytokine release [248], and $P2X_7$ channels appear to play a

role in microglial-dependent neurotoxicity in a rat co-culture system of microglia and embryonic cortical neurons [271]. The role of $P2X_7$ has also been investigated in models of spinal cord injury or cerebral ischemia to further assess the role of $P2X_7$ in neurodegeneration and cell death [187, 317].

$P2X_7$ channels are also expressed on osteoblasts and osteoclasts [101, 159, 220], but the physiological role of $P2X_7$ channels in bone development and remodeling is not entirely clear. $P2X_7$ does not appear to be critical for multinucleated osteoclast formation [99, 100, 197], and one recent study has suggested that $P2X_7$ channels may be important for osteoblastic responses to mechanical loading [197], as opposed to early suggestions of $P2X_7$-mediated osteoblast apoptosis [101]. In addition, studies of bone formation and resorption in two different strains of $P2X_7$-deficient mice have generated conflicting results, with one study demonstrating a phenotype of suppressed periosteal bone formation and excessive trabecular bone resorption [163] and the other showing no skeletal alterations [100].

Activation

$P2X_7$ channels are the least sensitive among P2X channels to activation by nucleotides. It has generally been established that BzATP is the most potent agonist at the rat $P2X_7$ channel (pEC_{50}=5.2–5.7). BzATP is ~10- to 30-fold more potent than ATP (pEC_{50}=3.7–4.1) when measuring inward currents in recombinantly expressed $P2X_7$ channels, while other common P2X agonists, such as 2-MeSATP, ATP-γ-S, α,β-MeATP and β,γ-MeATP, are even less potent or inactive altogether [54, 123, 249, 285]. By comparison, ATP has typically been reported to be 10- to 100-fold more potent at the other homomeric P2X channels [14, 20, 63, 80]. However, $P2X_7$ channels do show species differences in agonist potencies. BzATP at concentrations of 10–30 μM can evoke maximal inward currents or Ba^{2+} influx through rat $P2X_7$ channels, whereas, at least tenfold higher concentrations are required to evoke similar responses through human or mouse $P2X_7$ channels [74, 123, 249, 324]. In one study measuring inward currents through native $P2X_7$ channels in mouse NTW8 microglial cells, or through recombinant rat, human, or mouse $P2X_7$ channels expressed in HEK293 cells under identical conditions, the pEC_{50}s for BzATP were 5.7 (rat $P2X_7$), 4.3 (human $P2X_7$), 4.0 (mouse $P2X_7$), and 4.2 (NTW8) [54].

$P2X_7$ was also the first P2X channel that was shown to allow passage of larger molecular weight (≤900 Da) molecules, such as the fluorescent dyes YO-PRO-1 and ethidium bromide, after prolonged exposure to agonist [285]. This phenomenon presumably occurs by dilation of the channel pore, although this has recently become somewhat controversial [198, 231]. It has been shown that pore formation and dye uptake in mouse macrophages involve second messengers such as Ca^{2+} and MAP kinases [83], and in rat retinal microvascular cells, activation of $P2Y_4$ inhibits $P2X_7$-mediated pore formation [283]. Additionally, either alteration of the extracellular sodium concentration or deletion of an 18-amino acid domain in the C terminus of rat $P2X_7$ subunits expressed in HEK293 cells, resulted in markedly different permeabilities to NMDG and YO-PRO-1. These studies suggested that these molecules enter the cell through different pathways, and the authors concluded that NMDG probably enters through a pore intrinsic to the channel, whereas YO-PRO-1 most likely enters through a distinct, non-$P2X_7$ related pore [153]. Although the mechanism(s) of pore dilation are still unclear, BzATP tends to be more potent at evoking intracellular YO-PRO-1 accumulation than inward currents, with pEC_{50}s ranging from 6.6–7.1 at rat $P2X_7$, 6.0–6.3 at human $P2X_7$, and 4.7–4.9 at mouse $P2X_7$, again most potent at the rat ortholog [54, 123, 124, 213].

Inhibition

As with most of the other homomeric and heteromeric P2X channels, PPADS is an inhibitor of rat, human, and mouse $P2X_7$-mediated inward currents and Ca^{2+} influx with moderate, variable potencies (pIC_{50}=4.2–6.0) [14, 53, 74, 249, 285]. However, PPADS may be a more potent antagonist of BzATP-stimulated YO-PRO-1 accumulation, with reported pIC_{50}s of 7.8–7.9 and 6.9–7.1 in HEK293 cells expressing human and rat $P2X_7$, respectively [54, 124]. Interestingly, in the same studies, the mouse $P2X_7$ channel was significantly less sensitive to PPADS (pIC_{50}=5.0–5.2) [54, 124]. Suramin, another non-selective P2X (and P2Y) antagonist, has been reported to be a weak or inactive antagonist (pIC_{50}≤4.1) at $P2X_7$ channels of all species tested [14, 74, 285]. Oxidized ATP is an irreversible antagonist of $P2X_7$-mediated fluorescent dye uptake, but it requires long incubation times (1 to 3 h) and high concentrations (100–300 μM) to be effective [124, 213, 218, 285]. However, oATP may have utility for exploring the mechanism of action of various antagonists. For example, pre-incubation of HEK293 cells expressing human $P2X_7$ with either PPADS or suramin attenuated the irreversible antagonism of oATP, supporting the notion that these agents may be acting at the ATP binding site or a site that excludes this binding [213]. On the other hand, in curve shift experiments, increasing concentrations of PPADS results in a significant suppression of the BzATP concentration–response curve maxima, suggesting that it may be behaving as a non-competitive antagonist of the $P2X_7$ channel [53, 213]. However, this finding could also be explained by inadequate agonist–antagonist equilibrium at the receptor as PPADS is known to be very slowly reversible [53, 213]. Brilliant Blue G has been reported to be a $P2X_7$-selective antagonist of agonist-evoked inward currents in recombinant cell lines with pIC_{50}s of 8.0 and 6.6 at rat and human $P2X_7$ channels, respectively, compared to pIC_{50}s of 5.9 (rat $P2X_2$), 5.5 (human $P2X_4$), or <5.3 (rat $P2X_4$, rat $P2X_1$, human $P2X_1$, human $P2X_3$, rat $P2X_{2/3}$, and human $P2X_{1/5}$) [152].

Another class of $P2X_7$ antagonists is the large cationic inhibitors of Ca^{2+}/calmodulin-dependent protein kinase II

(CaMKII), including calmidazolium, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), and related compounds. Calmidazolium inhibits BzATP-evoked inward currents, but not YO-PRO-1 accumulation, in HEK293 cells expressing rat $P2X_7$ with a pIC_{50} of 7.9 [306], and has also been reported to inhibit inward currents through human $P2X_7$ [53]. KN-62 is among the most potent inhibitors of both inward currents and fluorescent dye uptake through human (pIC_{50}=7.3–8.0) and mouse $P2X_7$ channels (pIC_{50}=6.7), but is inactive at rat $P2X_7$ (pIC_{50}<5.5) [6, 53, 54, 98, 124, 134]. Although KN-62 is an inhibitor of CaMKII, a closely related compound, KN-04, also potently inhibits $P2X_7$-mediated Ba^{2+} uptake and ethidium influx but is inactive at CaMKII, thereby suggesting that these compounds do not inhibit $P2X_7$ function through the involvement of CaMKII [98, 134]. Many synthetic analogs of KN-62 have been tested, with the most potent being the fluoride derivative of KN-62 with a pIC_{50} of 8.9, almost 40-fold more potent than KN-62 in the same study [6].

$P2X_7$ channels are also very sensitive to their extracellular ionic environment. BzATP-evoked inward currents and YO-PRO-1 uptake have been shown to increase when extracellular concentrations of either monovalent or divalent cations (Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}) or anions (Cl^-) are decreased [53, 115, 211, 249, 306]. The most potent negative modulator of BzATP-evoked inward currents through rat $P2X_7$ among the divalent cations is Cu^{2+} (pIC_{50}=6.3), followed by Zn^{2+} (pIC_{50}=5.0), Mg^{2+} (pIC_{50}=3.3) and Ca^{2+} (pIC_{50}=2.5) [306]. Both acidic and basic conditions inhibit $P2X_7$-mediated inward currents [211, 306], but increasing the pH from 5.5 to 9.0 resulted in a progressive increase in the maximum YO-PRO accumulation in HEK293 cells expressing human $P2X_7$ [211].

Recent advances

In recent years, some of the most significant advances in purinergic pharmacology have been in the development of more potent and selective antagonists at certain P2X receptor subtypes, most notably $P2X_1$, $P2X_3$, $P2X_{2/3}$, and $P2X_7$. Some of these advances are limited to increases in potency and selectivity and not related to improving the other physicochemical characteristics required for a molecule to be advanced as a medicinal candidate. For example, suramin analogs with extremely high potency and selectivity for $P2X_1$-containing channels have been described in recent years. NF449 has pIC_{50}s of 9.5 and 9.2 (>3,000-fold more potent than suramin) at rat $P2X_1$ and $P2X_{1/5}$, respectively (expressed in oocytes) with 400–1,000,000-fold selectivity over rat $P2X_2$, $P2X_{2/3}$, $P2X_3$, and $P2X_4$ [251]. NF864 has been shown to inhibit α,β-MeATP-evoked human platelet shape change and intracellular calcium increase with pA_2 estimates of 8.49 and 8.17, respectively; approximately 5–7 fold more potent than NF449 and 200- to 540-fold more potent than suramin [129]. Although these compounds are potentially very useful as in vitro tools, their utility in vivo would be expected to be limited by poor pharmacokinetic properties.

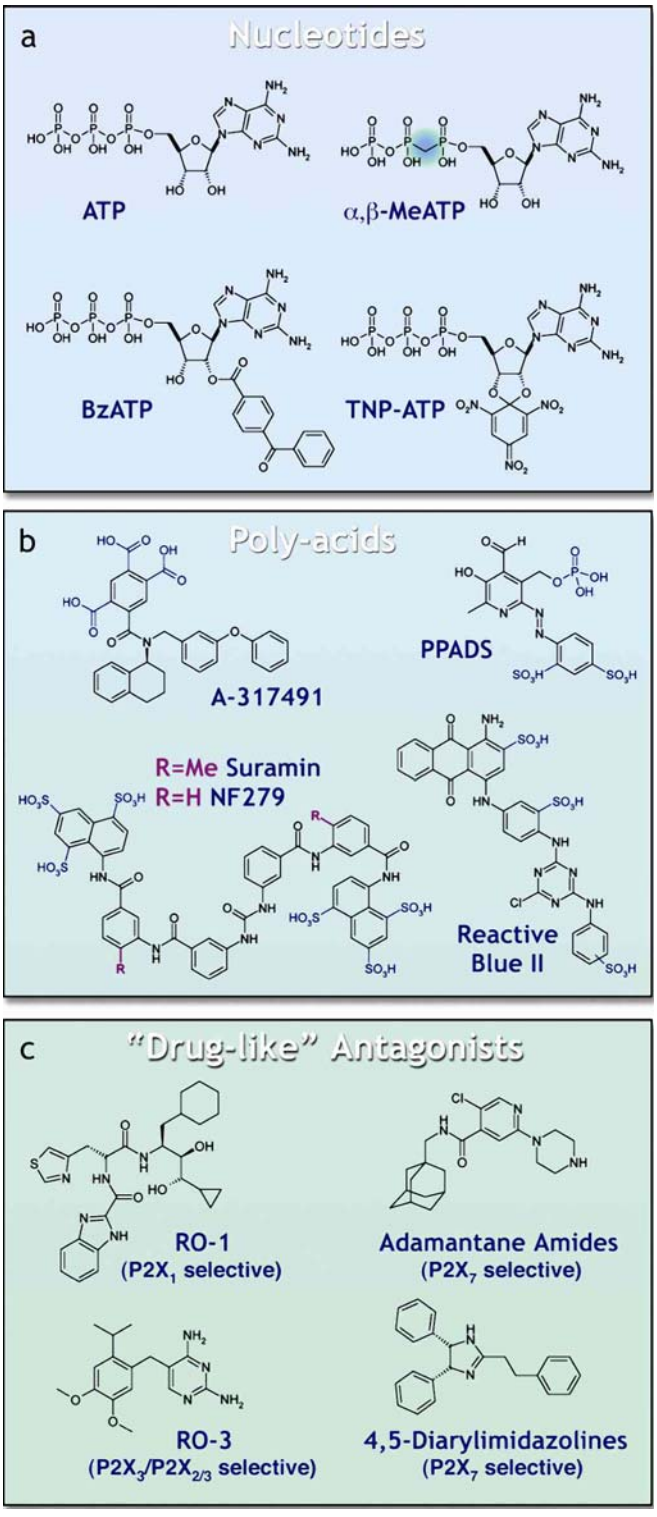

Fig. 2 Commonly used P2X agonists and antagonists: **a** Nucleotides related to the structure of ATP: ATP, α,β-MeATP, and BzATP are agonists, TNP-ATP is an antagonist. **b** Antagonists with multiple acidic functional groups imparting poor in vivo pharmacokinetic properties. **c** Selective antagonists with improved "drug-like" properties (e.g., oral bioavailability, improved metabolic stability)

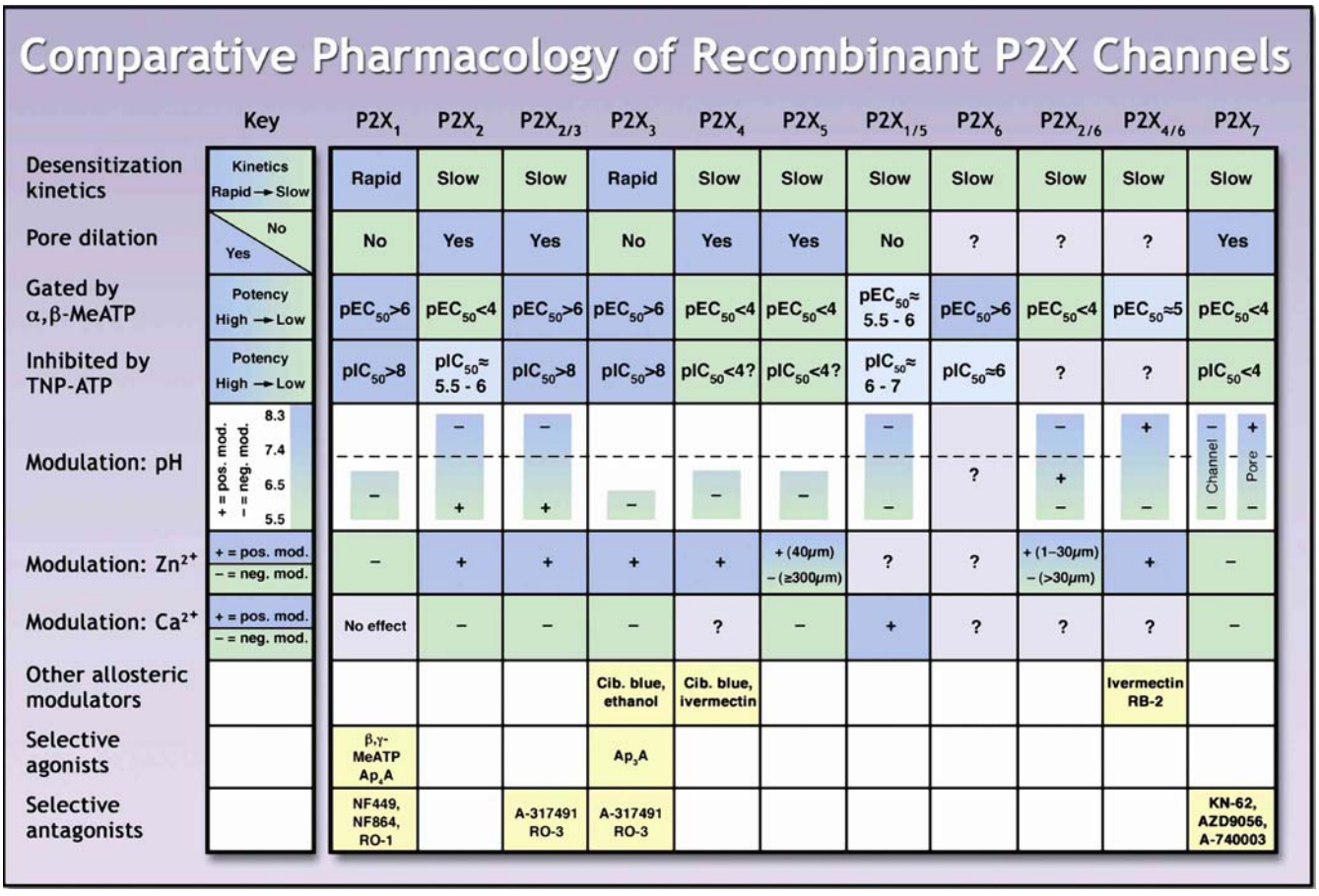

Fig. 3 Comparison of pharmacological and biophysical factors distinguishing homomeric and heteromeric P2X channels. The key on the *left side* of the table explains the use of *color*: *blue boxes* denote more rapid desensitization kinetics, ability of the channel to dilate to a larger pore, higher potency to agonism or antagonism or positive modulation of channel activation by an extracellular ion; *green boxes* denote slower desensitization kinetics, the inability of the channel to dilate to a larger pore, lower potency to agonism or antagonism or negative modulation of channel activation by an extracellular ion. A "?" appears where there are no published data. See text for references

All of the antagonists described above and in previous sections are either nucleotides that are acidic and rapidly degraded, or large polyanionic molecules (suramin, dyes, PPADS). None of these molecules represent an ideal starting point for medicinal optimization. However, one report has described a small molecule $P2X_1$ antagonist with drug-like properties, RO-1, derived from the optimization of dipeptide compounds synthesized originally as potential renin inhibitors [144]. Although it is moderately potent (pIC_{50}=5.5 at human $P2X_1$), it is selective over other homomeric and heteromeric P2X receptors (pIC_{50}<4 at $P2X_2$, $P2X_3$, and $P2X_{2/3}$) and effectively reduces rat detrusor smooth muscle contractions evoked by β, γ-MeATP or electrical field stimulation [88, 103].

In 2002, data were published for the first time on a selective $P2X_3$/$P2X_{2/3}$ "small molecule" antagonist from Abbott, A-317491 [146]: a tricarboxylic acid identified from random screening and originally patented in racemic form as an inhibitor of squalene synthetase and protein farnesyltransferase. Activation of recombinant and native $P2X_3$ and $P2X_{2/3}$ channels was inhibited by submicromolar concentrations of A-317491 (in human clones, pIC_{50}=7.0 at $P2X_3$, 6.8 at $P2X_{2/3}$, 5.0 at $P2X_1$, 4.3 at $P2X_2$, <4 at $P2X_4$ and $P2X_7$), and antinociceptive efficacy was demonstrated in several rodent models of chronic inflammatory and neuropathic pain. A-317491 was later shown to inhibit ATP-evoked Ca^{2+} influx through slowly desensitizing chimeric human $P2X_{2-3}$ channels (see above) in a manner consistent with competitive antagonism (pA_2=7.3) [225]. Long plasma half-life and demonstrated usefulness in some in vivo models make this a significant advance in this area. However, the poor pharmacokinetic properties of A-317491 (poor oral bioavailability, high protein binding, and poor tissue distribution) would likely make it unattractive for medicinal development.

More recently, the identification of a series of $P2X_3$/$P2X_{2/3}$ antagonists structurally related to the diaminopyrimidine antibacterial drug trimethoprim, exemplified by RO-3 (see Fig. 2), represents a step toward discovery of drug-like P2X antagonists [88]. RO-3 is a potent inhibitor of human homomeric $P2X_3$ (pIC_{50}=7.0) and heteromeric $P2X_{2/3}$ (pIC_{50}=5.9) channels with selectivity over other P2X channels (pIC_{50}<5 at $P2X_{1,\ 2,\ 4,\ 5,\ 7}$). Furthermore, RO-3 has moderate to high metabolic stability in rat and

human hepatocytes and liver microsomes, and is highly permeable, orally bioavailable (14%), and has a reasonable in vivo plasma half-life ($t_{1/2}$=0.41 h) in rats.

Several chemical series of $P2X_7$ antagonists with improved "drug-like" properties have also been reported. Aventis and AstraZeneca have published the syntheses of 4,5-diarylimidazolines (the most potent having a pIC_{50} of 8.0 vs BzATP-evoked YO-PRO-1 influx) and cyclic imides (the most potent having a pA_2 of 7.7 vs BzATP-evoked ethidium influx), respectively; selectivity or mechanism of action data was not provided in either case [2, 209]. Another class of $P2X_7$ antagonists reported by AstraZeneca is based on a series of adamantanes with affinity estimates (pA_2) as high as 8.8 [11]. The adamantane chemical series of $P2X_7$ antagonists was initially plagued with poor metabolic characteristics (high rat hepatocyte and human microsomal clearance), but this was reportedly overcome by the synthesis of an indazole amide derivative, which was deemed suitable for further lead optimization [11]. In fact, AstraZeneca have advanced a $P2X_7$ antagonist, AZD9056, into Phase II clinical trials for rheumatoid arthritis, although neither the structure nor the efficacy of this compound in humans has been announced up to the time of this writing. Abbott has recently published data showing the preclinical efficacy of a $P2X_7$ antagonist, A-740003, in rodent models of neuropathic pain [147]. A-740003 is reported to be a selective, competitive antagonist of agonist-evoked intracellular calcium flux with affinity estimates (pK_i) of 7.7, 8.0, and 6.8 at recombinant human, rat, and mouse $P2X_7$ channels. This compound was also reported to reduce hyperalgesia/allodynia in models of neuropathic pain produced by spinal nerve ligation (ED_{50}=41 μmol/kg, i.p.), chronic constriction injury of the sciatic nerve (54% effect at 300 μmol/kg, i.p.), and vincristine-induced neuropathy (51% reduction at 300 μmol/kg, i.p.) [147].

Antagonists with improved drug-like properties have only been identified for $P2X_1$, $P2X_3$, $P2X_{2/3}$, and $P2X_7$ channels. So why is this? The most parsimonious explanation is that these channels have been more clearly linked to specific pathological conditions (e.g., platelet and smooth muscle function, nociception, and inflammation), and may not be as broadly localized as other P2X channels. Consequently, they may have garnered the most attention as attractive targets for drug discovery and received greater focus from screening of compound libraries. The medicinal exploitability of the other homomeric and heteromeric P2X channels (Fig. 3) remains unknown for now.

In the decade since the seven known P2X subtypes were cloned, significant advances have been made in our understanding of their physiological roles, in part through the use of non-selective pharmacological agents in relevant animal models. As the selectivity and potency of these pharmacological tools have improved, so has our understanding of the biological function of the channels at which they act. For example, the role of $P2X_3$ and $P2X_{2/3}$ channels in the detection of noxious stimuli through sensory neurons has been elucidated, in part, through blockade of these stimuli in animal models by selective $P2X_3$/$P2X_{2/3}$ antagonists [88, 146]. Similarly, preclinical experiments using selective $P2X_7$ antagonists have supported the hypothesis that this channel may have an important role in inflammatory processes [147]. The challenge remains to advance candidate medicines targeting P2X channels through human clinical trials, and judging from recent progress, we are optimistic that safe and effective medicines for the treatment of disorders involving P2X channels will be reported in the coming years.

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