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Activation and Regulation of Purinergic P2X Receptor Channels

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Abstract—Mammalian ATP-gated nonselective cation channels (P2XRs) can be composed of seven possible subunits, denoted P2X1 to P2X7. Each subunit contains a large ectodomain, two transmembrane domains, and intracellular N and C termini. Functional P2XRs are organized as homomeric and heteromeric trimers. This review focuses on the binding sites involved in the activation (orthosteric) and regulation (allosteric) of P2XRs. The ectodomains contain three ATP binding sites, presumably located between neighboring subunits and formed by highly conserved residues. The detection and coordination of three ATP phosphate residues by positively charged amino acids are likely to play a dominant role in determining agonist potency, whereas an AsnPheArg motif may contribute to binding by coordinating the adenine ring. Nonconserved ectodomain histidines provide the binding sites for trace metals, divalent cations, and protons.

The transmembrane domains account not only for the formation of the channel pore but also for the binding of ivermectin (a specific P2X4R allosteric regulator) and alcohols. The N- and C- domains provide the structures that determine the kinetics of receptor desensitization and/or pore dilation and are critical for the regulation of receptor functions by intracellular messengers, kinases, reactive oxygen species and mercury. The recent publication of the crystal structure of the zebrafish P2X4.1R in a closed state provides a major advance in the understanding of this family of receptor channels. We will discuss data obtained from numerous site-directed mutagenesis experiments accumulated during the last 15 years with reference to the crystal structure, allowing a structural interpretation of the molecular basis of orthosteric and allosteric ligand actions.

I. Introduction

The potential relevance of extracellular ATP in synaptic transmission was originally introduced in 1972

(Burnstock, 1972) but was received with skepticism until the first receptor was cloned in 1993 (Webb et al., 1993). It is now well established that there are two families of receptors activated by extracellular nucleo-

¹Abbreviations: A317491, 5-[[[(3-phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate; A438079, 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride; A740003, N-1-[(cyanoimino)(5-quinolinylamino) methyl] amino-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide; A804598, 2-cyano-1-[(1S)-1-phenylethyl]-3-quinolin-5-ylguanidine; Ap_nA, diadenosine polyphosphate (where *n* = the number of phosphates); AR, adenosine receptor; ATP_γS, adenosine-5'-O-(3-thiotriphosphate); AZ10606120, N-[2-[[2-(2-hydroxyethyl)amino]ethyl]amino]-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide dihydrochloride; AZ11645373, 3-[1-[[[(3'-nitro[1,1'-biphenyl]-4-yl)oxy]methyl]-3-(4-pyridinyl)propyl]-2,4-thiazolidinedione; BBG, Coomassie brilliant blue G; BzATP, 2'-(3'-O-4-benzoylbenzoyl)-ATP; CaM, calmodulin; DRG, dorsal root ganglion; GW791343, N²-(3,4-difluorophenyl)-N¹-[(2-methyl-5-(1-piperazinyl)methyl)phenyl]glycinamide dihydrochloride; h, human; HEK, human embryonic kidney; Ip_nI, diinosine polyphosphate (where *n* = the number of phosphates); IVM, ivermectin; KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; LPC, lysophosphatidylcholine; LPS, lipopolysaccharide; m, mouse; meATP, methylene-ATP; 2-meSATP, 2-methylthio-ATP; MG50-3-1, 1-amino-4-(4-(4-chloro-6-(2-sulfonatophenylamino)-(1,3,5)triazine-2-ylamino)-2-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid trisodium salt; MK3, *ortho*-(2,2',2'',2''')-(carbonylbis(imino-5,1,3-benzenetriylbis (carbonylimino)))tetrakis-benzenesulfonic acid; MRS2159, 4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonoxy)methyl]-2-pyridinyl]azo]-benzoic acid; MRS2219, 1,5-dihydro-3-hydroxy-8-methyl[1,3,2]dioxaphosphepino[5,6-c]pyridin-9-ol-3-oxide; MRS2220, 6-azophenyl-2',5'-disulfonate derivative; MRS2257, pyridoxal-5'-phosphate-6-azophenyl-3',5'-bimethylenphosphonate; MRS2339, 1'S,2R,3S,4'R,5'S)-4-(6-amino-2-chloro-9H-purin-9-yl)-1-[phosphoryloxyethyl] bicyclo[3.1.0]hexane-2,3-diol; NF023, 8,8'-[carbonylbis(imino-3,1-phenylenecarbonylimino)]bis-1,3,5-naphthalene-

trisulfonic acid; NF110, 4,4',4'',4'''-[carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))]tetrakisbenzenesulfonic acid tetrasodium salt; NF279, 8,8'-[carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)]bis-1,3,5-naphthalenetrisulfonic acid; NF449, 4,4',4'',4'''-[carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))]tetrakis-1,3-benzenedisulfonic acid; NF770, 7,7'-[carbonylbis(imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene)carbonylimino)]bis(1-methoxynaphthalene-3,6-disulfonic acid) tetrasodium salt; NF776, 6,6'-[carbonylbis(imino-3,1-(4-methylphenylene)carbonylimino)]bis(1-methoxynaphthalene-3,5-disulfonic acid) tetrasodium salt; NF778, 6,6'-[carbonylbis(imino-3,1-phenylenecarbonylimino-3,1-(4-methyl-phenylene)carbonylimino)]bis(1-methoxy-naphthalene-3,5-disulfonic acid) tetrasodium salt; NF864, 8,8',8'',8'''-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino)))tetrakis-naphthalene-1,3,5-trisulfonic acid-dodecasodium salt; P2XRs, purinergic P2X cation-conducting receptor channels; P2YRs, G protein-coupled P2Y receptors; PIP₂, phosphatidylinositol-4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PPADS, pyridoxal 5-phosphate 6-azophenyl-2',4'-disulfonic acid; PPNDS, pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate; PSB-1011, disodium 1-amino-4-[3-(4,6-dichloro[1,3,5]triazine-2-ylamino)-4-sulfonylphenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate; PSB-10211, 1-amino-4-[3-(4,6-dichloro[1,3,5]triazine-2-ylamino)phenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate; r, rat; RB-2, reactive blue 2; Ro-0437626, N-[(1R)-2-[[[(1S,2R,3S)-1-(cyclohexylmethyl)-3-cyclopropyl-2,3-dihydroxypropyl]amino]-2-oxo-1-(4-thiazolylmethyl)ethyl]-1H-benzimidazole-2-carboxamide; RO-4, 5-[5-iodo-4-methoxy-2-(1-methylethyl)phenoxy]-2,4-pyrimidine diamine hydrochloride; RO-5, 5-(5-ethynyl-2-isopropyl-4-methoxy-phenoxy)-pyrimidine-2,4-diamine; RO-85, 1-methyl-3-phenyl-1H-thieno[2,3-c]pyrazole-5-carboxylic acid [(R)-2-(4-acetyl-piperazin-1-yl)-1-methyl-ethyl]-amide; ROS, reactive oxygen species; SB9,

tides: P2X receptors (P2XRs¹) and P2Y receptors (P2YRs). P2XRs are a family of ligand-gated receptor channels. Seven mammalian purinergic receptor subunits, denoted P2X1² through P2X7, and several spliced forms of these subunits have been identified (North, 2002). P2YRs are G protein-coupled receptors. Eight mammalian P2YRs, denoted P2Y₁R, P2Y₂R, P2Y₄R, P2Y₆R, P2Y₁₁R, P2Y₁₂R, P2Y₁₃R, and P2Y₁₄R, have been cloned (Fischer and Krügel, 2007). There are also four subtypes of nucleoside-activated G protein-coupled receptors known as P1 or adenosine receptors (ARs): A₁R, A_{2A}R, A_{2B}R, and A₃R (Ralevic and Burnstock, 1998). It has also been shown that nucleotides act not only as neurotransmitters but also as paracrine factors delivered by diffusion that requires several seconds, rather than a few milliseconds, to activate the receptors (Browne et al., 2010). The duration and distance of their actions are limited by several enzymes called ectonucleotidases (Yegutkin, 2008).

P2XRs are nonselective cation-conducting channels present in multiple species, from unicellular organisms to humans, but the phylogeny of these receptors remains to be established. The simplest organism that encodes a P2XR is the eukaryote green algae *Ostreococcus tauri* (Fountain et al., 2008). Although an ancestral prokaryotic P2XR has not been identified, these receptors are present in several invertebrate and vertebrate species (Fountain and Burnstock, 2009) and some of the properties of these channels (such as allosteric modulation) have been maintained evolutionarily (discussed in section IV). Some members of these channels provide not only a narrow conducting pathway for the passage of small ions but also a pathway for the passage of larger organic cations by dilation of the endogenous pore and/or integration of another channel or transporter. The native agonist for P2XRs is ATP, whereas both ATP and its metabolite ADP act as agonists for P2YRs in a receptor-specific manner. Other endogenous nucleotides, such as UTP, UDP, and UDP-glucose, are potent agonists for some P2YRs, but they have no activity at P2XRs (Jacobson et al., 2006). Diadenosine polyphosphates, known as dinucleotides, also act as agonists for P2XRs and P2YRs. These compounds are naturally occurring substances that are structurally related to ATP. They are composed of two adenosine moieties linked by their ribose 5' ends to a variable number of phosphates (Ap_nA) (Pintor et al., 2000). Ectonucleotidase-derived AMP does not act as an agonist, but its degradation product, adenosine, is a natural agonist for ARs. Inosine, formed by the deamination of adenosine, has also been shown to have agonist activity at ARs (Guinzbarg et al., 2006).

In this article, we review the current knowledge on orthosteric and allosteric regulation of P2XR function. Detailed literature on the expression, distribution, and function of P2XRs can be found elsewhere (see Burnstock and Knight, 2004; Burnstock, 2007; Surprenant and North, 2009). In contrast to G protein-coupled receptors, the wild-type P2XRs do not show obvious constitutive activity in the absence of agonist (North, 2002). The receptors probably have three classic agonist binding sites (Browne et al., 2010). Here we use the term "orthosteric sites" to describe all ATP binding sites on P2XRs, because they are the primary binding sites needed for the conformational changes that allow for the opening of the channels (gating).

The gating of P2XRs usually consists of three phases: a rapid rising phase of inward current induced by the application of agonist (activation phase), a slowly developing decay phase in the presence of an agonist (desensitization phase), and a relatively rapid decay of current after ATP is removed (deactivation phase). The main difference among receptors is in their sensitivity for agonists and their activation and desensitization rates. Figure 1A shows the profile of P2XR currents in response to supramaximal concentrations of ATP (10 μ M for P2X1R and P2X3R, 100 μ M for P2X2R and P2X4R, and 10 mM for P2X7R). P2X1R and P2X3R rapidly activate and desensitize, whereas P2X2R and P2X4R slowly desensitize. Rat P2X5Rs generate low amplitude nondesensitizing currents, whereas human and chick P2X5R respond with larger currents. The P2X6R does not express well at the plasma membrane (Collo et al., 1996). On the other hand, the gating of P2X7R is more complex, as indicated by the secondary current growth during sustained agonist application (Fig. 1A).

The dependence of activation, desensitization, and deactivation kinetics on agonist concentration was studied in detail using P2X4R as a receptor model (Yan et al., 2006). The activation efficiency increases with elevation in ATP concentration, and the activation time inversely correlates with ATP concentration (Fig. 1C). There is also an inverse relationship between desensitization time and ATP concentration (Fig. 1, D and E). In contrast, deactivation kinetics is independent of agonist concentration (Fig. 1B). The P2X2R exhibits a similar dependence of gating properties on agonist concentrations (Zemkova et al., 2004). Experiments with outside-out patches containing recombinant rP2X2Rs revealed an 80- μ s delay between the time when ATP arrives at the receptor and the opening of the channels. In addition, a brief pulse of saturating ATP leaves fully liganded channels without producing an opening at least 30% of the time (Moffatt and Hume, 2007). The deactivation properties of P2X1R and P2X3R are difficult to estimate because of the rapid receptor desensitization. The gating of P2X7R differs from that of other members of this family of channels (Yan et al., 2010). For details on the gating properties of P2XRs, see North (2002).

6-((4,6,8-trisulfo-1-naphthyl)iminocarbonyl-1, 3-(4-methylphenylene)iminocarbonyl-1,3-phenylene-azo)-pyridoxal-5'-phosphate; SS, disulfide bridges; TM, transmembrane; TNP-ATP, trinitrophenyl-ATP; z, zebrafish.

² According to the IUPHAR website (<http://www.iuphar-db.org>), the P2XR numbers should not be subscript as was commonly used in the past.

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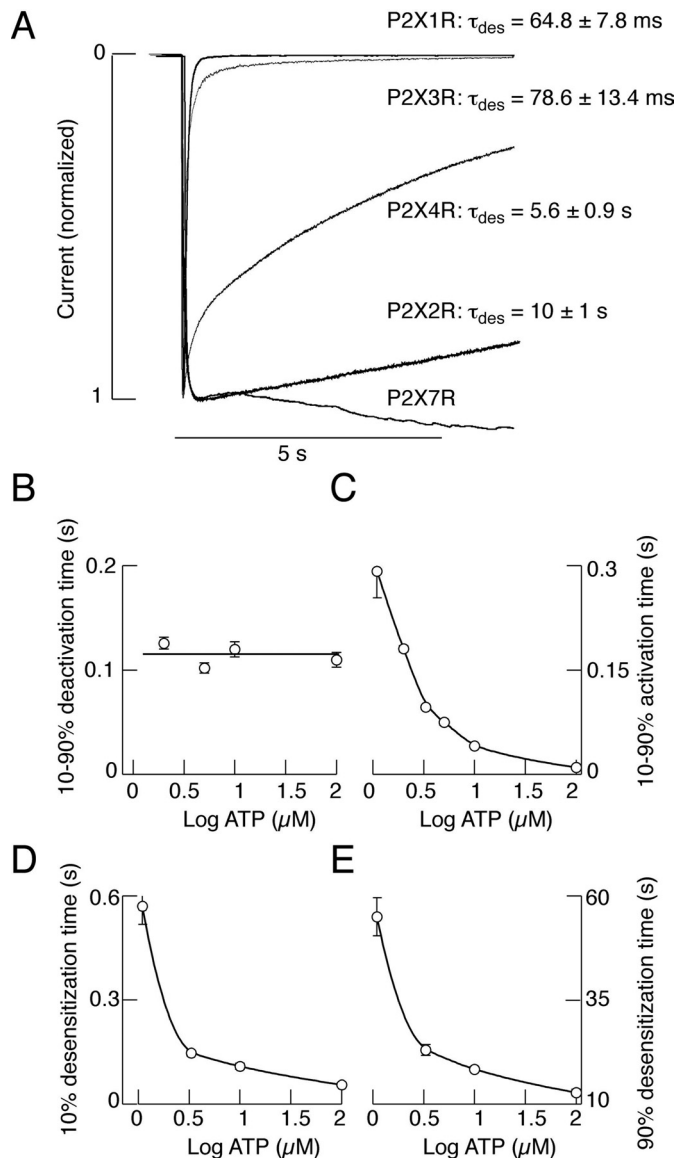


FIG. 1. Gating properties of P2XRs. A, profiles of P2XR currents induced by sustained agonist application. Recombinant rat receptors were expressed in HEK293 cells and stimulated with ATP (10 μ M for P2X1R and P2X3R, 100 μ M for P2X2R and P2X4R, and 3 mM for P2X7R). τ_{des} indicates the desensitization time constant derived from monoexponential fitting (mean \pm S.E.M.; values from at least five records per channel). B to E, characterization of P2X4R current. B, Deactivation time values (10–90%) are independent from the ATP concentration. C, inverse relationship between activation time and ATP concentration. D and E, inverse relationship between the values for 10% (D) and 90% (E) desensitization time and ATP concentrations.

The behavior of P2XRs can be altered by allosteric interactions. These interactions occur at additional sites that are linked conformationally or by other mechanisms to the orthosteric site(s) in such way that binding to one site can change the nature and extent of binding or signaling via the other site (Christopoulos et al., 2004; Gao and Jacobson, 2006). Thus, allosteric ligands bind to sites that are topographically distinct from the orthosteric sites recognized by the receptor's endogenous agonist. Allosteric perturbation arises not only from the binding of small or large molecules extracellularly but

also from changes in temperature, ionic strength, or concentration and from covalent modification tethering, glycosylation, phosphorylation, and ubiquitination, which could take place extracellularly in the transmembrane (TM) region or intracellularly (Tsai et al., 2009).

Allosteric systems are also much more versatile than orthosteric systems in that modulator ligands can augment, block, or potentiate the effects of the orthosteric agonist, and this effect can change with the nature of agonist (Kenakin, 2010). The term *cooperativity* is frequently used to describe the magnitude of an allosteric effect, which could be either positive or negative, leading to up- or down-regulation of receptor function. Thus, receptor allosterism can be defined as the effect on a receptor produced by simultaneous interactions with two distinct ligands: orthosteric and allosteric. The allosterically modified receptor exhibits different affinities and efficacies for all interactants. The term *affinity* is used to describe the ability of an agonist to bind to a receptor, whereas *potency* is a measure of agonist activity expressed in terms of the concentration required to generate an effect of a given intensity, usually expressed as the EC_{50} value. The term *efficacy* describes the degree to which different agonists (full and partial) produce maximal responses when occupying the same proportion of receptors, usually expressed as the E_{max} value.

In this review, we begin by summarizing the work concerning the pharmacology and structure of the orthosteric binding sites. This will be followed by a summary of the effects of trace metals (zinc, copper, cobalt, and nickel), heavy metals (mercury and cadmium), and macro metals (calcium, magnesium, and sodium) on receptor function and the identification of residues responsible for the binding of these metals. We will also review the literature pertaining to the effects of protons, ivermectin (IVM), neurosteroids, alcohols, and related clinically relevant anesthetic drugs, as well as the signaling pathways involved in the regulation of these receptor channels, such as reactive oxygen species, calmodulin (CaM), kinases, and phosphoinositides. Based on the crystal structure of the zebrafish P2X4.1R (zP2X4.1R), solved at a 3.5-Å resolution (Kawate et al., 2009), we have also generated a homology model of rat P2X4R (rP2X4R) and reanalyzed data published by our own group and other groups that have been working on the structural and functional characterization of this receptor. Throughout the text, we will use the homology model of rP2X4R to discuss orthosteric and allosteric binding sites.

II. Molecular and Crystal Structure of P2X2 Receptors

All members of extracellular ligand-gated ion channels contain two functional domains: an extracellular domain that binds a native agonist and a TM domain that forms an ion channel. Among ligand-gated receptor

channels, P2XRs are the simplest. Cloning of P2XRs revealed that all subunits have a large (~280 amino acids) extracellular loop (hereafter referred to as the ectodomain), two TM domains (TM1 and TM2), and intracellularly located N and C termini of variable lengths, resembling the topology of structurally unrelated epithelial sodium channels and the mechanosensitive degenerin channels (Stojilkovic, 2008).

The precise biophysical characterization of native and recombinant P2XRs was instrumental in generating the hypothesis that these channels are organized as trimeric homomers or heteromers. The concentration-dependence of P2XR activation measured by whole-cell (Bean et al., 1990; Jiang et al., 2003) and single-cell (Ding and Sachs, 1999) recording suggested that three molecules of ATP were required for channel gating. Biochemical studies showed that under nondenaturing conditions, P2XRs migrated as trimers in polyacrylamide gel electrophoresis, therefore suggesting that this was the conformation of native functional channels (Nicke et al., 1998). The same conclusion has been reached in a study with heteromeric P2XRs (Nicke et al., 2005). Other study showed that mutations that affect channel gating by methanethiosulfonate bromide block were effective only if they were contained in the three first subunits of concatenated P2XRs, further supporting a trimeric conformation (Stoop et al., 1999). Subsequently, other more sophisticated techniques, such as atomic force microscopy (Barrera et al., 2005; Nakazawa et al., 2005) and electron microscopy (Young et al., 2008), have corroborated the trimeric nature of P2XRs.

A structure for P2X2R at 15-Å resolution was also recently reported (Mio et al., 2009). Crystallization of the zP2X4.1R at 3.5-Å resolution showed that the receptor is indeed a trimer. As described by Kawate et al. (2009), each subunit rises from the plasma membrane, like a dolphin from the surface of the ocean, with its tail submerged within the lipid bilayer. The body regions of three subunits mutually intertwine, forming a central vertical cavity. The ectodomain projects 70 Å above the plasma membrane, and there are three vestibules in the center of the ectodomain (Kawate et al., 2009). Comparison of the zP2X4R with acid-sensing ion channel structure revealed similarity in pore architecture and aqueous vestibules (Gonzales et al., 2009).

Channels are organized as homotrimers or heterotrimers. Initial evidence for the formation of functional heteromers came from studies coexpressing the slowly desensitizing and $\alpha\beta$ -meATP-insensitive P2X2R with the rapidly desensitizing and $\alpha\beta$ -meATP-sensitive P2X3R, resulting in a slow desensitizing and $\alpha\beta$ -meATP-sensitive P2X2/3R (Lewis et al., 1995). A series of 11 heteromers was predicted by immunoprecipitation studies (Torres et al., 1999a). So far, six functional P2XR heteromeric receptors have been characterized: P2X1/2R (Brown et al., 2002; Aschrafi et al., 2004), P2X1/4R (Nicke et al., 2005), P2X1/5R (Torres et al., 1998; Haines et al., 1999; Lê et al.,

1999), P2X2/3R (Lewis et al., 1995; Liu et al., 2001a; Spelta et al., 2002; Jiang et al., 2003), P2X2/6R (King et al., 2000; Barrera et al., 2007), and P2X4/6R (Lê et al., 1998).

Studies using mutagenesis combined with functional expression have allowed for the initial designation of certain amino acids in specific receptor functions. The ectodomain contains 10 conserved cysteine residues that have been predicted to make bonds in the following order (P2X4R numbering): 116 to 165 (SS1), 126 to 149 (SS2), 132 to 159 (SS3), 217 to 227 (SS4), and 261 to 270 (SS5) (Clyne et al., 2002b; Ennion and Evans, 2002; Rokic et al., 2010). In P2X1R, the individual bonds are not essential for receptor function (Ennion and Evans, 2002). For P2X2R, however, the SS1 and SS4 bonds are individually required for proper receptor function (Clyne et al., 2002b). Disruption of the SS1, SS2, and SS4-P2X4R bonds by substituting both cysteines with threonine generated less sensitive receptors. Of these three bonds, mutation of the SS4 cysteine residues generated the most profound changes in receptor function (Rokic et al., 2010). The ectodomain also contains two to six asparagines that may contribute to N-linked glycosylation, an important process for the proper trafficking of P2XRs to the plasma membrane and receptor functions (North, 2002). As discussed in the following sections, SS bonds may participate in the formation of an allosteric regulatory domain, and other ectodomain residues may be important for the formation of ATP binding sites and several allosteric sites.

The crystal structure of zP2X4.1R provided direct evidence for the existence of these conserved disulfide bonds. The first three bonds are located in the head and beak regions of ectodomain, the fourth bond is located within the dorsal fin, and the fifth bond at the ends of two β -sheets in the low body region. The mammalian model of P2X4R, based on the crystal structure of zP2X4.1R, provides some rationale for the specific roles of the SS1 to -5 disulfide bonds in P2X4R function. The model shows that bonds SS1 to -3 are located within the head domain above the predicted ATP binding pocket, whereas SS4 is below the ATP binding pocket. The model also shows that the SS5 bond is located relatively far from the putative ATP binding site but close to the extracellular vestibule above the TM domains (Stojilkovic et al., 2010b). The SS4 bond is absent in the simple eukaryote *O. tauri*, and SS2, SS3, SS4 and SS5 bonds are absent in *Dictyostelium discoideum* (Surprenant and North, 2009). These receptors are functional, but a high concentration of agonist is required for their activation, suggesting that the formation of the SS bonds was an important step in the evolution of P2XR proteins. Together, these findings indicate that SS bonds provide the structural basis for the tridimensional organization of these receptors.

Perturbation of receptor function caused by scanning mutagenesis of the TM domains was useful in predicting that these regions adopt α -helical structures in activated

P2X2Rs (Rassendren et al., 1997a; Egan et al., 1998; Haines et al., 2001a,b; Jiang et al., 2001; Khakh and Egan, 2005) and P2X4Rs (Silberberg et al., 2005; Jelínková et al., 2008; Jindrichova et al., 2009). These experiments also helped identify the key regions involved in ion permeation. The helices of different subunits seem to move relative to each other during channel opening and closing (Egan et al., 1998), and TM2 seems to play a dominant role in receptor functions (Torres et al., 1999b; Duckwitz et al., 2006), channel assembly, gating, ion selectivity, and the permeability of divalent ions (Egan et al., 1998; Li et al., 2004; Khakh and Egan, 2005; Samways and Egan, 2007; Li et al., 2008). TM2 residues Thr³³⁶, Thr³³⁹, and Ser³⁴⁰ were suggested to contribute to the formation of the pore, gate, and selectivity filter of P2X2Rs (Migita et al., 2001; Egan and Khakh, 2004; Samways and Egan, 2007). It has been proposed that the external gate region Ile³³² to Ile³⁴¹ expands, and the pore-forming helices straighten, resulting in the opening of the channel pore (Li et al., 2010). None of the residues that seem to contribute to the formation of the P2X2R pore gate are conserved, clearly indicating that further structural information is required to understand the orientation of the TM2 residues in closed and open states.

Alanine and cysteine scanning of the P2X4R-TM1 domain indicated that Gly²⁹, Met³¹, Tyr⁴², Gly⁴⁵, and Val⁴⁹ residues are mutation-sensitive (Jelínková et al., 2008; Jindrichova et al., 2009). Among these residues, the conserved Tyr⁴² residue seemed to play the most important role in receptor function. At first, it was suggested that this residue affected the cation permeability of P2X2R only indirectly (Samways et al., 2008). The P2X4R-Y42 mutant showed an increased sensitivity to ATP and a decreased E_{\max} (Silberberg et al., 2005; Jindrichova et al., 2009). In P2X1R, replacement of this residue with alanine resulted in nonfunctional channels, further supporting the importance of this residue in receptor function. The sensitivity of the P2X3R-Y37A mutant to ATP was also increased. In addition, $\alpha\beta$ -MeATP was changed from a partial to full agonist for P2X2R and P2X4R with increased sensitivity. In contrast, mutation of the conserved TM1 tyrosine of P2X7R did not result in an increased sensitivity to ATP (Jindrichova et al., 2009).

Crystallization of zP2X4.1R (Kawate et al., 2009) confirmed the hypothesis that the TMs adopt α -helical structures. Consistent with the prediction that the TM2 region plays a dominant role in receptor function, channel assembly, gating, ion selectivity, and the permeability of divalent ions, the homology model of rat P2X4R shows that the TM2 domains form a triangle, which is the pore of the channel in a closed state, whereas the three TM1 domains are located more peripherally. The homology P2X4R model suggests that Tyr⁴² is located at the level of the membrane where the TM2 helices cross each other and the TM pore is the narrowest (Stojilkovic

et al., 2010b). The model shows the position of Trp⁵⁰ and Trp⁴⁶ residues, which have been shown to influence the function of the Tyr⁴² residue. Furthermore, the model suggests that Met³³⁶ of the second subunit may interact with Tyr⁴² in the closed state, a possibility that should be tested experimentally. The zebrafish structure also provided important information about residues that could account for the cation selectivity of P2XRs. These include acidic residues in the central vestibule that could concentrate cations in the extracellular part of the channel (Asp⁵⁹ and Asp⁶¹) and a residue that could directly interact with cations in the channel pore (Asn³⁴¹). It also gave some clues about which residues are important for the channel gate: Leu³⁴⁰ and Asn³⁴¹ in the extracellular part of the gate and Ala³⁴⁴, Leu³⁴⁶, and Ala³⁴⁷ as the hydrophobic residues that form the intracellular gate.

At present, we do not know the organization of the N and C termini in three dimensions. The N termini of all P2XRs is relatively short (around 25 amino acids), whereas the lengths of the C termini range from approximately 30 amino acids (P2X6R) to approximately 215 residues (P2X7R). Both the N and C termini serve as molecular targets for a series of post-transcriptional and post-translational modifications, including RNA splicing, phosphorylation, and protein-protein interactions (North, 2002). The physiological relevance of spliced channels has been well documented for P2X2R (Fig. 2). Two functional splice forms of these receptors, P2X2bR and P2X2eR, desensitize more rapidly than the full-sized receptor, P2X2aR (Brändle et al., 1997; Simon et al., 1997; Koshimizu et al., 1998b, 2006). In contrast, the C-terminal of P2X7R contains a unique Tyr³⁵⁸ to Glu³⁷⁵ sequence that contributes to the transition from the open to dilated state (Jiang et al., 2005a; Yan et al., 2008). The N and C termini also contain conserved phos-

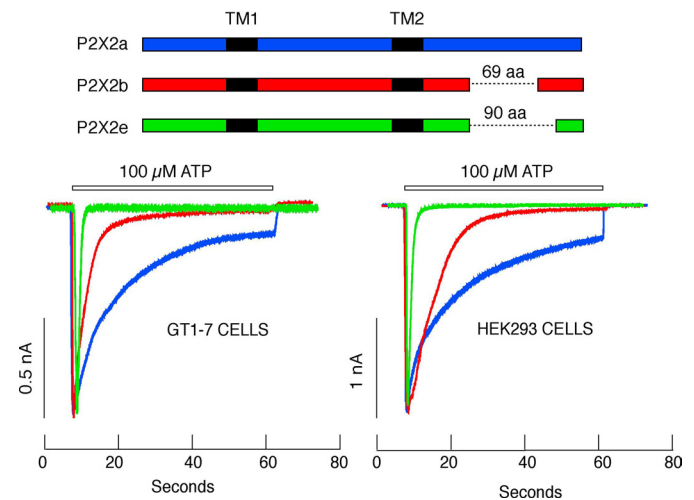


FIG. 2. Dependence of the mP2X2R current on the C-terminal structure. Top, schematic representation of the P2X2R splice forms. Bottom, typical patterns of ATP-induced current profiles for P2X2Rs expressed in GT1-7 (left) and HEK293 cells (right). Blue traces, P2X2aR; red traces, P2X2bR; green traces, P2X2eR.

phorylation sites for protein kinases A and C and other important regions responsible for the regulation of P2XRs, which is discussed in detail in the following sections.

III. Orthosteric Binding Sites and Receptor Function

A. P2X Receptor Agonism and Antagonism

All homomeric and heteromeric receptors are activated by ATP but in a receptor-specific manner, the EC₅₀ values ranging from nanomolar to submillimolar concentrations. As stated under *Introduction*, P2XRs are more structurally restrictive than P2YRs with regard to agonist selectivity. P2XRs are also activated by naturally occurring diadenosine polyphosphates (Ap_nAs, *n* = 3–7) and closely related dinucleotides (Ap_nGs, *n* = 3–6), but with a lower potency and efficacy than ATP. Alone, these agonists cannot be used to distinguish P2XRs. Other nucleoside triphosphates, such as CTP and GTP, can also activate some P2XRs. In contrast, ADP, AMP, adenosine, UTP, UDP, and UMP activate these receptors either weakly or not at all. The chemical structure of most common P2X agonists and antagonists can be found elsewhere (Ralevic and Burnstock, 1998; Kim et al., 2001; Lambrecht et al., 2002).

It has been difficult to develop subunit-specific agonists for P2XRs. The agonists that currently exist are analogs of ATP and act at several P2XRs with different potencies and efficacies. Triphosphate modification resulted in the generation of several potent P2XR agonist analogs. α,β -Methylene-ATP ($\alpha\beta$ -meATP) and β,γ -methylene-ATP ($\beta\gamma$ -meATP) are phosphonic acid analogs of ATP in which the bridging oxygen atom between corresponding phosphates is replaced with the methylene group. These analogs are metabolically more stable than ATP, do not activate P2YRs, exhibit higher potencies at P2X1R and P2X3R homomeric and heteromeric receptors, and serve as radioligands for these receptors. The thio substitution at the terminal phosphates resulted in several analogs, including adenosine-5'-O-(3-thiotriphosphate) (ATP γ S), which are relatively resistant to breakdown by ectonucleotidases. ATP γ S activates all P2XRs except P2X7R, as well as several P2YRs. Substitution of the adenine ring resulted in the formation of 2-methylthio-ATP (2-meSATP), the most potent agonist for P2XRs and P2YRs, and several other analogs. Because it does not activate adenosine receptors, 2-meSATP was an important compound in establishing the existence of two P2 receptor families. 2'-(3')-O-4-benzoylbenzoyl-ATP (BzATP), a modified ribose derivative, is a common agonist for P2XRs that, with the exception of P2Y₁₁R and P2Y₁₃R, does not activate P2YRs (Jacobson et al., 2002; Carrasquero et al., 2009; Jarvis and Khakh, 2009).

Several nucleotide derivatives also act as P2XR antagonists. Trinitrophenyl-ATP (TNP-ATP) and the corresponding di- and monophosphate derivatives inhibit

P2X1R, P2X3R, and P2X2/3R at nanomolar concentrations (Virginio et al., 1998b). The oxidized form of ATP has been suggested to act as an mP2X7R-specific blocker (Jacobson et al., 2002). Ip₅I, a diinosine polyphosphate, inhibits P2X1R currents at nanomolar concentrations, and Ip₄I and Ip₅I block P2X3R at micromolar concentrations (King et al., 1999).

Suramin is a large, complex, polysulfonated molecule and is one of the most widely used competitive P2R antagonists (Jacobson et al., 2002; Lambrecht et al., 2002). However, suramin is not specific for P2Rs and also antagonizes G proteins (Freissmuth et al., 1996; Hui and Nayak, 2002); inhibits several proteases, including HIV reverse transcriptase (Jentsch et al., 1987) and tyrosine phosphatase (Zhang et al., 1998); and stimulates ryanodine receptors (Hohenegger et al., 1996). Suramin inhibits ATP-induced currents in a receptor-specific manner (North, 2002). The replacement of Gln⁷⁸ residue with Lys at rP2X4R was sufficient to increase the sensitivity of this receptor for suramin (Garcia-Guzman et al., 1997a) but not to the extent observed in cells expressing human receptor (hP2X1R) (Roberts and Evans, 2004), indicating that other receptor regions are important for the recognition of this drug. Human and mouse P2X1R also exhibit marked differences in sensitivity to suramin, and the nonconserved Lys¹³⁸ residue may contribute to the antagonistic action of this drug and its derivatives (Braun et al., 2001; Sim et al., 2008).

A number of truncated forms of suramin, including 8,8'-[carbonylbis(imino-3,1-phenylenecarbonylimino)]bis-1,3,5-naphthalene-trisulfonic acid (NF023), 8,8'-[carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)]bis-1,3,5-naphthalenetrisulfonic acid (NF279), 4,4',4'',4'''-[carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))]tetrakis-1,3-benzenedisulfonic acid (NF449), and 8,8',8'',8'''-(carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino)))tetrakis-naphthalene-1,3,5-trisulfonic acid-dodecasodium salt (NF864) exhibit P2R antagonist activity. NF023 inhibits several homomeric and heteromeric P2XRs expressed in *Xenopus laevis* oocytes and is most potent at P2X1R (Soto et al., 1999). NF279 does not affect the activation of adenosine receptors, exhibits low inhibitory potency on P2YRs and ectonucleotidases, and antagonizes P2XRs (Damer et al., 1998), showing the greatest potency at P2X1R and P2X7R (Klapperstück et al., 2000; Rettinger et al., 2000; Donnelly-Roberts et al., 2009a). NF449 inhibits rP2X1R with subnanomolar potency (Braun et al., 2001; Kassack et al., 2004; Rettinger et al., 2005) but also inhibits G_s proteins (Hohenegger et al., 1998) and fibroblast growth factor receptor 3 signaling (Krejci et al., 2010). Three isomeric suramin analogs, 4,4',4'',4'''-[carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))]tetrakisbenzenesulfonic acid tetrasodium salt (NF110) and *ortho*-(2,2',2'',2'''-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino)))tetrakis-benzenesulfonic acid (MK3), are >200-fold less potent than NF449 in blocking P2X1R (Hausmann et al., 2006). NF864 selectively

blocks hP2X1R with low nanomolar potency (Horner et al., 2005).

Pyridoxal 5-phosphate 6-azophenyl-2',4'-disulfonic acid (PPADS) is another compound that is commonly used as a P2XR inhibitor. In contrast to suramin, the P2R specificity of PPADS is very high. It acts noncompetitively and reduces the E_{\max} value of ATP at high concentrations. PPADS blocks homomeric P2X1Rs, P2X2Rs, P2X3Rs, and P2X5Rs as well as heteromeric P2X2/3Rs, P2X1/5Rs, and P2Y1Rs, whereas it is weak or ineffective as an antagonist at rat P2X4Rs, P2X6Rs, P2X7Rs, and several P2YRs. Several attempts have been made to identify the ectodomain residues responsible for this receptor specificity. The sensitivity of P2X4R to PPADS was restored by replacing Glu²⁴⁹ with the lysine that occurs at the equivalent position in PPADS-sensitive P2X1R and P2X2R (Buell et al., 1996). However, the reverse mutation in the P2X2R did not remove inhibition, and this lysine is not present in PPADS-sensitive hP2X3Rs and hP2X7Rs. The human and mouse P2X4Rs have almost identical amino acid sequences around Glu²⁹⁴ but are sensitive to PPADS, indicating that other regions determine PPADS sensitivity (Garcia-Guzman et al., 1997a; Jones et al., 2000). Subsequent studies revealed that the 81-to-183 region of rP2X4R (Garcia-Guzman et al., 1997a), specifically Arg¹²⁶ (Michel et al., 2008b), contributes to PPADS insensitivity.

A chemical cousin to PPADS, pyridoxalphosphate-6-azophenyl-2',5'-disulfonic acid, has somewhat distinct pharmacological properties (Jacobson et al., 2002; Lambrecht et al., 2002). Pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate) (PPNDS) potently antagonized rP2XR-mediated responses in the vas deferens of rats and at recombinant rP2X1Rs expressed in *X. laevis* oocytes. PPNDS is approximately 50-fold less selective for P2Y₁R and does not interact with α_{1A} -adrenergic, adenosine A₁ and A_{2B}, histamine H₁, or muscarinic M₃ receptors (Lambrecht et al., 2000b). 1,5-dihydro-3-hydroxy-8-methyl[1,3,2]dioxaphosphepino[5,6-c]pyridin-9-ol-3-oxide (MRS2219) is a selective potentiator of ATP-evoked responses at rP2X1R, whereas the corresponding 6-azophenyl-2',5'-disulfonate derivative, known as MRS2220, is a selective antagonist of this receptor (Jacobson et al., 1998). 4-[(4-formyl-5-hydroxy-6-methyl-3-[(phosphonoxy)methyl]-2-pyridinyl)azo]-benzoic acid (MRS2159) is a potent inhibitor of rP2X1R (Brown et al., 2001; Kim et al., 2001) and mouse, rat, and human P2X7R (Donnelly-Roberts et al., 2009a). Pyridoxal-5'-phosphate-6-azophenyl-3',5'-bimethylenphosphonate (MRS2257) inhibits both P2X1R and P2X3R but with higher potency than PPADS (Brown et al., 2001). 6-[(4,6,8-Trisulfo-1-naphthyl)iminocarbonyl-1, 3-(4-methylphenylene)iminocarbonyl-1,3-phenylene-azo]-pyridoxal-5'-phosphate (SB9) is a high-affinity P2Y₁R antagonist with a 10-fold lower potency at P2X1R (Lambrecht et al., 2000a).

The anthraquinone class of compounds is also used to inhibit P2R. There has been some confusion concerning the identity and purity of commercially available reac-

tive blue 2 (RB-2) and the corresponding pure isomer, Cibacron blue 3GA. Here we use term RB-2 to describe the antagonistic actions of the mixture of terminal ring *meta*- and *para*-sulfonate, and we discuss separately the allosteric actions of Cibacron blue 3GA, the purified *ortho* isomer, at recombinant P2XRs. The antagonistic actions of RB-2 were observed in several diverse ATP-mediated physiological responses, such as rat urinary bladder smooth muscle contraction (Hashimoto and Kokubun, 1995), rat cecum inhibitory junction potentials (Manzini et al., 1986), and calcium influx in rat parotid acinar cells (Soltoff et al., 1989). It has also been suggested that RB-2 inhibits ectonucleotidase activity in *X. laevis* oocytes (Ziganshin et al., 1996) and ATP-induced inflammation of the mouse hind paw (Ziganshina et al., 1996). The inhibitory effects of RB-2 were also observed in currents and calcium measurements in single PC12 cells (Nakazawa et al., 1991; Michel et al., 1996) as well as in experiments with cells expressing recombinant rP2X1R and P2X2R (Surprenant, 1996). For a more detailed description of the antagonistic actions of RB-2, see Ralevic and Burnstock (1998).

Several other related compounds, such as RB-4, RB-5, 1519, and acid blue-25, -41, -80, and -129, have also been tested for their antagonistic actions at P2Rs (Tuluc et al., 1998). A series of RB-2 type anthraquinone derivatives were also synthesized, re-evaluated, and tested for their potency at P2Rs. Among them, 1-amino-4-(4-(4-chloro-6-(2-sulfonatophenylamino)-(1,3,5)triazine-2-ylamino)-2-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid trisodium salt (MG50-3-1) is the most potent antagonist for P2Y₁R, and none of the compounds tested exhibited specificity for P2XRs (Glänzel et al., 2003, 2005).

1. Homomeric P2X1 Receptor. One of the first pieces of evidence for the expression of P2X1R came from experiments indicating a role for ATP as a neurotransmitter involved in contractions of the guinea pig detrusor smooth muscle (Burnstock, 1972). Further studies found that $\alpha\beta$ -meATP could be substituted for ATP in these neurogenic contractions (Burnstock et al., 1978; Kasakov and Burnstock, 1982). Electrophysiological studies showed transient concentration-dependent effects of ATP and $\alpha\beta$ -meATP on inward currents causing membrane depolarization in isolated detrusor smooth muscle cells (Fujii, 1988; Inoue and Brading, 1990, 1991). Later, this receptor was cloned from the vas deferens of rats (Valera et al., 1994) and the human urinary bladder (Valera et al., 1995) and termed P2X1R.

In general, this receptor is densely localized in smooth muscle cells, including the urinary bladder, intestines, vas deferens, and arteries (Mulryan et al., 2000; Burnstock and Knight, 2004). Other tissues that express the P2X1R include the smooth muscle of small arteries, dorsal root, lung, central and peripheral nervous system, platelets, and megakaryocytes (Valera et al., 1994, 1995; Longhurst et al., 1996). In accordance with this, mice lacking the gene encoding P2X1R do not show

rapidly desensitizing inward currents in the detrusor smooth muscle, vas deferens, and mesenteric arteries (Mulryan et al., 2000). Furthermore, platelets from P2X₁R-deficient mice do not show normal aggregation, secretion, adhesion, and thrombus growth, and they have reduced mortality (Vial and Evans, 2000, 2002; Hechler et al., 2006). On the other hand, transgenic mice overexpressing human P2X₁R exhibit hypersensitive platelet responses *in vitro* and increased mortality (Oury et al., 2003).

Recombinant receptors respond to agonist application with a rapid rise in current, followed by a decline in the current amplitude during the sustained agonist application (Fig. 1A). The rates of receptor desensitization are augmented by increases in the agonist concentration. Repeated agonist applications lead to progressively smaller currents when applied less than 10 min apart (Valera et al., 1994). A similar response pattern is also observed in cells expressing recombinant P2X₃R (Fig. 1) and P2X_{2e}R, the splice form of mP2X₂R (Fig. 2). Run-down of current measured by the whole-cell recording was unaffected by changes in cytosolic calcium and was abolished when the amphotericin-perforated patch clamp was used for current recording (Lewis and Evans, 2000). Experiments using green fluorescent protein-attached P2X₁Rs (Dutton et al., 2000) or the biotinylation of surface P2X₁Rs (Ennion and Evans, 2001) suggest that P2X₁R is internalized after agonist activation. This behavior could contribute to both long-term desensitization and the recovery

from desensitization, although intrinsic channel properties could explain the fast desensitization. P2X₁R has been shown to exhibit both constitutive and agonist-induced recycling after photo-bleaching, suggesting that this could be important in the recovery from desensitization (Lalo et al., 2010).

The pharmacological profile of P2X₁R is shown in Table 1. Among P2XRs, this receptor has the highest affinity for ATP, with an EC₅₀ in the submicromolar concentration range (Valera et al., 1994; Wildman et al., 2002; Rettinger and Schmalzing, 2003). The real EC₅₀ value for ATP is probably lower, but it is masked by rapid receptor desensitization (Rettinger and Schmalzing, 2003). Other ATP analogs, including 2-meSATP, 2-chloro-ATP, ATP γ S and BzATP, also activate these receptors (Evans et al., 1995). hP2X₁R is activated by nanomolar concentrations of ATP, 2-meSATP, $\alpha\beta$ -meATP, and BzATP, as estimated by using calcium measurements (Bianchi et al., 1999). Electrophysiological measurements found EC₅₀ values for hP2X₁R to be comparable with those observed in rP2X₁R. In these experiments, ATP and 2-meSATP were full agonists at rP2X₁R, whereas other ATP analogs were partial agonists (Evans et al., 1995). Ap₆A is also a full agonist for rP2X₁R but is less potent than ATP, whereas Ap₅A, Ap₄A, Ap₆G, and Ap₅G act as partial agonists (Wildman et al., 1999a; Cinkilic et al., 2001). These diadenosine polyphosphates also activate hP2X₁R at submicromolar concentrations (Bianchi et al., 1999). At first, it was

TABLE 1
Pharmacological profile of P2X₁R

EC₅₀/IC₅₀ values are micromolar unless otherwise specified.

Compound	Method	EC ₅₀ /IC ₅₀	References
Full agonists			
ATP	Current	0.1–0.7	Evans et al., 1995; Wildman et al., 1999a, 2002
$\alpha\beta$ -meATP	Current	0.1–1	Evans et al., 1995; Wildman et al., 2002
2-meSATP	Current	0.1–1	Evans et al., 1995; Wildman et al., 2002
Ap ₆ A	Current	1	Wildman et al., 1999
Partial agonists			
$\beta\gamma$ -meATP	Current	2	Evans et al., 1995
ATP γ S	Current	1	Evans et al., 1995
BzATP	Current	0.7–24	Evans et al., 1995; Wildman et al., 2002
Ap ₅ A	Current	1–3	Evans et al., 1995; Wildman et al., 1999a, 2002
Ap ₄ A	Current	40 nM	Wildman et al., 1999a, 2002
Antagonists			
Suramin	Current	1–2	North and Surprenant, 2000; Wildman et al., 2002
PPADS	Current	1	North and Surprenant, 2000
PPNDS	Current	15 nM	Lambrech et al., 2000
TNP-ATP	Current	6 nM	Virginio et al., 1998
MRS2220	Current	10	Jacobson et al., 1998
MRS2159	Current	9 nM	Kim et al., 2001
NF449	Current	0.5 nM	Kassack et al., 2004
NF279	Current	20 nM	Rettinger et al., 2000; Klapperstück et al., 2000
NF023	Current	200 nM	Soto et al., 1999
Ro-0437626	Calcium	3	Jaime-Figueroa et al., 2005
IP ₅ I	Current	3 nM	King et al., 1999
Modulators			
MRS2219 (+)	Current	6	Jacobson et al., 1998
Protons (–)	Current	pK _a 6.3	Wildman et al., 1999b; Stoop et al., 1997
Zinc (–)	Current	1	Wildman et al., 1999
Cadmium (–)	Current	N.D.	Nakazawa and Ohno, 1997
Gadolinium (–)	Current	10	Nakazawa et al., 1997
PIPs (+)	Current	N.D.	Bernier et al., 2008

(+), positive modulator; (–), negative modulator; PIPs, phosphoinositides; N.D. not determined.

believed that ADP also activates this channel at high concentrations, but subsequent studies have shown that purified ADP is unable to activate hP2X1R (Mahaut-Smith et al., 2000).

P2X1R and P2X3R are distinct with regard to their activation by $\alpha\beta$ -meATP at relatively low concentrations (Valera et al., 1994). This agonist can activate other P2XRs as well, but only at high concentrations (He et al., 2003c). In cells expressing recombinant human P2X1R, $\alpha\beta$ -meATP is generally less potent than 2-meSATP and ATP (Valera et al., 1994; Evans et al., 1995; Torres et al., 1998; Bianchi et al., 1999). The defining pharmacological characteristic of P2X1R is the activation by $\beta\gamma$ -meATP with a potency comparable with $\alpha\beta$ -meATP. In contrast, $\beta\gamma$ -meATP is approximately 30- to 50-fold less potent at P2X3R (Evans et al., 1995; Garcia-Guzman et al., 1997b).

TNP-ATP is a potent (IC_{50} , 6 nM), but not selective, antagonist of hP2X1R. This compound also inhibits P2X3R (IC_{50} , 1 nM), as well as P2X2R, P2X4R, and P2X7R at micromolar concentrations (Virginio et al., 1998b). Native P2X1Rs expressed in rat mesenteric smooth muscle cells are also inhibited by TNP-ATP with an IC_{50} of 2 nM (Lewis et al., 1998). Ip_5I is also a highly potent antagonist at recombinant rP2X1Rs (IC_{50} = 3 nM) (King et al., 1999). Both antagonists are subject to degradation by ectonucleotidases, which limits their in vivo use. This is not the case with PPADS, which inhibits rat and human P2X1Rs (Valera et al., 1994; Evans et al., 1995; Bianchi et al., 1999). The naphthylazo derivative of the PPADS family, PPNDS, inhibits rP2X1R at nanomolar concentrations, with an IC_{50} of 15 nM (Lambrecht et al., 2000b) but behaves as an agonist for the ATP receptor in *Paramecium* spp. (Wood and Hennessey, 2003). Two other PPADS derivatives, MRS2159 and MRS2220, are highly selective for ATP-induced responses in rP2X1R, and are ectonucleotidase resistant (Jacobson et al., 1998; Kim et al., 2001).

Suramin inhibits P2X1R (Valera et al., 1994; Evans et al., 1995), and suramin derivatives show great promise as selective antagonists of P2X1R. NF023 inhibits human and rat P2X1R with an IC_{50} value of ~ 0.2 μ M (Soto et al., 1999). NF279 is a highly potent rat and human P2X1R antagonist with IC_{50} values of 20 to 50 nM with preincubation before ATP application and 2 μ M without preincubation. It also inhibits rP2X2R, hP2X7R, and rP2X3R, but with 40-, 60-, and 80-fold rightward shifts in the potency, respectively (Klapperstück et al., 2000; Rettinger et al., 2000). The more potent NF449 inhibits rP2X1R and hP2X1R with IC_{50} values of 0.03 and 0.5 nM (Braun et al., 2001; Hülsmann et al., 2003; Kassack et al., 2004). Both compounds have also been used to characterize the role of P2X1R in platelet activation (for review, see Hu and Hoylaerts, 2010). NF864 potently inhibits P2X1Rs expressed in platelets, as documented in a concentration-dependent study using calcium measurements (Horner et al., 2005). *N*-[(1*R*)-2-[(1*S*,2*R*,3*S*)-1-(cyclohexylmethyl)-3-cyclopropyl-

2,3-dihydroxypropyl]amino]-2-oxo-1-(4-thiazolylmethyl)ethyl]-1*H*-benzimidazole-2-carboxamide (Ro-0437626) is approximately 30-fold more selective for P2X1R over other purinergic receptor subtypes (Jaime-Figueroa et al., 2005).

2. Homomeric P2X2 Receptor and Heteromeric P2X1/2 Receptor. The P2X2R was first cloned from rat pheochromocytoma PC12 cells (Brake et al., 1994). Subsequent localization studies showed broad tissue distribution of this receptor subtype. It is present in different central nervous system regions, including cortex, cerebellum, striatum, hippocampus, nucleus of the solitary tract, and the dorsal horn of the spinal cord (Kidd et al., 1995; Kanjhan et al., 1996; Vulchanova et al., 1996; Simon et al., 1997; Vulchanova et al., 1997; Pankratov et al., 1998; Kanjhan et al., 1999; Scheibler et al., 2004). It is also present in the hypothalamus (Xiang et al., 1998; Stojilkovic, 2009) and retina (Greenwood et al., 1997) as well as in the peripheral nervous system (Collo et al., 1996; Robertson et al., 1996; Simon et al., 1997; Vulchanova et al., 1997; Xiang et al., 1998; Zhong et al., 1998; Zhong et al., 2000, 2001; Calvert and Evans, 2004; Ma et al., 2004; Cockayne et al., 2005; Ma et al., 2005). These receptors are also expressed in non-neuronal cells, including pituitary cells (Stojilkovic and Koshimizu, 2001; Zemkova et al., 2006; Stojilkovic et al., 2010a), the adrenal medulla (Vulchanova et al., 1996), skeletal cells (Ryten et al., 2001; Jiang et al., 2005b), cardiac cells (Hansen et al., 1999a), smooth muscle cells (Lee et al., 2000), endothelial and epithelial cells (King et al., 1998; Hansen et al., 1999b; Birder et al., 2004), and lymphocytes (Di Virgilio et al., 2001).

P2X2R is unique among P2XRs, because multiple splice variants exist in humans, rats, mice, and guinea pigs and are able to generate homomeric and heteromeric channels with different functional properties. In the anterior pituitary, inner ear, and other brain regions, the primary P2X2 gene transcript undergoes extensive alternative splicing, resulting in modified mRNA sequences (Stojilkovic et al., 2000). The spliced subunit, P2X2b, lacks a series of 69 C-terminal amino acids and creates a functional homomeric channel that desensitizes more rapidly than the full-sized receptor, P2X2a (Brändle et al., 1997; Simon et al., 1997; Koshimizu et al., 1998b; Parker et al., 1998; Housley et al., 1999; Lynch et al., 1999). The electrostatic charges of six amino-acid side chains located near the proximal splicing site play a critical role in controlling the rate of receptor desensitization (Koshimizu et al., 1998a, 1999). In mouse pituitary cells, an additional functional splice form has been identified that lacks 90 amino acids in the C-terminal and is termed P2X2e (Koshimizu et al., 2006). This receptor has desensitization rates comparable with the rapidly desensitizing P2X1R and P2X3R (Fig. 2).

The pharmacology of P2X2R is listed in Table 2. ATP is a full agonist at this receptor, although the estimated EC_{50} value for this agonist at rP2X2Rs varies from lab to lab: 2 μ M (Eickhorst et al., 2002), 3 μ M (Zemkova et al.,

TABLE 2
*Pharmacological profile of P2X2R*EC₅₀/IC₅₀ values are micromolar unless otherwise specified.

Compound	Method	EC ₅₀ /IC ₅₀	References
Full agonists			
ATP	Current	2–8	Evans et al., 1995; Eickhorst et al., 2002; Zemkova et al., 2004
2-meSATP	Current/Ca ²⁺	1	Evans et al., 1995; Lynch et al., 1999
Ap ₄ A	Current	15	Wildman et al., 1999a
Partial agonists			
αβ-meATP	Current	>100	Brake et al., 1994; Evans et al., 1995
βγ-meATP	Current	>300	Evans et al., 1995
ATPγS	Current	10	Evans et al., 1995; Liu et al., 2001
BzATP	Current	6–30	Evans et al., 1995; Lynch et al., 1999
Antagonists			
Suramin	Current	10	Brake et al., 1994; Evans et al., 1995
PPADS	Current	1–3	Evans et al., 1995; Lynch et al., 1999
TNP-ATP	Current	2	Virginio et al., 1998b
RB-2	Current	0.5	Liu et al., 2001a
NF279	Current	1	Rettinger et al., 2000
NF770	Current	19 nM	Wolf et al., 2011
NF776	Current	97 nM	Wolf et al., 2011
NF778	Current	140 nM	Wolf et al., 2011
PSB-1011	Current	79 nM	Baqi et al., 2011
PSB-10211	Current	86 nM	Baqi et al., 2011
Modulators			
Protons (+)	Current	pK _a 7.3	Stoop et al., 1997; Clyne et al., 2002a
Calcium (–) ^a	Current	5 mM	Evans et al., 1996; Ding and Sachs, 2000
Zinc (+) ^{b,c}	Current	30	Brake et al., 1994; Clyne et al., 2002a; Lorca et al., 2005
Cooper (+) ^c	Current	3	Xiong et al., 1999; Lorca et al., 2005
Cadmium (+)	Current	>100	Lorca et al., 2005
Lanthanum (–)	Current	10	Nakazawa et al., 1997
Gadolinium (–)	Current	1	Nakazawa et al., 1997
Mercury (+) ^d	Current	10	Lorca et al., 2005; Coddou et al., 2009
H ₂ O ₂ (+) ^d	Current	300	Coddou et al., 2009
Myxothiazol (+) ^d	Current	100 nM	Coddou et al., 2009
Rotenone (+) ^d	Current	600 nM	Coddou et al., 2009
CO (+)	Current	3	Wilkinson et al., 2009
Progesterone (+)	Current	10	De Roo et al., 2010
DHEA (+)	Current	10	De Roo et al., 2003
PIPs ^e	Current	N.D.	Fujiwara and Kubo, 2006
Ethanol (–)	Current	100 mM	Davies et al., 2002; Popova et al., 2010
Toluene (+)	Current	3 mM	Woodward et al., 2004
PKA (–)	Current		Chow and Wang, 1998
VILIP1 (–)	Current		Chaumont et al., 2008

(+) , positive modulator; (–) , negative modulator; DHEA, dehydroepiandrosterone; PIPs, phosphoinositides; N.D., not determined; VILIP1, visinin-like protein 1.

^a In addition, induces an increase in receptor desensitization/inactivation, also observed with Ba²⁺, Mn²⁺, and Mg²⁺.^b Inhibition at human P2X2R (Tittle and Hume, 2008).^c Biphasic effects, suggesting the existence of more than one allosteric site.^d Through the oxidation of the intracellular Cys⁴³⁰.^e Regulation of receptor desensitization by phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5-bisphosphate.

2004), 5 μM (Li et al., 2004), 8 μM (Evans et al., 1995), 7 to 37 μM (Clyne et al., 2003), and 60 μM (Brake et al., 1994). Shorter forms of this receptor exhibit ATP sensitivities comparable with that of the full-sized receptor (Koshimizu et al., 1998b, 2006). Under the same experimental conditions, mP2X2R is less sensitive to ATP than rP2X2R (Eickhorst et al., 2002). hP2X2R seems to be the most sensitive to ATP, with comparable (~1 μM) EC₅₀ values for ATP, 2-meSATP, ATPγS, and BzATP (Lynch et al., 1999). The higher sensitivity of hP2X2R was also shown in current measurements performed under comparable experimental conditions (Tittle and Hume, 2008). ATP, 2-meSATP, ATPγS, and BzATP are roughly equipotent as agonists at the rP2X2R (Brake et al., 1994). A more detailed analysis revealed a somewhat lower potency of ATPγS and BzATP and smaller *E*_{max} values, suggesting that these compounds act as partial agonists (Evans et al., 1995). In all studies, ADP, αβ-meATP, and βγ-meATP had very weak effects on P2X2R

activity. Ap₄A is a full agonist for rP2X2R, and other members of this family of agonists are inactive (Wildman et al., 1999a). The splice forms of human, rat and mouse P2X2R show similar agonist sensitivities to the full-sized receptors (Koshimizu et al., 1998b, 2006; Lynch et al., 1999). Thus, none of the agonists is selective for this receptor subtype.

For a long time, no P2X2R-specific antagonists were available. This has recently changed with the introduction of the anthraquinone derivatives sodium 1-amino-4-[3-(4,6-dichloro[1,3,5]triazine-2-ylamino)phenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (PSB-10211) and disodium 1-amino-4-[3-(4,6-dichloro[1,3,5]triazine-2-ylamino)-4-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (PSB-1011), which acts as P2X2R-selective antagonists in a nanomolar concentration range (Baqi et al., 2011). The P2X2R is also inhibited by suramin and PPADS at concentrations in the low micromolar range, showing less potency for these compounds than the

homomeric P2X1R and P2X3R (Brake et al., 1994; Evans et al., 1995). Other compounds, such as TNP-ATP, NF279, and RB-2, are potent but nonspecific antagonists for this channel. TNP-ATP inhibits this receptor with an EC_{50} value of 2 μ M, approximately 100-fold less potently than P2X1R and P2X3R (Virginio et al., 1998b). NF279 acts as a competitive antagonist at rP2X2R with an IC_{50} value of 0.76 μ M (Rettinger et al., 2000), whereas NF023 shows low sensitivity at this receptor ($IC_{50} > 50 \mu$ M) (Soto et al., 1999). The suramin derivatives 7,7'-(carbonylbis(imino-3,1-phenylenecarbonylimino-3,1-(4-methyl-phenylene)carbonylimino))bis(1-methoxy-naphthalene-3,6-disulfonic acid) tetrasodium salt (NF770), 6,6'-(carbonylbis(imino-3,1-(4-methylphenylene)carbonylimino))bis(1-methoxynaphthalene-3,5-disulfonic acid) tetrasodium salt (NF776), and 6,6'-(carbonylbis(imino-3,1-phenylenecarbonylimino-3,1-(4-methyl-phenylene)carbonylimino))bis(1-methoxy-naphthalene-3,5-disulfonic acid) tetrasodium salt (NF778) have been shown to act as nanomolar P2X2R antagonists (Wolf et al., 2011). RB-2 inhibits P2X2R with an IC_{50} value of 0.4 μ M (Liu et al., 2001a). When tested in the same experiment, RB-2 was more potent than TNP, which was more potent than suramin (Liu et al., 2001a). Native rP2X2Rs expressed in pituitary gonadotrophs are also inhibited by RB-2 (Zemkova et al., 2006).

P2X1 and P2X2 subunits can generate heteromeric receptors expressed at the plasma membrane (Aschrafi et al., 2004). Their biophysical and pharmacological profiles have been incompletely characterized. The profile of heteromers seems to resemble the current profile generated by homomeric P2X1R; however, the heteromers exhibit an acid sensitivity different from that of both P2X1R and P2X2R (Brown et al., 2002).

3. Homomeric P2X3 Receptor and Heteromeric P2X2/3 Receptor. The gene encoding the P2X3R subunit was originally cloned from dorsal root ganglion (DRG) sensory neurons (Chen et al., 1995; Lewis et al., 1995). These neurons also express mRNA transcripts for the P2X2 subunit, and the native channels are probably P2X2/3R heteromers (Lewis et al., 1995). The distribution of homomeric rP2X3R and heteromeric P2X2/3R is highly restricted, occurring in the dorsal root, trigeminal, and nodus sensory ganglia (Vulchanova et al., 1997; Bradbury et al., 1998; Dunn et al., 2001). The receptor expressed in trigeminal sensory neurons is up-regulated by calcitonin gene-related peptide (Fabbretti et al., 2006). The hP2X3R was cloned from heart tissue, but transcripts for this receptor are also present in the spinal cord (Garcia-Guzman et al., 1997b).

Homomeric and heteromeric P2X3Rs play an important role in nociceptive transmission and mechanosensory transduction within visceral organs (Galligan, 2004; Ford et al., 2006). Pharmacological studies have shown that peripheral and spinal P2X3Rs and P2X2/3Rs are involved in transmitting persistent, chronic inflammatory, and neuropathic pain signals (Gever et al., 2006). Studies of P2X3R gene knockouts and transient gene disruption by antisense P2X3R have revealed sim-

ilar findings (Cockayne et al., 2000; Souslova et al., 2000). The P2X2(−/−), P2X3(−/−), P2X2/P2X3^{Db1}(−/−) knockout mice were also used to investigate for the role of ATP signaling on the function of oxygen-sensitive chemoreceptor cells in the carotid body. These studies showed that a deficiency of the P2X2 subunit, but not P2X3, resulted in an attenuated ventilatory response to hypoxia and an impaired response of the afferent carotid sinus nerve to a oxygen decrease (Rong et al., 2003). Later, ATP was identified as the neurotransmitter linking taste buds to the gustatory nerves. This response is mediated through a combination of P2X2 and P2X3 subunits, because deleting either subunit alone did not result in the profound deficit in taste-mediated behaviors seen in the double deletion P2X2/P2X3^{Db1}(−/−) (Finger et al., 2005; Eddy et al., 2009). In other studies with knockout mice, heteromeric P2X2/3 receptors were shown to contribute to nociceptive responses and mechanosensory transduction within the urinary bladder (Cockayne et al., 2005), and P2X2 subunits were found to be important for fast synaptic excitation in myenteric neurons of the mouse small intestine (Ren et al., 2003).

P2X3Rs generate fast, rapidly activating, and acutely desensitizing inward currents. In contrast to rP2X1R currents, rP2X3R currents do not desensitize completely during sustained agonist application (Fig. 3A). The rate of receptor desensitization is determined by the agonist concentration (Fig. 3, A and B), and at low agonist concentrations, the deactivation kinetics of the receptor can be analyzed (Fig. 3B). These experiments clearly show that the deactivation of rP2X3R is relatively rapid, occurring on a time scale of seconds (Zemkova et al., 2004), and does not determine the recovery kinetics of the receptor, which occur on a time scale of minutes (Fig. 3, C and D). Further studies revealed the presence of two types of receptor desensitization (Sokolova et al., 2006). As with rP2X4R (Yan et al., 2006), the fluorescently labeled rP2X3 subunits form fully functional channels that have biophysical and pharmacological properties highly comparable with those of the wild-type receptor (Grote et al., 2005).

Table 3 summarizes the pharmacological profile of homomeric P2X3Rs. ATP is a full agonist for rP2X3R with estimated EC_{50} values of 1.2 μ M (Chen et al., 1995), 2.6 μ M (Pratt et al., 2005), 4.1 μ M (Asatryan et al., 2008), and 7.3 μ M (Grote et al., 2005), but the precision of these estimates is limited by profound desensitization (Khmyz et al., 2008). Similar to the rP2X1R, $\alpha\beta$ -meATP is a full agonist at rP2X3R, acting with a potency similar to (Pratt et al., 2005; Asatryan et al., 2008) or slightly lower than (Chen et al., 1995) that of ATP. 2-meSATP is also a full agonist for this receptor, whereas ATP γ S acts as a partial agonist (Lewis et al., 1995; Liu et al., 2001a). Native (Jarvis et al., 2001) and recombinant (He et al., 2002) rP2X3Rs are also activated by BzATP. Native receptors present in neurons of the rat DRG show a similar agonistic profile (Rae et al., 1998).

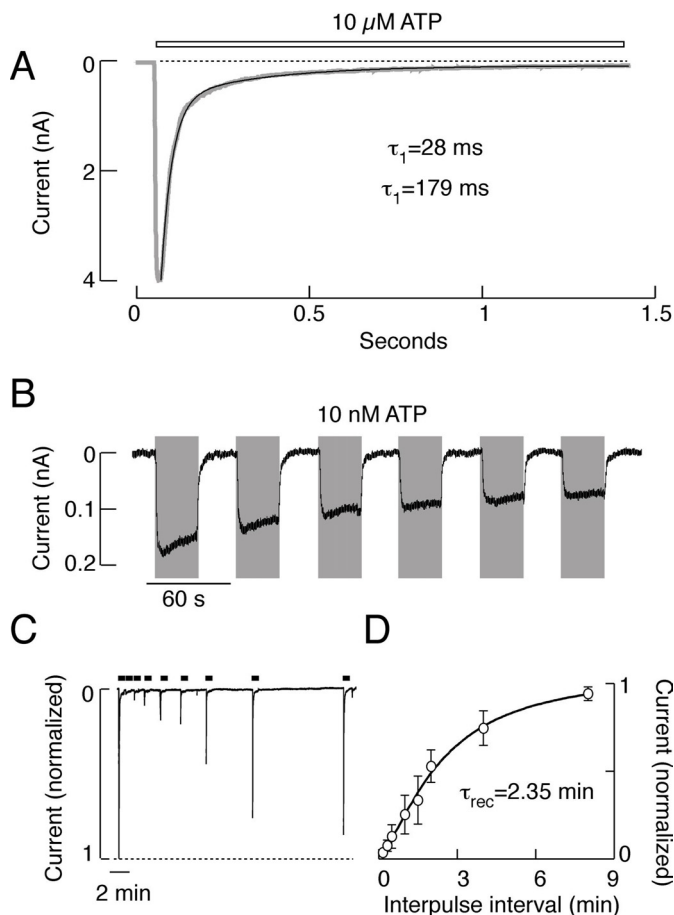


FIG. 3. Characterization of rP2X3R. A, the rates of receptor desensitization. Notice the presence of the residual current during sustained agonist application. B, patterns of P2X3R current responses during repetitive stimulation with 10 nM ATP. C, time course of recovery from desensitization.

Ap₄A, Ap₅A, and Ap₆A are also full agonists for rP2X3R with estimated EC₅₀ values of 0.8, 1.3, and 1.6 μM, respectively (Wildman et al., 1999a). Ap₅G and Ap₆G also activate rP2X3R (Cinkilic et al., 2001). At hP2X3Rs, 2-meSATP is the most potent agonist (320 nM), followed by ATP (780 nM), αβ-meATP (2.55 μM), CTP (17.9 μM), and βγ-meATP (>100 μM) (Garcia-Guzman et al., 1997b).

Suramin and PPADS inhibit P2X3R (Lewis et al., 1995), but these antagonists have relatively low potencies for this receptor compared with other P2XRs (Jacobson et al., 2006). The suramin derivatives NF023, NF279, and NF449 also antagonize P2X3R-mediated currents. P2X3Rs have an intermediate sensitivity to NF023, with IC₅₀ values of 8 and 29 μM for rat and human subtypes, respectively (Soto et al., 1999). NF279 inhibits rP2X3R with IC₅₀ values of 1.6 μM with preincubation and 85 μM when applied together with agonist (Rettinger et al., 2000). NF449 also inhibits rP2X3R with an IC₅₀ value of approximately 3 μM, in contrast to the subnanomolar concentrations needed to inhibit P2X1R, indicating that it could be used to distinguish between these biophysically comparable channels in na-

tive tissues (Braun et al., 2001; Kassack et al., 2004; Horner et al., 2005). Several PPADS derivatives also inhibit P2X3R currents (Brown et al., 2001; Kim et al., 2001).

Because of the involvement of P2X3R in pain pathways, there have been numerous efforts to develop P2X3R-selective drugs. TNP-ATP strongly inhibits rP2X3R currents with an IC₅₀ value of around 1 nM, and the effect of this compound was mimicked by TNP-ADP and TNP-AMP. TNP-ATP is approximately 1000 times less effective in cells expressing P2X2R, P2X4R, and P2X7R, indicating that it could be effectively used to inhibit P2X1R, P2X3R, and P2X2/3R-mediated responses (Virginio et al., 1998b). However, this compound is of limited use for in vivo experiments because of its rapid degradation by ectonucleotidases (Lewis et al., 1998). A compound named A317491 (5-[[[(3-phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate) seems to satisfy this requirement. The dissociation equilibrium constant for this compound is in the range of 10 to 100 nM. The compound also inhibits heteromeric P2X2/3Rs but is ineffective at a wide range of other receptors and channels; the R enantiomer, A317344, has no activity (Jarvis et al., 2002; North, 2004).

The heteromeric P2X2/3Rs exhibit pharmacological properties similar to those of P2X3R, including sensitivity to αβ-meATP and a similar rank order of agonist potencies, but they can be distinguished from homomeric P2X3Rs by the slow desensitization rate (Koshimizu et al., 2002). Selective agonists for P2X2/3R are Ap₅A > αβ-meATP >> βγ-ATP > UTP (Liu et al., 2001a). ATPγS and 2-meSATP also activate these heteromers (Lewis et al., 1995). The probable composition of this trimeric channel is P2X2(P2X3)₂ (Jiang et al., 2003), which probably explains why the heteromeric receptor is more tolerant of the αβ and βγ substitution (Spelta et al., 2003). The sensitivity of P2X2/3R to αβ-meATP provides an easy way to identify these channels, because this agonist does not activate P2X2Rs at low micromolar concentrations. In addition, P2X2/3Rs desensitize slowly in contrast to the rapid desensitization of P2X3R.

Selective antagonists for P2X2/3R are TNP-ATP >> suramin > RB-2 (Liu et al., 2001a). All inhibitors of P2X3R also inhibit P2X2/3R but are poor inhibitors of P2X2R. In most cases, the inhibition of P2X2/3 heteromers is slightly less effective than the inhibition of P2X3R homomers. rP2X2/3Rs have sensitivity to NF023 similar to that of homomeric P2X3Rs (Soto et al., 1999). In contrast to the homomeric rP2X2R, the activity of this heteromer is inhibited by TNP-ATP with an IC₅₀ value of 7 nM (Virginio et al., 1998b). Concentrations of NF449 that are 3 to 4 orders of magnitude higher are required to block heteromeric P2X2/3Rs compared with homomeric and heteromeric P2X1Rs (Rettinger et al., 2005). However, the αβ-meATP-induced currents and calcium signals through recombinant and native P2X2/3Rs are

TABLE 3
*Pharmacological Profile of P2X3R*EC₅₀/IC₅₀ values are micromolar unless otherwise specified.

Compound	Method	EC ₅₀ /IC ₅₀	References
Full agonists			
ATP	Current	1	Lewis et al., 1995; Chen et al., 1995
αβ-meATP	Current	1–2	Lewis et al., 1995; Chen et al., 1995
2-meSATP	Current	0.3	Lewis et al., 1995; Chen et al., 1995; Garcia-Guzman et al., 1997
Ap ₆ A	Current	1.5	Wildman et al., 1999a
Ap ₅ A	Current	1	Wildman et al., 1999a
Ap ₄ A	Current	1	Wildman et al., 1999a
Partial agonists			
ATPγS	Current	10	Liu et al., 2001
BzATP	Calcium	N.D.	Jarvis et al., 2001; He et al., 2002
βγ-meATP	Current	>300	Chen et al., 1995
Antagonists			
Suramin	Current	3	Lewis et al., 1995
PPADS	Current	1.5	Lewis et al., 1995
TNP-ATP	Current	1 nM	Virginio et al., 1998b
A-317491	Current/Ca ²⁺	20 nM	Jarvis et al., 2002
NF023	Current	8.5	Soto et al., 1999
NF279	Current	2	Rettinger et al., 2000
NF449	Current	3	Braun et al., 2001
RO-85	Calcium	30 nM	Brotherton-Pleiss et al., 2010
Ip ₄ I	Current	1	King et al., 1999
MRS2159	Current	150 nM	Kim et al., 2001
MRS2257	Current	30 nM	Kim et al., 2001
AF-353	Current/Ca ²⁺	10 nM	Gever et al., 2010
RO-4	Calcium	13 nM	Carter et al., 2009
RO-51	Calcium	10 nM	Jahangir et al., 2009
Modulators			
Protons (–)	Current	pK _a 6.0	Stoop et al., 1997; Gerevich et al., 2007
Calcium (–)	Current	90 mM	Virginio et al., 1998a
Zinc (+) ^a	Current	10	Wildman et al., 1999b
Cadmium (–)	Current	100	Nakazawa and Ohno, 1997
Ethanol (+)	Current	25 mM	Davies et al., 2005a
Cibracon Blue (+)	Current/Ca ²⁺	1.5	Alexander et al., 1999
Toluene (–)	Current	3 mM	Woodward et al., 2004
Tetramethylpyrazine	Current	1 mM	Gao et al., 2008
Cdk-5 (–)	Current		Nair et al., 2010
Csk (–)	Current		D'Arco et al., 2009

N.D., not determined; (+), positive modulator; (–), negative modulator; Cdk-5, cyclin-dependent kinase 5; Src, C-terminal Src kinase.

^a Biphasic effects, suggesting the existence of more than one allosteric site.

slowly desensitizing, in contrast to homomeric P2X3Rs (Lewis et al., 1995; Burgard et al., 1999; Liu et al., 2001a; Koshimizu et al., 2002).

Several new inhibitors have recently been introduced. Spinorphin, an endogenous antinociceptive peptide (LVVYPWT), seems to be a potent and noncompetitive antagonist at hP2X3Rs (IC₅₀ = 8.3 pM). The antagonistic properties are sustained when single alanine substitutions were made from the 1st to 4th amino acids and when the cyclic form of LVVYPWT was introduced (Jung et al., 2007). Two diaminopyrimidines, 5-[5-iodo-4-methoxy-2-(1-methylethyl)phenoxy]-2,4-pyrimidine diamine hydrochloride [RO-4, also known as AF-353 (Carter et al., 2009)] and 5-(5-ethynyl-2-isopropyl-4-methoxy-phenoxy)-pyrimidine-2,4-diamine [RO-5, also known as AF-729] (Jahangir et al., 2009)], inhibit rP2X3Rs and hP2X2/3Rs at nanomolar concentrations. RO-5 also inhibits native presynaptic P2X3Rs and P2X2/3Rs (Kaan et al., 2010). 1-Methyl-3-phenyl-1*H*-thieno[2,3-*c*]pyrazole-5-carboxylic acid [(*R*)-2-(4-acetyl-piperazin-1-yl)-1-methyl-ethyl]-amide (RO-85) has demonstrated selectivity for P2X3Rs (IC₅₀ = 30 nM) over heteromeric P2X2/3Rs (IC₅₀ = 400 nM) and other P2XRs (IC₅₀ > 10 μM), indicating the pharmacological possibility to distinguish between homomeric and heteromeric P2X3Rs

(Brotherton-Pleiss et al., 2010). Finally, AF-353 inhibits hP2X3R (IC₅₀, 10 nM), rP2X3R (IC₅₀, 10 nM), and hP2X2/3R (IC₅₀, 38 nM) at low nanomolar concentrations (Gever et al., 2010).

4. Homomeric P2X4 Receptor and Heteromeric P2X1/4 Receptor. This receptor was identified as a distinct member of the P2XR family when it was shown that a single gene product was sufficient to generate ion channels with distinct patterns and pharmacological properties (Bo et al., 1995; Buell et al., 1996; Soto et al., 1996). Homomeric rP2X4Rs bathed in physiological solutions activated rapidly, desensitized at a moderate rate, and displayed inwardly rectifying current-voltage relationships that reversed at 0 mV (Khakh et al., 1999a; Fountain and North, 2006). Figure 1 shows a typical pattern of ATP-induced current (A) and the dependence of receptor activation, desensitization, and deactivation kinetics on agonist concentration (B).

P2X4Rs are widely expressed in the brain, spinal cord, and autonomic and sensory ganglia (Rubio and Soto, 2001; Burnstock and Knight, 2004; Burnstock, 2007; Surprenant and North, 2009). They are also expressed in the anterior pituitary gland, specifically in lactotrophs, and their activation leads to the stimulation of

electrical activity, promotion of voltage-gated and voltage-insensitive Ca^{2+} influx and prolactin release (He et al., 2003a; Zemkova et al., 2010). Recent studies have also shown that P2X4Rs are expressed in microglia and alveolar macrophages (Bowler et al., 2003) and that the up-regulation of these receptors in activated microglia located in the dorsal horn of the spinal cord contributes to neuropathic pain (Tsuda et al., 2003; Ulmann et al., 2008). Immortalized C8-B4 cells derived from cerebellar microglia also express P2X4Rs and were used to show that antidepressants indirectly inhibit the receptor-mediated responses by interfering with lysosomal trafficking (Toulme et al., 2010). In addition, P2X4Rs are present in human lung mast cells (Wareham et al., 2009) and PC12 cells (Sun et al., 2007). The P2X4R knockout mice have high blood pressure, probably reflecting the role of these channels in the regulation of the vascular tone on endothelial cells (Yamamoto et al., 2006) and show reduced amplitudes in long-term potentiation in the hippocampus (Sim et al., 2006). Experiments with the P2X4R-deficient mouse line also revealed the potential involvement of this receptor in ATP-mediated brain-derived neurotrophic factor microglial secretion and neuropathic pain (Ulmann et al., 2008). The native P2X4R and P2X7R currents are seen in recruited peritoneal macrophages of wild-type mice, and P2X4R inactivation in a mouse line eliminated the P2X4-like current, suggesting an immunologic role for this receptor (Br ne et al., 2007).

Green fluorescent protein-tagged receptors were used to show that P2X4Rs, but not P2X2Rs, undergo rapid constitutive internalization and subsequent reinsertion into the plasma membrane in a dynamin-dependent manner. Internalization of P2X4/6 heterodimers was also observed, suggesting that one or two P2X4 subunits are sufficient to govern the trafficking properties of the receptor (Bobanovic et al., 2002). The C-terminal YXXGL motif serves as a noncanonical tyrosine-based sorting signal that is necessary for efficient endocytosis of this receptor (Royle et al., 2002). P2X4R rundown is evident after repetitive stimulation in a whole-cell configuration. This effect is completely prevented with the use of a perforated patch, indicating that small cytosolic factors that are lost during intracellular dialysis could be important in the trafficking of this receptor (Fountain and North, 2006). Endogenous P2X4Rs in cultured rat microglia, vascular endothelial cells, and freshly prepared peritoneal macrophages are localized predominantly to lysosomes, where the receptors can retain their function and subsequently travel out to the plasma membrane (Qureshi et al., 2007). Unstimulated macrophages express very low levels of functional P2X4Rs, but expression of these receptors at the plasma membrane was enhanced by the activation of phagocytosis (Stokes and Surprenant, 2009).

The pharmacological profile of P2X4R is shown in Table 4. This receptor is activated by ATP with esti-

mated EC_{50} values of 3 μM (Yan et al., 2005; Yan et al., 2006), 4 to 5 μM (Zemkova et al., 2007; Jel nkova et al., 2008; Jindrichova et al., 2009), 7 μM (Soto et al., 1996), and 10 μM (Bo et al., 1995; Buell et al., 1996). Under similar experimental conditions, the potency of ATP at the mouse, rat, and human P2X4R was comparable, ranging between 1 and 8 μM (Garcia-Guzman et al., 1997a; Bianchi et al., 1999; Jones et al., 2000). 2-meSATP also activates rP2X4Rs with a potency similar to or lower than ATP (Buell et al., 1996; Soto et al., 1996). $\alpha\beta$ -meATP and Ap_4P are partial agonists for mouse, rat, and human receptors (Jones et al., 2000). The rP2X4R is activated by nucleotide analogs with the following order of efficacy: $\text{ATP} > \text{ATP}\gamma\text{S} > 2\text{-meSATP} > \text{CTP} > \alpha\beta\text{-meATP}$. In calcium measurements, the potency order was $\text{ATP} > \text{BzATP} > \alpha\beta\text{-meATP}$ (He et al., 2003c). The human receptor displays an agonist potency profile similar to that of rP2X4R (Garcia-Guzman et al., 1997a; Jones et al., 2000). Ap_4A acts as a partial agonist for mouse, rat, and human P2X4Rs, whereas other members from this family are inactive (Wildman et al., 1999a; Jones et al., 2000). Experiments that measure the displacement of [^{35}S]ATP γS from rP2X4Rs also indicated that ATP is the most potent agonist, followed by ATP γS , 2-meSATP, and $\alpha\beta$ -meATP (Michel et al., 1997). A methanocarba derivative of AMP, 1'*S*,2*R*,3*S*,4'*R*,5'*S*)-4-(6-amino-2-chloro-9*H*-purin-9-yl)-1-[phosphoryloxymethyl] bicyclo[3.1.0]hexane-2,3-diol (MRS2339), has been found to act as a potent agonist of heart purinergic receptors; the authors suggested that this compound could be a P2X4R agonist (Zhou et al., 2010).

Mouse and rat P2X4Rs are insensitive to suramin. The rP2X4R is also insensitive to PPADS, whereas the mouse and human orthologs are inhibited by this compound (Garcia-Guzman et al., 1997a; Jones et al., 2000). TNT-ATP antagonizes the ATP-mediated currents with an IC_{50} of 15 μM (Virginio et al., 1998b). Rat and human P2X4Rs are not sensitive to NF023 in concentrations up to 100 μM (Soto et al., 1999), and BBG is a weak receptor antagonist (Jiang et al., 2000a). 5-(3-Bromophenyl)-1,3-dihydro-2*H*-benzofuro[3,2-*e*]-1,4-diazepin-2-one has been suggested to act as a specific P2X4R inhibitor with an IC_{50} of 0.5 μM (Donnelly-Roberts et al., 2008). RB-2 and Coomassie blue are the most potent antagonists of this channel, producing an inhibition of [^{35}S]ATP γS binding (Michel et al., 1997). Recent studies have also suggested the potential role of some serotonergic antidepressants as P2X4R antagonists (Nagata et al., 2009; Sim and North, 2010). Paroxetine inhibits P2X4R-mediated currents and calcium increases with an IC_{50} of 2.5 and 1.9 μM at the rat and human receptor, respectively (Nagata et al., 2009). In contrast, amitriptyline modestly and noncompetitively inhibits rat and mouse P2X4Rs and has no effect on hP2X4Rs. However, this modest inhibition was not observed in P2X2R or P2X7R (Sim and North, 2010), suggesting an interesting possibility for the development of specific P2X4R antagonists.

TABLE 4
*Pharmacological profile of P2X4R*EC₅₀/IC₅₀ values are micromolar unless otherwise specified.

Compound	Method	EC ₅₀ /IC ₅₀	References
Full agonists			
ATP	Current	1–10	Buell et al., 1996; Soto et al., 1996; Jones et al., 2000
BzATP	Calcium	3	He et al., 2003a
Partial agonists			
2-meSATP	Current	10–100	Buell et al., 1996; Soto et al., 1996; Jones et al., 2000
$\alpha\beta$ -meATP	Current	4–300	Buell et al., 1996; Soto et al., 1996; Jones et al., 2000
$\beta\gamma$ -meATP	Current	>300	Soto et al., 1996
CTP	Current	200	Soto et al., 1996
Ap ₄ P	Current	2–10	Wildman et al., 1999a; Jones et al., 2000
Antagonists			
Suramin	Current	>300	Buell et al., 1996; Soto et al., 1996
PPADS	Current	>300	Buell et al., 1996; Soto et al., 1996
TNP-ATP	Current	15	Virginio et al., 1998b
5-BDBD	Current	0.5	Donnelly-Roberts et al., 2008
NF023	Current	>100	Soto et al., 1999
BBG	Current	3–100	Jiang et al., 2000a
Paroxetine	Current/Ca ²⁺	2	Nagata et al., 2009
Modulators			
Protons (–)	Current	pK _a 7.0 ^c	Stoop et al., 1997; Clarke et al., 2000; Yan et al., 2005
Calcium ^a	Current	1 mM	Khakh et al., 1999a
Zinc (+) ^b	Current	5	Soto et al., 1996; Xiong et al., 1999; Acuña-Castillo et al., 2000; Coddou et al., 2007
Cooper (–)	Current	10	Acuña-Castillo et al., 2000; Coddou et al., 2003; Coddou et al., 2007
Cadmium (+) ^b	Current	20	Acuña-Castillo et al., 2000; Coddou et al., 2005
Mercury (–)	Current	10	Coddou et al., 2005; Coddou et al., 2009
H ₂ O ₂ (–)	Current	300	Coddou et al., 2009
CO (–)	Current	N.D.	Wilkinson et al., 2009
IVM (+)	Current	3	Khakh et al., 1999b; Priel and Silberberg, 2004; Jelínková et al., 2006; Silberberg et al., 2007
Alfaxalone (+)	Current	0.4 nM	Codocedo et al., 2009
ALP (+)	Current	0.3 nM	Codocedo et al., 2009
THDOC (+)	Current	0.1 nM	Codocedo et al., 2009
PIPs (+)	Current	N.D.	Bernier et al., 2008b
Ethanol (–)	Current	60 mM	Xiong et al., 2000; Davies et al., 2002;
Cibacron blue (+)	Current	3	Miller et al., 1998
Propofol (+)	Current	50	Tomioka et al., 2000
Toluene (+)	Current	1 mM	Woodward et al., 2004
PKA (+)	Current/Ca ²⁺		Brown and Yule, 2010

(+), positive modulator; (–), negative modulator; N.D., not determined; ALP, allopregnanolone; THDOC, 3 α ,21-dihydroxy-5 α -pregnan-20-one; PIPs, phosphoinositides; 5-BDBD, 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one.

^a In the absence of calcium, a secondary current is developed in P2X4Rs.

^b Biphasic effects, suggesting the existence of more than one allosteric site.

Heteromeric assembly of P2X1 and P2X4 subunits results in a functional channel with kinetic properties resembling homomeric P2X4Rs and a pharmacological profile similar to homomeric P2X1R. Specifically, heteromers activated, desensitized, and deactivated slower than P2X1Rs and at rates comparable with those observed in cells expressing P2X4R. On the other hand, a leftward shift in the sensitivity of heteromers to $\alpha\beta$ -meATP was observed compared with P2X4R. Furthermore, suramin and TNP-ATP blocked both P2X1R and P2X1/4R currents but not P2X4R currents (Nicke et al., 2005). It is currently unclear whether the native channels assemble as heteromers.

5. Homomeric P2X5 Receptor and Heteromeric P2X1/5 Receptor. P2X5R was first cloned from rat celiac ganglia (Collo et al., 1996). The expression of P2X5R mRNA and protein transcripts is restricted to the trigeminal mesencephalic nucleus of the brainstem, sensory neurons, cervical spinal cord, and some blood vessels. A limited amount of the receptor was also detected in the heart, skeletal muscle, kidney, adrenal gland, and retina (Collo et al., 1996; Garcia-Guzman et al., 1996; Brändle et al., 1998; Phillips et al., 1998; Gröschel-Stewart et al., 1999; Phillips

and Hill, 1999; Taylor et al., 1999; Gitterman and Evans, 2000; Ryten et al., 2001). The receptor was also detected in carcinomas of the skin and prostate (Greig et al., 2003; Calvert et al., 2004). In humans, the expression and function of this receptor is still unclear. The mRNA transcripts have been detected predominantly in tissues related to the immune system (Lê et al., 1997). The recombinant rat and zP2X5R generates a low-amplitude nondesensitizing current, whereas P2X5Rs from other species respond to ATP with large, rapidly activating and slowly desensitizing currents. The recovery from desensitization is also slow (Collo et al., 1996; Garcia-Guzman et al., 1996; Bo et al., 2000; Jensik et al., 2001; Diaz-Hernandez et al., 2002; Wildman et al., 2002; Bo et al., 2003). Like P2X7Rs (see section III.A.7), the pore of human, chick, and bullfrog P2X5Rs dilates during prolonged receptor activation (Bo et al., 2000, 2003; Jensik et al., 2001).

ATP is a full agonist for this receptor (Table 5) with estimated EC₅₀ values of 0.4 μ M (Wildman et al., 2002) and 8 μ M (Garcia-Guzman et al., 1996) for rP2X5R, 0.3 μ M (Kotnis et al., 2010) and 4 μ M (Bo et al., 2003) for hP2X5R, and 2 μ M for chicken P2X5R (Ruppelt et al., 2001). The potency order at the rP2X5R is ATP = 2-

TABLE 5
*Pharmacological profile of P2X₅R*EC₅₀/IC₅₀ values are micromolar unless otherwise specified.

Compound	Method	EC ₅₀ /IC ₅₀	References
Full agonists			
ATP	Current	0.5–4	Wildman et al., 2002; Bo et al., 2003
2-meSATP	Current	0.5	Wildman et al., 2002
ATP γ S	Current	0.5	Wildman et al., 2002
Partial agonists			
$\alpha\beta$ -meATP	Current	1–12	Wildman et al., 2002; Kotnis et al., 2010
$\beta\gamma$ -meATP	Current	10	Wildman et al., 2002
BzATP	Current	1–6	Wildman et al., 2002; Bo et al., 2003
Ap ₃ A	Current	5	Wildman et al., 2002
Ap ₄ A	Current	0.3	Wildman et al., 2002
Ap ₅ A	Current	0.7	Wildman et al., 2002
Ap ₆ A	Current	5	Wildman et al., 2002
Antagonists			
Suramin	Current	2–3	Wildman et al., 2002; Bo et al., 2003
PPADS	Current	200–600 nM	Wildman et al., 2002; Bo et al., 2003; Kotnis et al., 2010
TNP-ATP	Current	600–700 nM	Wildman et al., 2002; Kotnis et al., 2010
BBG	Current	0.5	Bo et al., 2003
Modulators			
Protons (–)	Current		Wildman et al., 2002
Calcium (–) ^a	Current	10 mM	Wildman et al., 2002
Zinc (+) ^b	Current	10	Wildman et al., 2002

(–), positive modulator; (–), negative modulator.

^a In addition, a sensitization of ATP-evoked currents is observed with calcium.^b Biphasic effects, suggesting the existence of more than one allosteric site.

MeSATP = ATP γ S > $\alpha\beta$ -meATP = BzATP, with $\alpha\beta$ -meATP and BzATP acting as partial agonists. Ap₂A, Ap₄A, Ap₅A, and Ap₆A are also partial agonists for this receptor (Wildman et al., 2002). At hP2X₅R, concentration-response curves for ATP, BzATP, and $\alpha\beta$ -meATP yield EC₅₀ values of approximately 4, 6, and 161 μ M, and BzATP, $\alpha\beta$ -meATP, and 2-meSATP are partial agonists (Bo et al., 2003). Others observed a higher potency for $\alpha\beta$ -meATP at hP2X₅R with an EC₅₀ value of approximately 12 μ M (Kotnis et al., 2010). At the rP2X₅R, the potency order for five antagonists was PPADS > TNP-ATP > suramin > RB-2 >> Ip₅I (Wildman et al., 2002). At hP2X₅R, PPADS, BBG, and suramin inhibited the ATP-evoked currents with IC₅₀ values of 0.2, 0.5, and 2.9 μ M, respectively (Bo et al., 2003). TNP-ATP and suramin also inhibited the ATP-evoked currents at hP2X₅R (Kotnis et al., 2010).

Many of the tissues expressing P2X₅R also express other isoforms, including the P2X₁ subunit, which raised the possibility of heteromeric expression. Consistent with this, several laboratories have found expression of heteromeric P2X₁/5Rs (Torres et al., 1998; Haines et al., 1999; Lê et al., 1999; Surprenant et al., 2000). From a biophysical standpoint, this heteromer more closely resembles the P2X₅R, because it generates a nondesensitizing plateau in current. Like P2X₁R, however, the heteromers are activated by $\alpha\beta$ -meATP (Torres et al., 1998; Lê et al., 1999). ATP activates these heteromers with an EC₅₀ value of 0.7 μ M (Haines et al., 1999). 2-meSATP is also the full and equipotent agonist for heteromeric P2X₁/5R, whereas ATP γ S and $\alpha\beta$ -meATP are partial agonists. Suramin and PPADS are equipotent at P2X₁R and P2X₁/5R, but the heteromer is less sensitive to TNP-ATP (Haines et al., 1999). NF449 also

potently inhibits rP2X₁/5R with an IC₅₀ value of 0.7 nM (Rettinger et al., 2005). The native channel in guinea pig submucosal arterioles is biophysically and pharmacologically similar to the recombinant P2X₁/5R (Surprenant et al., 2000). Mouse cortical astrocytes express mRNAs for P2X₁R and P2X₅R subunits, and the high sensitivity to ATP, biphasic kinetics, and inhibition by PPADS were also comparable with the responses seen in recombinant P2X₁/5Rs (Lalo et al., 2008).

6. Homomeric P2X₆ Receptor and Heteromeric P2X₂/6 and P2X₄/6 Receptors. The expression and immunoreactivity of P2X₆R mRNA are seen throughout the central nervous system, including Purkinje cells in the cerebellum and pyramidal cells in the hippocampus (Collo et al., 1996; Rubio and Soto, 2001; Burnstock and Knight, 2004). Expression of this receptor has also been reported in sensory ganglia (Xiang et al., 1998), skeletal muscle (Meyer et al., 1999), uterus and granulosa cells of the ovary (Collo et al., 1996), thymus (Glass et al., 2000), and the human salivary gland (Worthington et al., 1999). Up-regulation of this receptor has been detected in human heart tissue from patients with congestive heart failure (Banfi et al., 2005). The P2X₆R subunit expressed alone generates functional membrane receptors very inefficiently (Collo et al., 1996; Lê et al., 1998; King et al., 2000; Koshimizu et al., 2000b; Jones et al., 2004).

This receptor seems to have higher sensitivity for ATP and 2-meSATP and is inhibited by TNP-ATP and PPADS (Jones et al., 2004). The current response was described as nondesensitizing and may be either sensitive (Jones et al., 2004) or insensitive (Collo et al., 1996) to $\alpha\beta$ -meATP. This could reflect differences in heteromerization with endogenous P2XR subunits (Jones et al., 2004). At first, it was believed that the low expres-

sion in the plasma membrane was due primarily to a failure to form the proper homomers (Aschrafi et al., 2004). It has also been suggested that the protein could be expressed in the plasma membrane in a partially glycosylated state and that further glycosylation would yield a functional channel (Jones et al., 2004). Later studies indicated that an uncharged region of the P2X6R N terminus is responsible for receptor retention at the endoplasmic reticulum. When the charges were introduced by mutagenesis, the mutant receptors were able to form homotrimers and to glycosylate and were delivered to the membrane (Ormond et al., 2006).

Subsequent studies confirmed that rat P2X2 and P2X6 subunits form functional heteromers that have a somewhat different phenotype from homomeric P2XRs. This includes a reduction in ATP potency, a significant loss of Ap_4A activity, and a distinct pattern of pH regulation. The rank order of agonist activation for these heteromers is highly comparable with that observed for P2X2Rs: $\text{ATP} = \text{ATP}\gamma\text{S} = 2\text{-meSATP} \gg \text{BzATP} = \alpha\beta\text{-meATP}$. The heteromer is also sensitive to inhibition by suramin, similar to the homomeric P2X2R (King et al., 2000). Both $(\text{P2X2})_2\text{P2X6}$ and $\text{P2X2}(\text{P2X6})_2$ compositions of heteromeric channels have been detected (Barrera et al., 2007). The rat P2X6 subunit also forms functional heteromers with the P2X4 subunit that are pharmacologically similar to P2X4R. These heteromers exhibit sensitivity to ATP and 2-meSATP similar to that of homomeric P2X4Rs, are activated by low micromolar $\alpha\beta\text{-meATP}$ concentrations, and are blocked by suramin and RB-2 (Lê et al., 1998). Allosteric regulation of this heteromer resembles the regulation of P2X4Rs.

7. Homomeric P2X7 Receptor. The P2X7R subunit, initially cloned from a rat brain cDNA library, shares an overall membrane topology with the other members of this family of receptors (Surprenant et al., 1996). The receptor is distinguished structurally from other members of P2XRs by its long intracellular C terminus tail containing multiple protein and lipid interaction motifs and a cysteine-rich 18 amino acid segment. These receptors are expressed on cells of the immune system, such as macrophages/monocytes, dendritic cells, lymphocytes, and mast cells, as well as in glia cells, including microglia, astrocytes, oligodendrocytes, and Schwann cells (Collo et al., 1997; Rassendren et al., 1997b; Di Virgilio et al., 2001, 2009; Franke et al., 2001; Chessell et al., 2005; Skaper et al., 2010). Epithelial cells, fibroblasts, osteoblasts, pituitary cells, and some neuronal populations also express P2X7Rs (Gröschel-Stewart et al., 1999; Koshimizu et al., 2000a; Deuchars et al., 2001; Gartland et al., 2001; Sim et al., 2004).

Two different genetically engineered P2X7-deficient mice from Pfizer (New York, NY) and GlaxoSmithKline (Brentford, Middlesex, UK) (Solle et al., 2001; Chessell et al., 2005) were used to study P2X7R physiology. However, a “P2X7-like” receptor that is similar, but not identical to, “native” P2X7Rs was found in the Pfizer knock-

out mice (Sánchez-Nogueiro et al., 2005; Marín-García et al., 2008), and a novel functional P2X7 splice variant with an alternative exon 1 and translation start was found in the GlaxoSmithKline knockout mice (Nicke et al., 2009). The Pfizer P2X7R knockout mice established the role of P2X7R in ATP-induced interleukin-1 post-translational processing, the inflammatory response (Solle et al., 2001; Labasi et al., 2002) and the regulation of bone formation and absorption (Ke et al., 2003). In the GlaxoSmithKline P2X7 knockout mice, inflammatory (in an adjuvant induced model) and neuropathic (in a partial nerve ligation model) hypersensitivity was abolished (Chessell et al., 2005). These findings and multiple subsequent studies became the basis for synthesizing and developing new P2X7 antagonists into therapeutic drugs to treat inflammatory and neuropathic pain (Perez-Medrano et al., 2009). Clinical studies combined with experiments using P2X7 knockout mice also highlighted the relevance of ATP-P2X7 cryopyrin/NALP3 caspase-1 inflammasome activation to the therapeutic efficacy for anthracycline-treated breast cancer patients (Ghiringhelli et al., 2009; Lamkanfi and Dixit, 2009).

Although numerous studies have been performed with the recombinant P2X7R since it was cloned, the gating properties of this receptor are not well understood. P2X7R operates as a nonselective cationic channel during initial agonist application, but with prolonged application, the receptor also provides a permeation pathway for molecules with molecular masses up to ~ 800 Da, including the fluorescent dye YO-PRO-1, a process known as cell “permeabilization.” At first, it was suggested that the bifunctional permeation properties of P2X7R reflect a dilation of the integral pore of the channel (Surprenant et al., 1996; Chessell et al., 1997; Michel et al., 1999; Gudipaty et al., 2001). It was further suggested that a progressive dilation of the ion-conducting pathway during prolonged agonist application is not unique to the P2X7R, but also occurs in cells expressing P2X2R and P2X4R (Khakh et al., 1999a; Virginio et al., 1999).

However, this hypothesis has been questioned. One group noticed the uptake of fluorescent dyes was not affected in cells expressing P2X7R in which the receptor-specific C-terminal 18 amino acid sequence was deleted, although the mutant was not permeable to *N*-methyl-D-glucamine, suggesting that YO-PRO-1 entry occurs through a pathway separate from that of *N*-methyl-D-glucamine (Jiang et al., 2005a). Others observed that cells expressing a mutant P2X7R that was truncated at residue 581 had negligible ethidium uptake and normal current responses (Smart et al., 2003). It was also reported that the coupling of P2X7R to pannexin-1 channels accounts for cellular entry of fluorescent dyes (Pelegri and Surprenant, 2006).

Recent studies have shown that naive rP2X7R activates and deactivates biphasically at higher agonist concentrations (Fig. 4A), and the slow secondary growth of

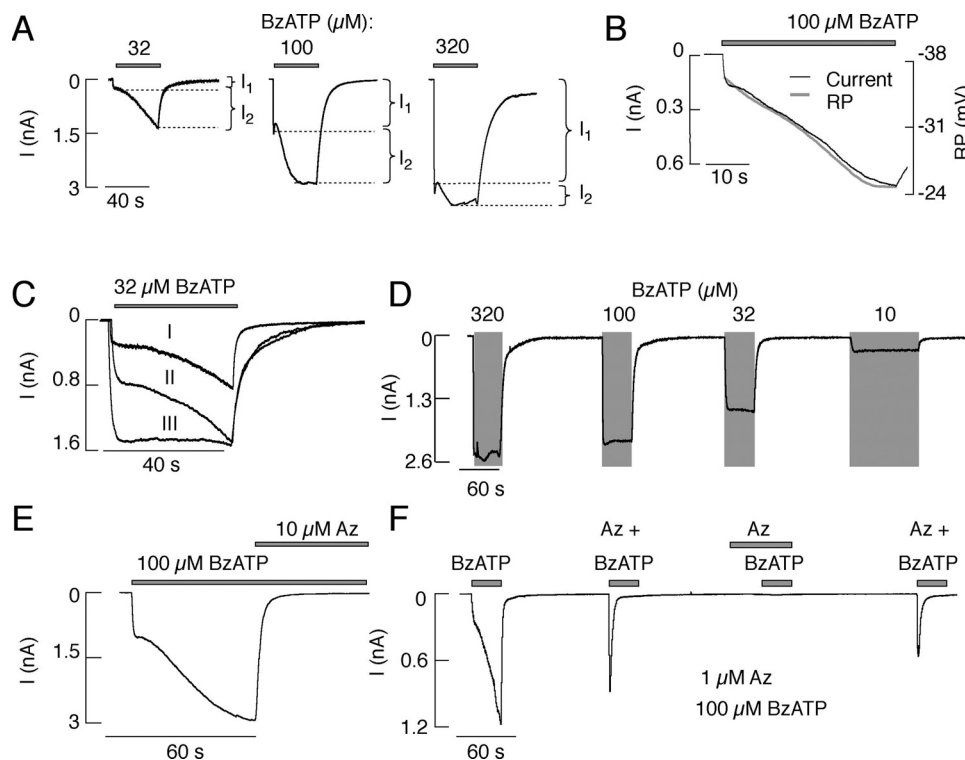


FIG. 4. Characterization of rP2X7R. A, concentration-dependent effects of BzATP on biphasic receptor activation. B, comparison of kinetics of the secondary current growth and changes in the reversal potentials. C, sensitization of receptors induced by repetitive application of agonist. D, sensitized receptors respond with monophasic currents with the peak amplitude of current determined by agonist concentration. E, abolition of rapid and sustained P2X7R currents by the P2X7R-specific antagonist AZ10606120 (Az). F, repetitive stimulation of receptors with 100 μ M BzATP in the presence and absence of AZ10606120.

current in the biphasic response coincides temporally with pore dilation (Fig. 4B). The secondary rise in current was observed under different ionic conditions as well as in cells with blocked pannexin channels and in cells not expressing these channels endogenously. Experiments with several mutants further indicated that the P2X7R dilates under physiological ion conditions, leading to generation of biphasic current, and that this process is controlled by residues near the intracellular side of the channel pore (Yan et al., 2008). It has also been shown recently that P2X2R display permeability dynamics, which are correlated with conformational changes in the cytosolic domain remote from the selectivity filter itself (Chaumont and Khakh, 2008). Several laboratories have also suggested that pannexins contribute to P2XR signaling by providing a pathway for release of intracellular ATP (Locovei et al., 2006; Iglesias et al., 2009; MacVicar and Thompson, 2010; Li et al., 2011).

In contrast to other homomeric and heteromeric P2XRs, repetitive stimulation with the same agonist concentration causes sensitization of P2X7R, which manifests as a progressive increase in the current amplitude accompanied by a slower deactivation rate (Fig. 4C). Once a steady level of the secondary current is reached, responses at high agonist concentrations become monophasic (Fig. 4D). Both phases of response are abolished by the application of Az10606120, a P2X7R-

specific antagonist (Fig. 4, E and F). These results further support the conclusion that pore dilation accounts for the secondary current growth (Yan et al., 2010). Sensitization of the receptors caused by repetitive agonist applications could be partially explained by calcium-CaM signaling (Roger et al., 2008).

The pharmacological profile of mammalian P2X7Rs is shown in Table 6. This is the least sensitive member of the P2XR family to activation by nucleotides. BzATP is the most potent agonist for this receptor with an EC_{50} value of 50 μ M, compared with 3 to 4 mM for ATP. At the sensitized receptor, the EC_{50} values for BzATP and ATP are approximately 25 μ M and 2 mM, respectively (Roger et al., 2008; Donnelly-Roberts et al., 2009a; Yan et al., 2010). Similar values have been suggested by others (Rassendren et al., 1997b; Hibell et al., 2000; Duan et al., 2003). The potency of ATP at the human receptor increases when calcium and magnesium are removed from the bath medium (Klapperstuck et al., 2001). 2-meSATP and ATP γ S are partial agonists of this receptor, whereas $\alpha\beta$ -meATP and $\beta\gamma$ -meATP have very little effect on activation.

An interesting feature of P2X7R is its activation by ADP-ribosylation, a reaction that requires the enzyme ADP-ribosyltransferase and NAD as a substrate. In T-regulatory cells, both P2X7R and ADP-ribosyltransferase are expressed, and the activation of P2X7R by ADP-ribosylation observed in these cells was also mim-

TABLE 6
Pharmacological profile of P2X7R

EC₅₀/IC₅₀ values are micromolar unless otherwise specified.

Compound	Method	EC ₅₀ /IC ₅₀	References
Full agonists			
ATP	Current/Ca ²⁺	2–4 mM	Yan et al., 2010; Donnelly-Roberts et al., 2009a; Roger et al., 2008
BzATP	Current/Ca ²⁺	10	Yan et al., 2010; Donnelly-Roberts et al., 2009a
Partial agonists			
αβ-meATP	Current	>300	Surprenant et al., 1996
βγ-meATP	Current	>300	Surprenant et al., 1996
2-meSATP	Current	200	Surprenant et al., 1996
Antagonists			
Suramin	Current	>300	Surprenant et al., 1996
NF279	Ca ²⁺	3–20	Donnelly-Roberts et al., 2009a; Klapperstück et al., 2000
PPADS	Current	10–45	Surprenant et al., 1996
PPNDS	Ca ²⁺	1–10	Donnelly-Roberts et al., 2009a
MRS2159	Calcium	5	Donnelly-Roberts et al., 2009a
KN-62	Ca ²⁺	10	Donnelly-Roberts et al., 2009a
BBG	Current	15–250 nM	Jiang et al., 2000a
oATP	Current	100–300	Surprenant et al., 1996; Michel et al., 2000; Hibell et al., 2001
A740003	Calcium	20–700 nM	Honore et al., 2006; Donnelly-Roberts et al., 2009a
A438079	Current/	0.2–1	Nelson et al., 2006; Donnelly-Roberts et al., 2009a
A804598	Ca ²⁺	10 nM	Donnelly-Roberts et al., 2009b
AZ10606120	Ethidium/Ca ²⁺	10–200 nM	Michel et al., 2007
AZ11645373	Current	5–10 nM	Stokes et al., 2006
Modulators			
Protons (–)	Current	pK _a 6.1	Virginio et al., 1997; Acuña-Castillo et al., 2007; Liu et al., 2009
Calcium (–)	Current	3 mM	Surprenant et al., 1996; Virginio et al., 1997
Magnesium (–)	Current	500	Virginio et al., 1997; Acuña-Castillo et al., 2007
Zinc (–)	Current	80	Virginio et al., 1997; Acuña-Castillo et al., 2007; Liu et al., 2008a
Cooper (–)	Current	2	Virginio et al., 1997; Acuña-Castillo et al., 2007; Liu et al., 2008a
PIPs (+)	Current	N.D.	Zhao et al., 2007a,b
Propofol (+)	Current	100	Nakanishi et al., 2007
Ketamine (+)	Current	3 mM	Nakanishi et al., 2007
GW791343 (+) ^a	Ethidium	10	Michel et al., 2008
Calmodulin (+)	Current		Roger et al., 2008
LPC (+)	Ca ²⁺		Takenouchi et al., 2007
Polymyxin B (+)	Ca ²⁺	0.5 μg/ml	Ferrari et al., 2006

(+), positive modulator; (–), negative modulator; oATP, oxidized ATP; PIPs, phosphoinositides; N.D., not determined.

^a Positive modulator at rat and negative modulator at human P2X7R (Michel et al., 2008).

icked in HEK293 cells cotransfected with both proteins (Schwarz et al., 2009). The same group also showed that the P2X7R-Arg¹²⁵ residue is the target for ADP-ribosylation and that this chemical modification promotes channel gating (Adriouch et al., 2008; Schwarz et al., 2009). It has also been suggested that this mechanism could be relevant in regulating the functions of T-regulatory cells and in treatments of some tumors (Hubert et al., 2010).

As with rP2X4R, the rP2X7R also shows a resistance to the generally used P2 antagonist suramin (Surprenant et al., 1996; Bianchi et al., 1999; Duan et al., 2003). NF279, a suramin analog, inhibits hP2X7R with an IC₅₀ of ~3 μM, but concentrations above 100 μM are required to inhibit rP2X7R (Klapperstück et al., 2000; Donnelly-Roberts et al., 2009a). PPADS antagonizes the currents and calcium signals mediated by mammalian receptors with variable potencies ranging from 1 to 60 μM (Chessell et al., 1998; Bianchi et al., 1999; Gunosewoyo et al., 2007; Guile et al., 2009). MRS2159, a potent inhibitor of rP2X1Rs, also inhibits mouse, rat, and human P2X7Rs (Donnelly-Roberts et al., 2009a). Oxidized ATP is an irreversible antagonist that requires several hours of preincubation and concentrations of 100 to 300 μM to be effective (Murgia et al., 1993; Surprenant et al., 1996; Michel et al., 2000; Hibell et al., 2001). BBG also inhibits

rat and human P2X7Rs with IC₅₀ values of approximately 15 and 250 nM, respectively (Jiang et al., 2000a; Jacobson et al., 2002). Several blockers of calcium/CaM-dependent protein kinase II, including calmidazolium and 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), also inhibit the BzATP-induced hP2X7R current. This effect is probably kinase-independent, because the KN-04 compound is inactive toward kinases but inhibits P2X7R current (Gargett and Wiley, 1997; Chessell et al., 1998; Humphreys et al., 1998; Hibell et al., 2001; Jacobson et al., 2004).

More recently, several new compounds have been introduced as potent P2X7R inhibitors. N-(1-((cyanoimino)(5-quinolinylamino) methyl) amino)-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide (A740003) is a selective and competitive antagonist of P2X7R-mediated calcium influx with IC₅₀ values of 18 and 40 nM for the rat and human receptor and submicromolar potency at mP2X7R (Honore et al., 2006; Donnelly-Roberts et al., 2009a). The same group also introduced 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride (A438079). This compound inhibits mouse, rat, and human P2X7Rs at submicromolar concentrations (Nelson et al., 2006; Donnelly-Roberts et al., 2009a). A novel series of cyanoguanidine-piperazine P2X7R antagonists was designed based upon the structure of A740003 that focused on the piperazine

moiety and the right-hand side substitution (Morytko et al., 2008). The compound 2-cyano-1-[(1*S*)-1-phenylethyl]-3-quinolin-5-ylguanidine (A804598) inhibits mouse, rat, and human P2X7Rs at low nanomolar concentrations (IC_{50} = 9–11 nM) (Donnelly-Roberts et al., 2009b).

Figure 4 shows that *N*-[2-[[2-(2-hydroxyethyl)amino]ethyl]amino]-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide dihydrochloride (AZ10606120) also inhibits BzATP-induced currents at submicromolar concentrations (Yan et al., 2010). It has been suggested that AZ10606120 binds at separate, interacting sites on the P2X7R, which could suggest the allosteric nature of its action (Michel et al., 2007). *N*²-(3,4-difluorophenyl)-*N*¹-((2-methyl-5-(1-piperazinylmethyl)phenyl)glycinamide dihydrochloride (GW791343) acts as a positive allosteric regulator of rP2X7R (Michel et al., 2008a). In contrast, 3-[1-[(3'-nitro[1,1'-biphenyl]-4-yl)oxy]methyl]-3-(4-pyridinyl)propyl]-2,4-thiazolidinedione (AZ11645373) is a highly selective and potent (IC_{50} 5–10 nM) allosteric antagonist at the human but not rat receptor (Stokes et al., 2006).

Initial coimmunoprecipitation assay suggested that the rP2X7R does not form heteromeric assemblies (Torres et al., 1999a). This conclusion was questioned by others, who suggested formation of functional P2X4/X7 heterotrimeric receptors (Guo et al., 2007). However, another group indicated that either heteromerization between P2X4R and P2X7 subunits results not in stable heteromeric complexes or P2X4/X7 heteromers do not represent a dominant subtype in native tissues (Nicke, 2008). In immune cells, expressing P2X4R and P2X7R, the preferred assembly pathway for both receptors is also the formation of homotrimers. Furthermore, the authors suggested that an interaction could occur between rather than within receptor complexes (Boumeche et al., 2009).

B. Ligand Binding Domain

1. Identification of Residues Contributing to ATP Binding. The ectodomain contains ~280 amino acids, the majority of which are conserved among the receptor subtypes, but does not contain the consensus sequences for agonist binding present in other ATP-sensitive proteins (Evans, 2009). In an attempt to identify the candidate residues responsible for binding sites in this domain, the secondary structure similarities between rP2X4R and class II aminoacyl-tRNA synthases were investigated (Freist et al., 1998). This study revealed that the receptor function was practically lost in the K190A, R278A, and D280A mutants. However, the model of the ATP binding site that emerged from this study also included nonconserved residues, arguing against a common sequence accounting for the ligand binding (Yan et al., 2005).

Experiments with variable chimeric receptors have provided useful information about the positions of the ATP binding site on P2XRs. The P2X2/X3R chimeras containing the Val⁶⁰-Phe³⁰¹ sequence of P2X3R instead

of the Ile⁶⁶-Tyr³¹⁰ sequence of P2X2R not only preserved ATP binding but also developed two intrinsic functions of P2X3R: sensitivity to $\alpha\beta$ -meATP and ecto-ATPase-dependent recovery from endogenous desensitization but not the rate of receptor desensitization (Koshimizu et al., 2002; Zemkova et al., 2004). The corresponding P2X2/X4R chimera showed the gain of function, as indicated by increased sensitivity to ligands and faster desensitization compared with both parental receptors (He et al., 2003c). The P2X2/X7R chimera exhibited a sensitivity to ATP that fell between the sensitivities observed in cells expressing the parental receptors (He et al., 2002). These experiments indicated that the Lys⁶⁷-Lys³¹³ ectodomain sequence not only contains an ATP binding domain but also accounts in part for the receptor agonist specificity of these sites (Stojilkovic et al., 2005).

The contribution of the majority of the conserved amino acids in this region has been studied using alanine scanning mutagenesis. The most systematic investigation in this field was performed by the Evans laboratory using the hP2X1R as a receptor model in which over 120 ectodomain amino acids were replaced with other residues. At first, they analyzed the relevance of positively charged ectodomain residues in ATP recognition (Ennion et al., 2000). Later, they also studied the relevance of aromatic (Roberts and Evans, 2004), structural (Ennion and Evans, 2002; Digby et al., 2005; Roberts and Evans, 2005), polar (Roberts and Evans, 2006), and negatively charged ectodomain residues (Ennion et al., 2001). The main conclusion reached in these experiments was that the majority of individual alanine substitutions have little or no effects on ATP potency at the receptor. They identified Lys⁶⁷, Lys⁶⁹, Arg²⁹⁵, and Lys³¹³ (P2X4R numbering) as potential residues involved in coordinating the negatively charged phosphate group of ATP and that the adenine ring may be sandwiched between Phe¹⁸⁵/Thr¹⁸⁶ and Asn²⁹³/Phe²⁹⁴/Arg²⁹⁵ residues (Roberts and Evans, 2006). Alanine and arginine substitutions of the positively charged ectodomain residues in rP2X2R revealed that mutations at Lys⁶⁷ (P2X4R numbering) were least tolerant, followed by Lys³¹³, Arg²⁹⁵, Arg³⁰⁹, and Lys⁶⁹ (Jiang et al., 2000b). For rP2X4R, the order was Lys⁶⁷, Lys³¹³, Arg²⁹⁵, Lys¹⁹⁰, and Phe¹⁸⁵ (Zemkova et al., 2007). Replacement of Lys⁶⁹ with alanine also affected the receptor function (Roberts et al., 2008). The relevance of Lys⁶⁷, Lys⁶⁹, Arg²⁹⁵, and Lys³¹³ (P2X4R numbering) with regard to agonist potency was also identified in hP2X3R (Fischer et al., 2007).

The breakthrough in understanding the structure of the ATP binding site came with the finding that it is localized between two subunits. The first evidence supporting this view was provided by Wilkinson et al. (2006) using the wild-type and K67A and K313A mutants (P2X4R numbering). The work of Marquez-Klaka et al. (2007) further supported the hypothesis that the interface between the subunits is important for the formation

of the ATP binding site. Specifically, this group showed that a disulfide bond could form between K67C and F294C from two adjacent subunits.

The pharmacology of the P2XRs discussed above provides additional insights into the structure of P2XRs. For example, the lack of ADP effects at P2XRs indicates that the coordination/detection of the three phosphate residues, but not the adenine ring or ribose, plays a dominant role in determining the affinity of drugs for the ATP binding site. Consistent with this view, longer chain phosphates, such as adenosine tetraphosphate and the diadenosine polyphosphates, also act as agonists (Lewis et al., 2000). The Lys¹³⁸ residue of hP2X1R seems to account for the high potency of suramin and its analog NF449 at this receptor compared with rP2X1R (Sim et al., 2008). The exchange of one Lys²⁴⁶ to the equivalent position in the P2X2R (Glu²⁴⁹) transfers the PPADS sensitivity (Buell et al., 1996). In hP2X7R, Phe⁹⁵ is required for the high sensitivity to the allosteric modulators GW791343 and SB203580 (Michel et al., 2009). The ADP-ribosylation of Arg¹²⁵ is sufficient to activate this receptor (Adriouch et al., 2008).

Together, these extensive investigations suggest that P2XRs share a similar agonist binding site with some variations in the binding pocket or gating, which account for receptor specificity in agonist and antagonist binding/receptor gating. Eight residues have the potential to contribute to the formation of the ATP binding site: Lys⁶⁷, Lys⁶⁹, Phe¹⁸⁵, Thr¹⁸⁶, Asn²⁹³, Phe²⁹⁴, Arg²⁹⁵, and Lys³¹³ (P2X4R numbering). All of these residues are also present in distantly related P2XRs (algae, slime mold amoebae, and choanoflagellates) (Surprenant and North, 2009). Positively charged lysines seem to coordinate the binding of the negatively charged phosphate tail of ATP, whereas aromatic phenylalanine residues could coordinate the binding of the ATP adenine ring directly or indirectly.

2. Number of Occupied Binding Sites Necessary to Channel Activation. An interesting question about P2XRs is how many molecules of ATP are necessary to open a channel. Early studies of ATP-evoked currents in rat and bullfrog neurons suggested that three molecules of ATP were required for channel gating. This was based on the superlinearity of currents versus agonist concentration observed at low ATP concentrations (Bean et al., 1990). Single-channel experiments also suggested that the binding of three ATP molecules was required to activate the P2X2R (Ding and Sachs, 1999). However, other reports have suggested that the binding of two (Friel and Bean, 1988; Friel, 1988) or even one molecule of ATP (Krishtal et al., 1983) is sufficient to activate channels.

After the cloning of P2XRs, a great effort was made to determine the stoichiometry of the functional channel. Diverse experimental approaches suggested that the channel was composed of three P2X subunits (Nicke et al., 1998; Stoop et al., 1999; Jiang et al., 2003; Barrera et

al., 2005, 2007; Young et al., 2008). The crystallization of the zP2X4.1R confirmed that the channel is composed of three subunits (Kawate et al., 2009), suggesting that it should contain three binding sites for ATP. Moreover, as discussed in the previous section, ATP binding sites are located at the interface of neighboring subunits, each contributing residues that are necessary for adenine and phosphate interactions (Marquez-Klaka et al., 2007; Roberts and Evans, 2007).

However, the heteromeric P2X2/3R requires only two functional binding sites. This has been derived from separate studies in which the Hill slope at low ATP concentrations indicates the binding of two ATP molecules (Jiang et al., 2003). In addition, the mutation of ATP-binding residues on the P2X2 subunit results in functional channels when they are coexpressed with P2X3 subunits (Wilkinson et al., 2006), suggesting that the disruption of only one binding site is not sufficient to shut down channel function (P2X2/3Rs are composed from two P2X3 and one P2X2 subunits). The same group also showed that mutations at Lys⁶⁸ or Lys³⁰⁸ (P2X2R numbering) resulted in nonfunctional receptors. Surprisingly, when these two mutants were coexpressed, small but consistent ATP-mediated currents were observed, suggesting that channels containing subunits with different mutations can form at least one intersubunit binding site, and these channels account for the small currents (Wilkinson et al., 2006). We have characterized the gating of P2X7R, combining experimental data with a mathematical model that could explain the different activation states of this channel with the occupancy of two or three ATP binding sites (Yan et al., 2010).

3. Orthosteric Sites in the Context of the P2X4.1 Structure. Although the reported crystal structure of zP2X4.1R was solved in the absence of ATP, it revealed a potential location for the ligand binding site (Kawate et al., 2009; Browne et al., 2010; Evans, 2010; Stojilkovic et al., 2010b; Young, 2010). This putative ATP binding site, shaped like an open jaw, is ~42 Å from the extracellular limit of the plasma membrane on the interface between adjacent subunits. To discuss the location and the structure of this putative ligand binding site, we have generated a homology model of rat P2X4R with docked ATP molecule (Fig. 5). Homology models based on the crystal structure of zP2X4.1R suggest the position of three ATP binding sites embedded within the interfaces of the trimer (Browne et al., 2010; Stojilkovic et al., 2010b; Young, 2010). The bottom of the predicted ATP binding site is formed by the upper part of the body domain of two neighboring subunits, whereas the head domain and the left flipper of one subunit and the dorsal fin of the other create its walls. Molecular docking suggested that ATP binds with its adenine ring and ribose moiety positioned at the bottom of the ligand binding pocket, making several van der Waals and polar contacts with both the main chain and side-chain atoms of the body and head domains, whereas the phosphate groups are ori-

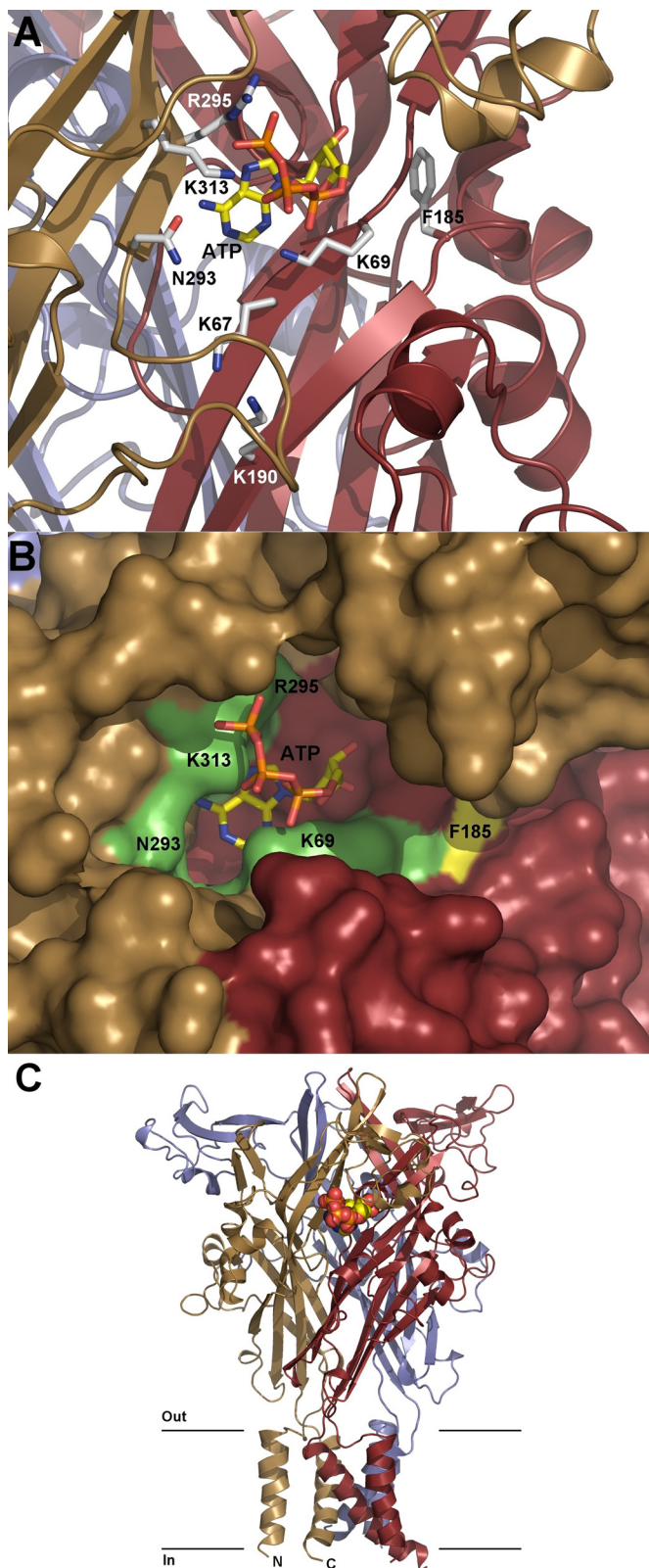


FIG. 5. Homology model of rat P2X4R with docked ATP. A, predicted structure of the ATP binding site. Residues suggested to be involved in ATP binding or located in the close vicinity of putative ATP-binding site are shown as sticks. Each subunit is shown in a different color. B, surface representation of the ATP binding pocket with a docked molecule of ATP. C, homology model of homotrimeric rat P2X4 viewed parallel to the membrane. Each subunit is depicted in a different color. The ATP molecule is shown as a Corey-Pauling-Koltun model. The black lines suggest the boundaries of the outer (out) and inner (in) layers of the plasma

ented toward the surface of the receptor and surrounded by basic residues, including Lys⁶⁹, Arg²⁹⁵, and Lys³¹³ (in rat P2X4R numbering). The predicted ligand binding cleft also includes residues Lys⁶⁷, Asn²⁹³, and Phe²⁹⁴ in good agreement with the site-directed mutagenesis experiments (Zemkova et al., 2007). Residues Phe¹⁸⁵, Thr¹⁸⁶, and Lys¹⁹⁰ are located in close vicinity of the binding site. It is likely that upon the ATP-induced conformational change, these residues might be an integral part of the ligand-binding site.

It has been speculated, based on the crystal structure of zP2X4.1R, that ATP binding induces movement of the head, right flipper, and dorsal fin domains, resulting in a conformational change within and between subunits and channel opening (Kawate et al., 2009). The bottom of the predicted ATP binding site is in the upper part of the rigid β -sandwich motif, where the residues Lys⁶⁷, Lys⁶⁹, Asn²⁹³, Arg²⁹⁵, and Lys³¹³ are located. This transthyretin-like β -sandwich motif forms the skeleton of the extracellular body domain and seems to be very rigid because of an extensive number of hydrogen bonds between β -strands (Blake et al., 1978; Kawate et al., 2009). Therefore, it is reasonable to speculate that this rigid β -structure transmits the ATP-induced conformational change from the ligand-binding site to the base of the extracellular domain, resulting in the repositioning of the connected TM helices and channel opening.

The structure of zP2X4.1R does not provide a rationale for the differences in ligand sensitivity between receptors (e.g., the 200-fold difference in ATP potency at P2X1R and P2X7R, the selectivity of $\alpha\beta$ -meATP for P2X1R and P2X3R, or the mechanism of action of the receptor-specific antagonists). However, mutagenesis studies have revealed receptor-specific effects of the replacement of some conserved residues. Mutations in Lys¹⁸⁸, Asp²⁵⁹, and Arg³⁰⁴ residues decreased the potency of ATP for rP2X2R more than 300-fold (Jiang et al., 2000b) but did not significantly affect hP2X1R function (Ennion et al., 2000, 2001). Likewise, replacement of Lys¹⁹⁰ and Phe²³⁰ with alanines generated practically nonresponsive rP2X4R receptors, whereas the function of equivalent mutants in hP2X1R was only slightly affected (Ennion et al., 2000; Roberts and Evans, 2004). Mutation of mP2X7-Arg²⁰⁶ resulted in increased agonist potency, but this mutation was ineffective at hP2X1R (Ennion et al., 2000). These differences could contribute to the heterogeneity of the pharmacological properties of

membrane. The homology model of rat P2X4R (sequence Arg³³-Val³⁵⁵) was generated using the Modeler 9v7 package (Sali and Blundell, 1993) and the crystal structure of zP2X4.1R (Kawate et al., 2009) as a template. Missing side chains were built using the DeepView/Swiss-PdbViewer v4.0.1 program (Guex, 1997). Bad contacts were corrected manually and the model was energy-minimized using the DeepView/Swiss-PdbViewer with the GROMOS96 43B1 parameter set. The AutoDock v4.2 (Morris et al., 2009) was used to predict the position and the conformation of ATP within the putative ligand-binding site at the interface between two neighboring subunits. The figure was generated using PyMol v0.99 (<http://www.pymol.org>).

these receptor subtypes. In the absence of a P2XR crystal structure with bound ATP; however, this evidence should be considered circumstantial and does not prove the precise localization of the ATP binding site or the specificity of these sites for various agonist analogs.

IV. Allosteric Binding Sites and Receptor Function

Allosteric modulators can change the affinity of receptors for native agonists and/or affect agonist efficacy in different ways. Allosteric antagonists may reduce agonist affinity without affecting agonist efficacy. Such a pattern of action resembles the effect of competitive antagonists at orthosteric binding sites, but in a limited way. The allosteric antagonistic effect is always saturable, because the allosteric effect will reach a maximum when all allosteric binding sites are occupied. In contrast, orthosteric ligands can have almost infinite competitive effects as long as the concentrations of the competitive ligands are changed in the correct manner. Allosteric agents may reduce both affinity and efficacy, producing a mixture of competitive/noncompetitive effects, or may act by reducing efficacy only, producing noncompetitive effects. Other combinations are also possible, such as increasing the affinity of an agonist and inhibiting its efficacy, potentiating the endogenous response through an increase in the receptor's affinity, or efficacy for its agonist (Kenakin and Miller, 2010). In this review, we talk about allosteric-induced changes in the potency and efficacy of ATP for particular receptor subtypes.

The majority of allosteric regulation of P2XRs occurs at extracellular allosteric binding sites. This includes binding sites for essential trace metals and other micro and macro metals, as well as binding sites for protons. Allosteric binding sites around the TM region include sites for IVM and alcohols. Regulation of P2XR function by calcium binding proteins, protein kinases, lipids, and reactive oxygen species also occurs through allosteric modulatory sites located at the N and C termini, whereas the sites affected by carbon monoxide, steroids, propofol, ketamine, and toluene, if they exist, have not been identified.

A. Metals as Allosteric Modulators

Numerous metals have profound effects on P2XR function, acting as potent allosteric modulators (Huidobro-Toro et al., 2008). The effects of essential trace metals, heavy metals, trivalent cations, and macro metals on P2XR functions are summarized in Tables 1 to 6 and are discussed in detail in the following sections.

1. Essential Trace Metals. Nine metals (chromium, cobalt, copper, iron, manganese, molybdenum, nickel, selenium, and zinc) are now included in the group of essential trace elements. Some other metals, such as vanadium, may also be nutritionally important, but

their essentiality has not been fully established. These elements occur in very small amounts (usually less than 1–10 parts per million) as constituents of living organisms. Their deficiency induces specific biochemical changes causing abnormalities in function, and these abnormalities can be corrected by supplementation with the elements (Reilly, 2004). Among the trace metals, zinc, copper, and iron have particular relevance (Mathie et al., 2006; Madsen and Gitlin, 2007). Zinc and copper are transported intracellularly and stored in synaptic vesicles together with transmitters through variable transporters (Kuo et al., 2001; Palmiter and Huang, 2004). After release to the synaptic cleft, these metals can reach concentrations as high as 250 to 300 μM (Assaf and Chung, 1984; Kardos et al., 1989). The released trace metals can bind to numerous receptors and channels, including voltage-gated sodium, calcium, or potassium channels, store-operated Ca^{2+} channels, transient receptor potential channels and ligand-gated channels, where they act as allosteric modulators (Mathie et al., 2006). Trace metals are also allosteric modulators of P2XRs.

a. P2X1, P2X3, and P2X5 receptors. The function of rP2X1R is inhibited by zinc in a concentration-dependent manner without affecting E_{max} values, whereas rP2X3R function is potentiated by this metal, causing a leftward shift in the concentration-response to ATP application with a maximal effect at 10 μM . Higher zinc concentrations have been shown to reduce P2X3R current, suggesting the possible existence of two allosteric sites at this receptor, a high-affinity site with a potentiating effect and a low-affinity site with an inhibitory role (Wildman et al., 1999b). The rP2X5R is also modulated in a biphasic fashion by zinc, again suggesting the existence of at least two allosteric sites for this metal (Wildman et al., 2002). There is no information about the amino acid residues involved in the binding of trace metals at these receptors. In addition, effects of other trace metals on the function of these receptors have not been reported.

b. P2X2 receptor. Zinc and copper both exhibit a biphasic effect on rP2X2R function: the ATP-induced responses are significantly potentiated at 10 to 100 μM and inhibition at millimolar concentrations (Xiong et al., 1999; Clyne et al., 2002a; He et al., 2003b; Lorca et al., 2005). Zinc also potentiates the ATP responses of native rP2X2Rs and rP2X2/3Rs expressed in neurons from parasympathetic ganglia (Ma et al., 2005), as well as in neurons from the rat hypothalamus (Vorobjev et al., 2003) and dorsal motor nucleus (Ueno et al., 2001). Cobalt and nickel also potentiate the activity of rP2X2R, but the potential inhibitory effect at high concentrations was not tested (Lorca et al., 2005). Site-directed mutagenesis revealed the critical role of extracellular His¹²⁰ and His²¹³ in the positive modulation by zinc and copper (Clyne et al., 2002a; Lorca et al., 2005). His¹⁹², His²⁴⁵, and His³¹⁹ are also partially involved, probably

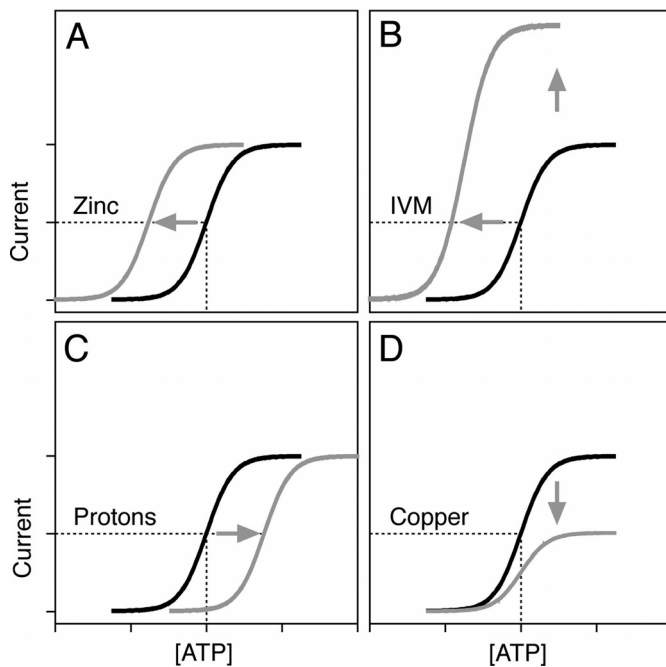


FIG. 6. Patterns of rP2X₄R allosteric modulations. A and C, zinc increases and protons decrease the sensitivity of receptors for agonist without affecting E_{\max} . B, IVM treatment causes both a leftward shift in the sensitivity of receptors for agonist and an increase in the E_{\max} value. D, copper decreases the E_{\max} without affecting the sensitivity of receptors for agonist.

contributing to the stabilization of the allosteric site (Lorca et al., 2005). Nagaya et al. (2005), using concatamers, showed that the allosteric sites for zinc and probably copper are formed by His¹²⁰ and His²¹³ of adjacent subunits. These results have been recently confirmed with the crystallization of P2X₄.1R (Kawate et al., 2009). The flexibility of the zinc binding site of rP2X₂R was also tested by shifting two essential histidines 13 residues upstream or downstream from their original position. The ability of zinc to potentiate the mutated channels followed the order His¹²⁰ > His¹²¹ > His¹¹⁹ and His²¹² > His²¹³ > His²¹¹ (Tittle et al., 2007). In contrast, it has been difficult to identify the residues that compose the zinc/copper inhibitory site (Clyne et al., 2002a; Friday and Hume, 2008). Tittle and Hume (2008) also showed that, unlike rat and mouse P2X₂Rs, hP2X₂R is inhibited by zinc and copper, revealing the species-specific modes of metal modulation. This group also found that none of the nine histidines in the extracellular domain of hP2XR were required for zinc inhibition, suggesting that the replacement of His²¹³ with arginine in human receptors at least partially accounts for the change in the receptor behavior. Cobalt and nickel seem to act at the same allosteric site, as deduced from experiments using mutant receptors (Lorca et al., 2005).

c. P2X₄ receptor. The unique characteristic of P2X₄R is its differential modulation by trace metals. Zinc and cobalt potentiate receptor activity and copper inhibits it. Figure 6 shows that zinc potentiation is due to an in-

crease in ATP potency at P2X₄R, whereas copper affects the E_{\max} value without affecting the potency of the agonist. At higher concentrations (30 μ M–1 mM), zinc inhibits P2X₄R function via a reduction in ATP efficacy (Wildman et al., 1999b; Acuña-Castillo et al., 2000). Residues that are commonly present in copper binding sites include histidines, aspartic acids, glutamic acids, and cysteines, although other residues could also contribute (Aitken, 1999). Among the three ectodomain histidines, only the mutation of His¹⁴⁰ abolished the inhibitory action of copper and modified the pattern of zinc action from a bell-shaped curve, typical of biphasic modulation, to a sigmoid curve, accompanied by an increase in the amplitude of response (Coddou et al., 2003). Subsequent studies focused on the Thr¹²³-Thr¹⁴⁶ ectodomain sequence in a search for other residues that contribute to the regulatory actions of copper and zinc. These experiments revealed that Asp¹³⁸ is a second component of the P2X₄R inhibitory site, whereas Cys¹³² is suggested to account for the positive zinc allosteric effect. These experiments also ruled out the involvement of Thr¹²³, Ser¹²⁴, Asp¹²⁹, Asp¹³¹, Thr¹³³, and Thr¹⁴⁶ residues in the allosteric actions of copper and zinc (Coddou et al., 2007). Cobalt also potentiates P2X₄R activity, but this action is long lasting, as indicated by the lack of recovery during a 45-min washout period (Coddou et al., 2005).

To visualize the location of these three residues, we built the homology model of rat P2X₄R (sequence Arg³³-Val³⁵⁵) using the Modeler 9v7 package (Sali and Blundell, 1993) and the crystal structure of zP2X₄.1R (Kawate et al., 2009) as a template. Figure 7 shows that the His¹⁴⁰ residue is located close to the proposed ATP binding site that includes Asn²⁹³, Arg²⁹⁵, and Lys³¹³

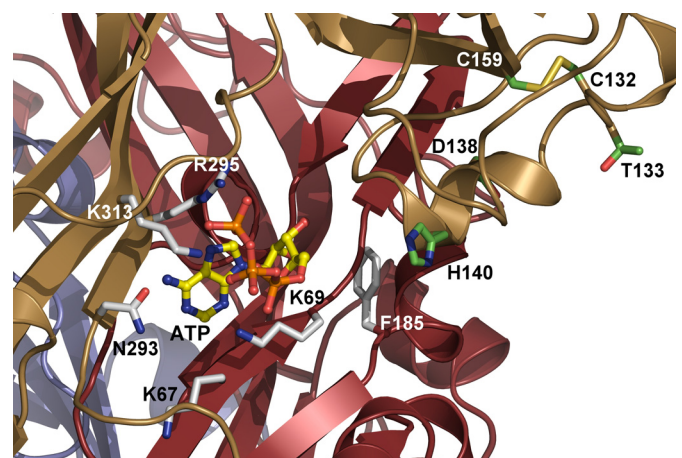


FIG. 7. Residues of the rat P2X₄R potentially involved in the modulatory effects of copper and zinc cations (Wildman et al., 1999; Acuña-Castillo et al., 2000; Coddou et al., 2003, 2007). Structural model of rat P2X₄R shows the location of residues Asp¹³⁸, His¹⁴⁰, Thr¹³³, Cys¹³², and Cys¹⁵⁹ (shown as sticks) with respect to the predicted ATP-binding site. All these residues are present within the head domain of one subunit, which creates one wall of the predicted ATP-binding pocket and is probably involved in the ATP-induced conformational change. The figure was generated using PyMol v0.99 (<http://www.pymol.org>).

from the same subunit and Lys⁶⁷, Lys⁶⁹, and Phe¹⁸⁵ from the adjacent subunit. Thus, it is reasonable to speculate that Asp¹³⁸ and His¹⁴⁰ from the same subunit represent the P2X4R-specific region accounting for the inhibitory effects of copper and high concentrations of zinc. The finding that the replacement of Cys¹³² abolishes the potentiating effect of zinc also raises the possibility that the SS3 bond is important for zinc potentiation or that this bond is not permanent but instead breaks and reforms.

The zinc-induced potentiation of P2X4R does not depend on the preapplication time, whereas copper-inhibition is time dependent, and the inhibition persists when the metal is washed out before the channel is opened by ATP (Acuña-Castillo et al., 2000). This suggests that the zinc binding site appears on the receptor in the open state, whereas copper binds to the receptor in the closed state. This provides a rationale for the easy prediction of which residues participate in copper binding based on the crystal structure of the closed receptor (Kawate et al., 2009).

d. P2X7 receptor. Our group examined effects of copper and zinc in *X. laevis* oocytes expressing rP2X7R (Acuña-Castillo et al., 2007). Copper noncompetitively inhibited the receptor-mediated current with an IC₅₀ of 4 μ M. Zinc also inhibited the ATP-gated current, but was 20-fold less potent. Other groups have also observed inhibition of rP2X7R currents when the receptor was expressed in HEK293 cells (Liu et al., 2008a). Differences in metal modulation across species have also been reported for this receptor. Unlike rP2X7R, copper, but not zinc, inhibited the mouse receptor (Moore and Mackenzie, 2008). Mutagenesis of six ectodomain histidines revealed that the H267A mutation resulted in a copper-resistant receptor, whereas the H201A and H130A mutants were less sensitive to copper than the wild-type receptor, and the H62A, H85A, and H219A mutants exhibited normal copper sensitivity. The zinc-induced inhibition of receptor function was reduced in H267A and H219A mutants (Acuña-Castillo et al., 2007). Other groups have reported some effects of replacing His²⁰¹ and His²⁶⁷ on the copper- and zinc-mediated inhibition of P2X7R currents, but they also detected a dramatic reduction or almost a complete loss of inhibition by the two metals in cells expressing the H62A and D197A double mutant (Liu et al., 2008a). Further studies should clarify whether the differences are due to the expression system (oocytes versus HEK293 cells), the agonist used (ATP versus BzATP), or some other factors.

e. Other P2X receptors. Native P2XRs expressed in bullfrog DRG neurons are also inhibited by zinc (Li et al., 1997). Five P2X-like genes are expressed in *D. discoideum*, and ATP-activated channels are expressed in the plasma membrane. The responses of these channels are inhibited by copper and zinc with IC₅₀ values of 0.9 and 6.3 μ M, respectively (Ludlow et al., 2008). A P2XR cloned from the parasitic platyhelminth *Schistosoma*

mansoni is permeable to fluorescent dyes and is inhibited by extracellular zinc with an IC₅₀ of 0.4 μ M (Raouf et al., 2005). Zinc and copper also inhibited the ATP-induced current in cells expressing a P2XR cloned from the tardigrade *Hypsibius dujardini* with IC₅₀ values of 63 and 20 μ M, respectively. In contrast to vertebrate P2XRs, none of the ectodomain histidines plays a major role in coordinating metal binding at this receptor (Bavan et al., 2009).

f. Summary. A common aspect of P2XRs is their regulation by essential trace metals. The nature of trace metal actions (positive or negative) depends on the subtype of receptor and species differences. This indicates that relatively small changes in the structure of receptors are sufficient to change the direction of modulatory action of trace metals. Furthermore, in contrast to the ATP binding sites, the amino acids involved in P2XR trace metal coordination do not seem to be aligned among P2X subunits. This may provide additional rationale for the opposing effects of allosteric regulators, such as zinc and copper, on receptor function. Two distinct and separate binding sites seem to exist for trace metals, and it seems that both sites can be occupied by different elements with variable affinities. In P2X4R, these sites provide opposite receptor modulation, positive for zinc and negative for copper. The three-dimensional structure of the P2XRs indicates that coordination sites of trace metals at the P2X4R are spatially close to the ATP binding site, allowing us to propose that these metals modify the ligand binding. For P2X2R, the ATP binding site and the zinc allosteric sites are not only close by but are also formed at the interface between neighboring subunits.

2. Heavy Metals. The term heavy metal refers to any metallic element that has a relatively high density and is toxic or poisonous at low concentrations. Examples of heavy metals include cadmium, lead, mercury, and thallium, as well as lighter metals, such as arsenic and chromium. These metals affect the function of numerous voltage- and ligand-gated channels and ionic pumps (Kiss and Osipenko, 1994). Mercury acts as a positive modulator of P2X2R. It causes a leftward shift in the potency of ATP and does not change the E_{\max} value (Lorca et al., 2005). In contrast, mercury acts as a negative modulator of P2X4R (Coddou et al., 2005). In both cases, the actions of mercury resemble the actions of copper, suggesting that these metals may bind to the same allosteric site. However, this hypothesis was discarded when it was reported that the copper-resistant mutants were still modulated by mercury (Coddou et al., 2005; Lorca et al., 2005). Using chimeric P2X2/X4Rs, we recently found that the primary site of action for mercury is an intracellular cysteine at position 430 (Coddou et al., 2009). The residual action of mercury could be mediated by still unidentified ectodomain binding sites. Cadmium potentiates P2X2R and P2X4R (Coddou et al., 2005; Lorca et al., 2005) but inhibits P2X1R, P2X3R, and

P2X7R (Nakazawa and Ohno, 1997; Virginio et al., 1997). This metal seems to bind to the allosteric sites identified for zinc in the P2X2R and P2X4R. This was inferred from competition studies using zinc and cadmium and from experiments with zinc-resistant mutants (Coddou et al., 2005; Lorca et al., 2005). Other metals, including lead, barium, palladium, silver, platinum, and gallium, had no effect on the ATP-evoked currents in P2X2R- and P2X4R-expressing cells, indicating that the allosteric sites have strict structural requirements (Coddou et al., 2005; Lorca et al., 2005).

3. Lanthanides. These rare earth elements are commonly known for their ability to inhibit ion permeation by blocking the pore of various ion channels and causing toxicity (Kiss and Osipenko, 1994). These trivalent cations also inhibit P2XRs. Lanthanum, cerium, neodymium, and gadolinium inhibit P2X1R and P2X2R expressed in PC12 cells and *X. laevis* oocytes at 3 to 300 μ M. This inhibition is due, at least in part, to allosteric mechanisms (Nakazawa et al., 1997). Gadolinium also inhibits *D. discoideum* P2XRs (Ludlow et al., 2008). The existence of allosteric binding sites for gadolinium was recently confirmed with the crystallization of zP2X4.1R. Using a gadolinium derivative, three sites located in the periphery of each subunit were identified, each site composed of Asp¹⁸⁴ and Asn¹⁸⁷ residues. The fourth site is formed by the Glu⁹⁸ residues of each channel subunit (Kawate et al., 2009). At zP2X4.1R, Asn¹⁸⁷ is adjacent to Phe¹⁸⁸ and Thr¹⁸⁹, two residues that were predicted to participate in the formation of the ATP binding pocket, suggesting that gadolinium binding reduces ATP affinity. At rP2X4R, an Asn residue is also present, but the effects of gadolinium on the function of this receptor have not been tested. The use of 1 mM gadolinium during receptor crystallization may explain the lack of a solved structure for an ATP-bound form. Other groups have shown that gadolinium increases recovery from desensitization in P2X3Rs (Cook et al., 1998).

4. Macro Metals. Calcium, potassium, sodium, and magnesium belong to macro elements, which are present in human tissues in 14, 2, 1.4, and 0.27 g/kg body weight, respectively (Reilly, 2004). There is no information about the potential role of potassium in the regulation of P2XR function, and very limited information exists supporting a role for sodium in P2XR gating. Early evidence for the role of sodium comes from experiments in human lymphocytes showing that the ATP-evoked calcium influx is strongly inhibited by this cation (Wiley et al., 1992). A possible role of extracellular sodium in the regulation of P2X7R permeability to large molecules has been proposed by two groups (Jiang et al., 2005a; Li et al., 2005). In another study, it was demonstrated that sodium could regulate the motility of airway cilia by inhibiting native P2XRs, presumably P2X4R and/or P2X7R by binding to an extracellular allosteric site (Ma et al., 1999, 2006).

On the other hand, there is a large body of information concerning the effects of divalent calcium and magnesium ions on channel function. Calcium and magnesium inhibit recombinant P2XRs at millimolar concentrations (North, 2002). Native receptors are also sensitive to modulation by calcium (Nakazawa and Hess, 1993; Cook and McCleskey, 1997) and magnesium (Tomić et al., 1996; Miyoshi et al., 2010). It is well known that these cations make complexes with ATP, reducing the free acid form of ATP (or ATP⁴⁻) in solution. It has also been suggested that ATP⁴⁻ acts as an agonist for histamine secretion by mast cells (Dahlquist and Diamant, 1974). This conclusion was further supported by experiments on the concentration dependence of ATP-induced histamine secretion in cells bathed in different calcium and magnesium concentrations (Cockcroft and Gomperts, 1979). More recently, the effect of ATP on P2X7R currents was studied in the presence and absence of calcium, and the same conclusion was reached (Klapperstück et al., 2001). Although it has never been supported by direct evidence, this hypothesis has prevailed in the literature during the last decade. However, numerous data strongly support alternative hypotheses.

Surprenant et al. (1996) reported that extracellular calcium inhibits both the cationic current and dye uptake. The inhibitory effect of this cation on rP2X7R function was mimicked by other divalent cations in the following order: Cu²⁺ > Cd²⁺ ~ Zn²⁺ > Ni²⁺ >> Mg²⁺ ~ Co²⁺ > Mn²⁺ > Ca²⁺ = Ba²⁺ >> Sr²⁺. For each of these metals, the IC₅₀ values for current and dye uptake were similar. This inhibition was voltage-independent, a finding inconsistent with the hypothesis that divalent cations cause functional inhibition by blocking the channel pore. The same group also observed that the major inhibitory effect of divalent cations was observed in relatively low concentrations (1–3 mM), a pattern typical for allosteric modulation (Virginio et al., 1997). The rP2X7R-H130A mutant has been shown to be resistant to magnesium inhibition, reinforcing the hypothesis that this metal acts through an allosteric mechanism (Acuña-Castillo et al., 2007).

The inhibitory effects of calcium and magnesium were also observed in recombinant rP2X2Rs with single-channel recording. These effects manifest as a fast block, visible as a reduction in amplitude of the unitary currents (Ding and Sachs, 1999). In further work on this topic, this group showed that bath calcium concentrations also increase the rates of receptor desensitization, a finding inconsistent with ATP⁴⁻ acting as an agonist. Furthermore, they showed a similar phenomenon with magnesium, barium, and manganese, but the calcium-induced desensitization exhibited a higher affinity and cooperativity, also not consistent with divalent cations determining ATP⁴⁻ concentration as the sole mechanism of calcium modulation. The authors have not clarified whether this effect is due to a direct allosteric interaction of calcium with the receptor or occurs

through the activation of an accessory calcium-dependent protein (Ding and Sachs, 2000).

Other groups have also observed a strong inhibitory effect of bath calcium on rP2X2Rs with a half-maximal block of the current at about 5 mM, but not on hP2X1Rs (Evans et al., 1996). rP2X3R was more than 10-fold less sensitive to blockade by bath calcium (IC_{50} , 89 mM), whereas heteromeric rP2X2/3Rs were more sensitive (IC_{50} 15 mM). Thus, the rank of order for inhibition by calcium at these receptors is P2X2R > P2X2/3R > P2X3R > P2X1R (Virginio et al., 1998a). A stimulatory role of calcium in P2X3R recovery from desensitization has also been proposed (Cook et al., 1998). A distinct role of calcium and magnesium in the modulation of native P2X3Rs from cultured rat DRG neurons was also reported. Calcium positively modulated these channels, whereas magnesium had an inhibitory effect (Giniatullin et al., 2003). Single-channel recordings of P2X4R further revealed that magnesium reversibly decreases the amplitude of ATP-evoked single channel currents in a concentration-dependent manner that is independent of the membrane potential. In addition, they found that this cation shortens the mean open time without affecting the mean closed time. The authors concluded that magnesium inhibits the function of human P2X4R by means of an open-channel block via a binding site located at the exterior surface of the pore (Negulyaev and Markwardt, 2000).

Calcium may also regulate P2XR function by acting as an intracellular messenger. All P2XRs conduct calcium through their pores, but these channels also produce cell depolarization and promote calcium influx indirectly by activating voltage-gated calcium channels. Elevated intracellular calcium concentrations can trigger many cellular functions, including the activation and inhibition of adenylyl and guanylyl cyclases, leading to up- and down-regulation of protein kinase A and G, as well as the activation of some protein kinase C isoforms. Calcium also binds to numerous signaling proteins, such as CaM, which can alter cellular functions either directly or through CaM-dependent kinases. A recent report describes the role of intracellular calcium through CaM on P2X7R function. In cells expressing this receptor, CaM contributes to current facilitation and membrane blebbing. The proposed CaM-binding site corresponds to the Ile⁵⁴¹-Ser⁵⁵² sequence located in the C-terminal tail of P2X7R (Roger et al., 2008). Single-nucleotide polymorphisms of hP2X7R, specifically Q521H, also affect receptor sensitivity to extracellular calcium inhibition (Roger et al., 2010). Calcium-CaM-dependent protein kinase II potentiates the ATP response in primary sensory DRG neurons, not in an allosteric manner but by promoting trafficking of P2XRs (Xu and Huang, 2004). A recent report also described a novel interaction of the P2X2R with the neuronal calcium sensor VILIP1. The authors found that VILIP1 constitutively binds to P2X2Rs, altering channel properties such as ATP-sensitivity and

peak currents. This opens the field to study if such interactions occur in other P2XRs (Chaumont et al., 2008).

B. Protons as Allosteric Modulators

Extracellular and intracellular pH, determined by proton concentration, affects the gating properties of both voltage- and ligand-gated ion channels, including acid-sensing ion channels and the transient receptor potential vanilloid receptor channels (Caterina et al., 1997; Tominaga et al., 1998; Wemmie et al., 2006). With the cloning of P2XRs, it became obvious that these receptors can also sense hydrogen ions in the bath medium and respond by modulating the ATP-induced currents in a receptor-specific manner.

For rP2X2R, the potency of all agonists, but not antagonists, is enhanced 5- to 10-fold by acidification without affecting the maximal agonist response (King et al., 1996, 1997; Stoop et al., 1997; Clyne et al., 2002a). The receptor-specific sensitivity to pH is also obvious in single-cell calcium measurements (He et al., 2003b). This effect was also seen in cells expressing heteromeric rP2X2/3Rs (Stoop et al., 1997), whereas rP2X1/2Rs exhibit unique pH properties (Brown et al., 2002). The agonist-induced response of P2X2/3R is also extremely sensitive to small changes in extracellular pH (approximately 7.1–7.2) (Li et al., 1996a,b). The pH range was narrowed for the proton enhancement of the ATP response at the heteromeric rP2X2/6Rs (King et al., 2000). The basic His³¹⁹ residue was identified as a putative pH sensor for rP2X2R (Clyne et al., 2002a). This residue is in a receptor region that has been suggested to operate as a linker region between the ligand binding domain and the channel pore in other P2XR subunits (Yan et al., 2006; Roberts and Evans, 2007).

All other homomeric P2XRs are inhibited by acidification. The ATP potency for rP2X1R was reduced 2-fold at pH 6.5 and 6-fold at pH 5.5 without altering the maximum current amplitude (Stoop et al., 1997; Wildman et al., 1999b). Effects of extracellular protons and zinc are additive for rP2X1R (Wildman et al., 1999b). Acidification also reduces the peak amplitude of the rP2X3R current at an EC_{50} concentration of agonists (Stoop et al., 1997), and it right-shifts the ATP concentration-response curve without altering E_{max} (Wildman et al., 1999). Another study performed with hP2X3R revealed a dual effect of acidification. This group found an inhibitory effect that shifts the concentration-response curves to the right and a stimulatory effect that increases the current peak amplitude and activation time constant and accelerates in the recovery from desensitization. The authors also identified the His²⁰⁶ residue as the responsible for these effects (Gerevich et al., 2007). It is interesting that only fully N-glycosylated hP2X3Rs recognize external protons (Wirkner et al., 2008). Acidification also decreased the potency of ATP for rP2X4R without a change in the maximum response (Stoop et al.,

1997; He et al., 2003b), as summarized in Fig. 6. Alkalization, on the other hand, enhanced the current amplitude (Clarke et al., 2000). Mutation of His²⁸⁶ removes the extracellular pH sensitivity of this receptor subtype (Clarke et al., 2000; Yan et al., 2005). This residue is located far from the putative ATP binding site, suggesting that protons affect gating rather than ATP binding.

Acidification also inhibits the function of P2X5R and P2X7R, but with a pattern of inhibition that differs from other members of this family. In rP2X5R, acidification reduced both the potency and efficacy of ATP (Wildman et al., 2002), whereas in rat and human P2X7R, it causes a reduction in the peak amplitude of current without altering the agonist sensitivity (Liu et al., 2009). The inhibitory effect of protons on hP2X7R was also observed in single-channel recordings (Flittiger et al., 2010). Changes in the EC₅₀ value of agonists caused by acidification of P2X7R could be masked by the complex gating properties of these receptors, including the generation of biphasic currents (Klapperstück et al., 2001; Yan et al., 2008) and the facilitation of responses during repetitive agonist application (Roger et al., 2008). Several residues seem able to contribute to the pH sensitivity of P2X7R, His¹³⁰ and Asp¹⁹⁷ playing major roles (Acuña-Castillo et al., 2007; Liu et al., 2009).

Native P2XRs are also sensitive to changes in pH. Acidic pH potentiates the ATP-gated currents in guinea pig cochlear outer hair cells (Kanjhan et al., 2003), mice DRG neurons (Light et al., 2008), and in neurons from different parasympathetic ganglia (Ma et al., 2005). The pH sensitivity of P2XRs seems to be conserved in evolution, in that *D. discoideum* P2XRs are also modified by acidification (Ludlow et al., 2008). These results are consistent with the hypothesis that acidic conditions in the synaptic cleft could modify purinergic transmission, which could be physiologically relevant in certain events, such as pain sensation during inflammation.

C. Ivermectin as the P2X4 Receptor-Specific Modulator

IVM, a semisynthetic derivative of the natural fermentation products of *Streptomyces avermitilis*, is a member of a class of lipophilic compounds known as avermectins. It is a relatively large molecule, spanning a distance of approximately 20 Å, as documented by nuclear magnetic resonance and X-ray crystallographic analysis (Springer et al., 1981; Hu et al., 1998). IVM is widely used in human and veterinary medicine as an antiparasitic drug, predominantly to treat river blindness caused by *Onchocerca volvulus* (Burkhart, 2000). The therapeutic effects of IVM are mediated by the action of this compound as an agonist and allosteric modulator of glutamate-gated chloride channels expressed in the nerves and muscles of the parasite, leading to muscle paralysis and starvation (Cully et al., 1994; Dent et al., 1997; Ikeda, 2003). IVM was also found to modulate other extracellular ligand-gated channels, including nicotinic and GABA receptors (Sigel

and Baur, 1987; Krůsek and Zemková, 1994; Krause et al., 1998; Shan et al., 2001).

IVM also acts as a reversible allosteric modulator at mammalian homomeric and heteromeric P2X4Rs, but not at homomeric P2X2Rs, P2X3Rs, or P2X7Rs (Khakh et al., 1999b). Extracellularly applied IVM has multiple effects on the ATP-induced current. It increases both the current amplitude in response to supramaximal agonist concentration and the sensitivity of receptors to agonists (Fig. 6), reduces the desensitization rate, and greatly prolongs the deactivation of current after ATP removal (Khakh et al., 1999b; Priel and Silberberg, 2004; Jelínková et al., 2006). Single-channel analysis showed that IVM increases the channel conductance for approximately 20% and the probability of channel opening and prolongs the mean channel open time (Priel and Silberberg, 2004). In addition to human and rat P2X4R, *S. mansoni* P2XR is also potentiated by IVM (Agboh et al., 2004). More recently, a P2XR from the tardigrade species *H. dujardini* was cloned, and the gating properties of this receptor were also affected by IVM treatment (Bavan et al., 2009). Unlike rP2X4R, IVM does not affect the kinetics of desensitization for *S. mansoni* and *H. dujardini* P2XR currents. IVM alone does not open any of these channels. It has also been suggested that potentiation of P2X4R by IVM reflects an increase in the number of cell surface receptors, resulting from a mechanism dependent on the endocytosis of this receptor (Toulmé et al., 2006), but further work is needed to clarify this hypothesis.

Initial experiments also indicated that IVM is effective when applied extracellularly but not intracellularly, suggesting that the ectodomain contains the binding site for this compound (Priel and Silberberg, 2004). Experiments with chimeric receptors revealed that the TM domains and nearby residues of P2X4R are important for the effects of IVM on channel deactivation (Jelínková et al., 2006). This conclusion was confirmed by Silberberg et al. (2007) using TM domain chimeras between P2X4R and P2X2R. Their data also showed that there is widespread rearrangement of the TMs during the opening of P2X4 receptors. These conclusions were based on experiments with chimeric channels and tryptophan-scanning mutagenesis. To evaluate IVM effects in such mutants and to identify residues that alter the effects of IVM application, the authors used the EC₅₀ concentrations of ATP, a saturating concentration of IVM, and between 2-fold and 5-fold increases in the amplitude of current in the presence of IVM. Their study revealed weakened effects of IVM at V28A, I39W, V43W, and V47W TM1 mutants as well as at G340W, G342W, L345W, and V348W TM2 mutants and enhanced effects of IVM at G29W, V35W, L37W, L40W, A41W, S341A, G347W, A349V, V351W, and C353A mutants. Helical net diagrams of the TM domains showed a random distribution of these residues, and none of these residues were P2X4R-specific (Silberberg et al., 2007).

In our hands, the IVM-sensitive residues were Gln³⁶, Leu⁴⁰, Val⁴³, Val⁴⁷, Trp⁵⁰, Asn³³⁸, Gly³⁴², Leu³⁴⁶, Ala³⁴⁹, and Ile³⁵⁶. Replacing these residues with cysteine and alanine attenuated the allosteric action of IVM, but none of these mutations alone accounted for all the effects of this compound on the receptor function (Jelínková et al., 2008; Jindrichova et al., 2009). The pattern of these predominantly nonpolar residues, which are also present in the IVM-sensitive *S. mansoni* P2X subunit, was consistent with the helical topology of both TM domains. Every third or fourth amino acid was affected by substitution (Fig. 8). It is unlikely that these residues directly contribute to IVM binding, but they could reflect a change in the IVM binding pocket. The most probable location of the IVM allosteric site is at the interface of the TM1 and TM2 domains, and its mechanism of action could involve the rotation of TM1 relative to TM2, leading to an increased potency of ATP (Silberberg et al., 2007).

The specificity of the allosteric actions of IVM was used to identify native P2X4Rs in rat pituitary lactotrophs (Zemkova et al., 2010), hepatocytes (Emmett et al., 2008), sensory neurons (De Roo et al., 2003), alveolar macrophages (Bowler et al., 2003), human lung mast cells (Wareham et al., 2009), porcine tracheal smooth muscle cells (Nagaoka et al., 2009), and rabbit airway ciliated cells (Ma et al., 2006). IVM-sensitive currents were also detected in numerous mouse cell types, includ-

ing macrophages (Sim et al., 2007), cortical neurons (Lalo et al., 2007), glial cells (Raouf et al., 2007), ventricular myocytes (Shen et al., 2006), submandibular gland ductal cells (Pochet et al., 2007), and Leydig cells (Antonio et al., 2009). IVM was also used to study the functional interactions of P2X4R with other members of this family of channels (Alqallaf et al., 2009; Casas-Pruneda et al., 2009). We have also frequently used the allosteric control of rP2X4R in structural-functional characterization of these receptors (Zemkova et al., 2007; Jelínková et al., 2008; Rokic et al., 2010).

D. Alcohols Influence P2X Receptor Gating

Alcohols can modulate the function of several voltage-gated and ligand-gated ionic channels, most likely through interactions with the TM domains or hydrophobic regions within the channel structure (Crews et al., 1996). Alcohols also affect the gating properties of both recombinant and native P2XRs. Experiments with recombinant rP2X4R expressed in *X. laevis* oocytes revealed a dose-dependent inhibitory effect of ethanol on receptor function, causing a 2-fold rightward shift in the sensitivity of this receptor to ATP without altering the E_{\max} . This effect is not dependent on membrane potential, and ethanol does not change the reversal potential of ATP-activated currents (Xiong et al., 2000). Ethanol also inhibits rP2X2R function but does so less effectively than rP2X4R (Davies et al., 2002). In the case of rP2X3R, ethanol potentiates the ATP-gated currents in a concentration-dependent manner and increases the maximal response to zinc, indicating that ethanol and zinc act at different sites (Davies et al., 2005a). In contrast, the function of hP2X3R was unaffected by 100 mM ethanol (Köles et al., 2000).

In the native P2XRs of amphibian DRG neurons, ethanol also inhibits P2XR function by shifting the agonist concentration-response curve to the right in a parallel manner, increasing the EC_{50} without affecting E_{\max} . To distinguish whether this inhibition involves competitive antagonism or a decrease in the affinity of the agonist binding site, the authors also studied the kinetics of activation and deactivation of the P2XR current. These studies revealed that ethanol decreases the time-constant of P2XR deactivation without affecting the time-constant of activation, indicating that ethanol acts as an allosteric regulator, causing a decrease in the affinity of receptors for ATP. They also compared the potency of different alcohols for inhibiting ATP-activated current. The following rank order was reported: 1-propanol = trifluoroethanol > monochloroethanol > ethanol > methanol (Li et al., 1993, 1998; Weight et al., 1999). The effect of ethanol was not specific for this type of neuron but has also been observed in freshly isolated rat hippocampal CA1 neurons (Li et al., 2000) and ventral tegmental dopaminergic neurons (Xiao et al., 2008). The relevance of these findings to the pharmacology of ethanol and alcoholism remains elusive.

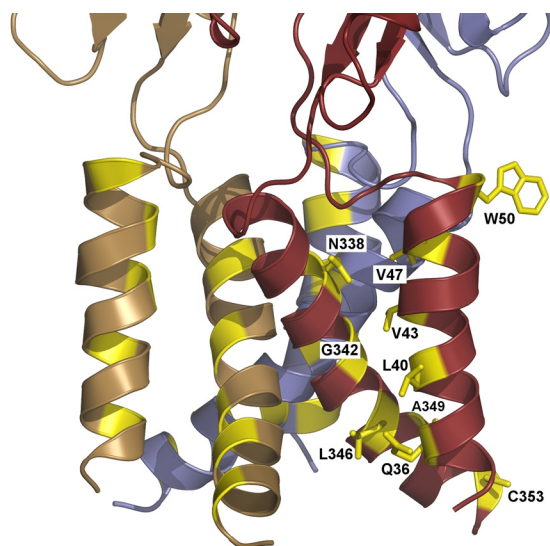


FIG. 8. Cartoon representation of the TM domains of rat P2X4 viewed parallel to the plasma membrane plane. Residues sensitive to the presence of IVM are shown in yellow. Replacement of these residues with cysteine and alanine attenuated the allosteric action of IVM, but none of these mutations alone accounted for all the effects of this compound on the receptor function (Jelínková et al., 2008; Jindrichova et al., 2009). For better clarity, the IVM-sensitive residues of only one subunit are shown as sticks. It is clear that the pattern of these predominantly nonpolar residues is consistent with the helical topology of both TM domains. The process of channel opening probably involves the tilting and/or rotation of TM helices (Doyle, 2004). Therefore, the mechanism of IVM action could involve facilitating the reorientation of the TM segments during the channel opening. The figure was generated using PyMol v0.99 (<http://www.pymol.org>).

Several studies have focused on the identification of the binding site responsible for the allosteric actions of ethanol. The intracellular application of ethanol does not enhance the inhibition of current induced by extracellular ethanol, suggesting that the alcohol inhibition of ATP-gated ion channel function involves the extracellular domain of the receptor (Weight et al., 1999). Conserved extracellular cysteines in the P2X4R differentially regulate the inhibitory effect of ethanol in this receptor (Yi et al., 2009). The effects of mutating the conserved cysteine residues on ethanol modulation are probably indirect because of changes in the structure of the receptor, which in turn affects agonist binding and the molecular changes associated gating. In another study, His²⁴¹ was identified as a residue responsible for the ethanol sensitivity of P2X4Rs (Xiong et al., 2005). Experiments with chimeric P2X2R and P2X3R revealed that ectodomain segments at the TM interfaces play key roles in determining the qualitative and quantitative responses to ethanol (Asatryan et al., 2008). Further experiments by the same group identified Asp³³¹ and Met³³⁶ as critical TM residues for the ethanol sensitivity of P2X4R (Popova et al., 2010). It has recently been suggested that IVM could antagonize the ethanol-dependent inhibition of P2X4R current and that mutation of the Met³³⁶ residue affects both IVM and ethanol effects. A homology model built from the crystallized zP2X4.1R revealed that a pocket formed by Asp³³¹, Met³³⁶, Trp⁴⁶, and Trp⁵⁰ could play a role in the allosteric action of ethanol and IVM (Asatryan et al., 2010).

E. Roles of Protein Kinases in Regulation of P2X Receptor Function

Protein kinase A (PKA) has been suggested to play a role in the prostaglandin E₂-induced potentiation of P2X3R currents in DRG neurons (Wang et al., 2007). A role for PKA in the glucocorticoid-induced inhibition of P2XR current in the HT4 neuroblastoma cells of mice has also been proposed (Han et al., 2005). Both 8-bromo-cAMP and the PKA catalytic subunit cause a reduction in the amplitude of rP2X2R current, presumably through the phosphorylation of the C-terminal residue Ser⁴³¹ (Chow and Wang, 1998). More recently, the potentiation of P2X4R function by this kinase was also reported and involves the phosphorylation of an accessory protein that interacts with the YXXGL endocytosis motif located in the C-terminal, resulting in an augmentation of P2X4Rs in the plasma membrane (Brown and Yule, 2010).

The ATP-induced P2X1R and P2X3R currents (Paukert et al., 2001; Vial et al., 2004; Brown and Yule, 2007), but not P2X4R and P2X7R currents (Brown and Yule, 2007), are potentiated by the phorbol ester phorbol 12-myristate 13-acetate, an activator of protein kinase C (PKC). Potentiation of P2X1R currents by 5-hydroxytryptamine 2A receptors is mediated by diacylglycerol- and calcium-dependent kinases (Ase et al., 2005). Other

groups have suggested that P2X7R function is potentiated by PKC γ in the type-2 astrocyte cell line RBA-2 (Hung et al., 2005). The inhibitory effects of phorbol esters on agonist-induced P2XR currents have been observed in isolated DRG neurons from adult rats (Bie et al., 2009). A critical role of the cAMP sensor Epac in the sensitization of native P2X3R was also reported (Wang et al., 2007).

P2XRs share a putative PKC binding motif (TXR/K) at the receptor N-terminal that has been proposed as a site for modulation by phosphorylation. At first, mutation of the N-terminal Thr¹⁸ residue of rP2X2R was found to dramatically affect receptor desensitization, and the addition of the PKC activator phorbol 12-myristate 13-acetate recovers the desensitization profile in chimeric receptors (Boué-Grabot et al., 2000). Mutation of the respective threonine residues resulted in a receptor with either significantly smaller amplitudes or an absence of P2X1R- and P2X3R-gated currents (Liu et al., 2003; Franklin et al., 2007). However, there is no biochemical evidence of direct phosphorylation of P2X2R and at this residue (Franklin et al., 2007; Vial et al., 2004; Brown and Yule, 2007). The function of P2X7R is also affected by replacing Thr¹⁵ with other residues in a PKC-independent manner (Yan et al., 2008). These observations do not exclude the potential phosphorylation of other residues by PKC. In hP2X3R, the substitution of Ser/Thr residues situated within the PKC consensus phosphorylation sites with Ala either abolished (T134A, S178A) or did not alter (T196A, S269A) the UTP-induced potentiation of current (Wirkner et al., 2005; Stanchev et al., 2006).

It has also been suggested that the P2X3R current is down-regulated by the C-terminal SRC kinase and cyclin-dependent kinase-5-dependent receptor phosphorylation. For the C-terminal SRC kinase-dependent regulation, the C-terminal Tyr³⁹³ residue has been proposed as a target for phosphorylation (D'Arco et al., 2009; Nair et al., 2010). In mouse trigeminal sensory neurons, the role of this kinase is controlled by nerve growth factors (D'Arco et al., 2007).

F. Regulation of P2X Receptors by Lipids

Although phosphoinositides constitute only a small fraction of cellular phospholipids, their importance in the regulation of cellular functions is enormous. Among others, phosphatidylinositol-4,5-bisphosphate (PIP₂) and phosphatidylinositol-3,4,5-trisphosphate control the function of numerous membrane transport proteins, including K_v and Ca_v channels, ion channels that mediate sensory and nociceptive responses, epithelial transport proteins, and ionic exchangers (Gamper and Shapiro, 2007). Phosphoinositides also play a role in the regulation of P2XRs. At first, it was shown that PI3K inhibitors accelerate the P2X2R desensitization and that two positively charged residues, Lys³⁶⁵ and Lys³⁶⁹, located in the C-domain, are critical for the interaction of membrane phosphoinositides

with the P2X2R (Fujiwara and Kubo, 2006). Immediately thereafter, it was demonstrated that PIP₂ directly interacts with Lys³⁶⁴ of the P2X1R and that the inhibition of this interaction, by either the mutagenesis of Lys³⁶⁴ or reducing the abundance of membrane phosphoinositides by PI3K/PI4K blockade, resulted in a decrease of current amplitude and the recovery from desensitization (Bernier et al., 2008b). In heteromeric P2X1/5Rs, PIP₂ modulates the receptor function through the P2X1 lipid-binding domain (Ase et al., 2010). The same group also showed similar effects of PIP₂ and PIP₃ on P2X4R activity. Using an in vitro binding assay, they found that phosphoinositides interact with the Cys³⁶⁰-Val³⁷⁵ sequence located in the C terminus of the receptor (Bernier et al., 2008a). Likewise, P2X3R and P2X7R are inhibited by decreases in PIP₂ induced either by PI4K inhibition or phospholipase C activation by coexpressed G protein coupled receptors (Zhao et al., 2007a,b). Native and recombinant P2X2/3Rs are also regulated by phosphoinositides (Mo et al., 2009).

A lipopolysaccharide (LPS) is a large molecule consisting of a lipid and a polysaccharide joined by a covalent bond. LPSs are found in the outer membrane of Gram-negative bacteria act as endotoxins and elicit strong immune responses in animals. P2X7R has been shown to modulate the LPS-induced macrophage production of numerous inflammatory mediators. The P2X7 C-terminal domain contains several apparent protein-protein and protein-lipid interaction motifs that are potentially important for macrophage signaling and LPS action, including a conserved LPS-binding domain. Peptides derived from this P2X7 sequence bind LPS in vitro and neutralize the ability of LPS to activate the extracellular signal-regulated kinases and to promote the degradation of inhibitor of nuclear factor κ B, α isoform, in vivo. Taken together, these data suggest that P2X7R, through its C terminus, may directly coordinate several signal transduction events related to macrophage function and LPS action (Denlinger et al., 2001). Other groups have reported effects of LPS on native P2X4Rs in microglia (Raouf et al., 2007).

Lysophosphatidylcholine (LPC), an inflammatory phospholipid that promotes microglial activation, may also contribute to P2X7R signaling. LPC was found to facilitate agonist-induced Ca²⁺ signaling in P2X7R-expressing microglial cell lines and to enhance the P2X7R-associated formation of membrane pores and the activation of the p44/42 mitogen-activated protein kinase. These results suggest that LPC may regulate microglial functions in the brain by enhancing the sensitivity of P2X7R (Takenouchi et al., 2007). Serum constituents can also affect P2X7R function (Michel et al., 2001). Polymyxin B, specifically its hydrophobic tail, positively modulates P2X7R-mediated ethidium uptake (Ferrari et al., 2006). In further support of the role of lipids in P2X7R activity, it has been shown that a diverse range of lipids increase agonist potency at the P2X7R in functional and binding studies (Michel and Fonfria, 2007).

Cholesterol-rich lipid rafts (Pike, 2003, 2004) may also play a role in P2XR function. It has been suggested that P2X3R localizes into lipid rafts in primary cultures of cerebellar granule neurons as well as in brain and DRG extracts (Vacca et al., 2004) and that the receptor undergoes rapid constitutive and cholesterol-dependent endocytosis (Vacca et al., 2009). Some reports have suggested that P2X3R does not require cholesterol for its function (Liu et al., 2006). P2X7R is also localized in lipid rafts (Gonnord et al., 2009), interacts with caveolin-1 (Barth et al., 2007), and controls the cellular levels of several lipid messengers through the modulation of phospholipases A2, C, and D (Garcia-Marcos et al., 2006). P2X1R is also expressed in lipid rafts (Vial and Evans, 2005; Vial et al., 2006). Cholesterol-depleting agents reduce P2X1R current by approximately 90%, but do not change P2X2R, P2X3R, and P2X4R currents. The N-terminal 20 to 23 and 27 to 19 residues seem to play a role in the cholesterol-dependent gating of this receptor (Allsopp et al., 2010).

G. Multiple Roles of Steroid Hormones on P2X Receptor Function

In addition to their genomic effects, which require a longer exposure time, steroids can also exhibit rapid effects, either by activating cell membrane G protein-coupled steroid receptors or by their allosteric actions on signaling molecules. The effect of neurosteroids on the GABA_A receptor channel is well established (Harrison and Simmonds, 1984). There are also reports of allosteric actions of neurosteroids on nicotinic α 4 β 2, *N*-methyl-D-aspartate, and glycine receptors (Paradiso et al., 2001; Ahrens et al., 2008; Johansson et al., 2008). Sex and adrenal steroid hormones influence P2XR expression by activating cytosolic receptors, leading to changes in transcriptional regulation (Holbird et al., 2001; Wang et al., 2008; Fan et al., 2009; Urabe et al., 2009). As discussed in detail below, steroids also exhibit rapid, nongenomic effects on P2XR function. In the absence of information about the binding site(s) for steroids, however, we cannot rule out the possibility that neuroactive steroids might interact with a cell membrane G protein-coupled steroid receptor that directly or indirectly modifies the ATP-gated currents.

The first report concerning the nongenomic effects of 17 β -estradiol on the activity of native P2XRs expressed in rabbit detrusor was reported in 1999 (Ratz et al., 1999). 17 β -estradiol also inhibits P2X2Rs expressed in PC12 cells in a nongenomic manner (Kim et al., 2000). Dehydroepiandrosterone sulfate and estrone also inhibit P2XRs expressed in PC12 cells (Liu et al., 2001b). A fast potentiating effect of dehydroepiandrosterone on native P2XRs expressed in rat sensory neurons, but only at submaximal concentrations of ATP, has also been reported (De Roo et al., 2003). In the same cells, progesterone also rapidly and reversibly potentiates submaximal, but not saturating, responses evoked by ATP. A

similar effect was observed in cells expressing recombinant P2X2Rs, but not P2X1Rs, P2X3Rs, or P2X4Rs, indicating the receptor specificity of steroid action (De Roo et al., 2010). A nongenomic inhibitory effect of testosterone on P2XR function in isolated rat urinary bladder has also been observed (Hall et al., 2002).

It has also been reported that the neurosteroid alfaxalone potentiates the ATP-evoked P2X4R currents within 60 s, independent of the expression system used (HEK293 or oocytes). Likewise, allopregnanolone and 3 α ,21-dihydroxy-5 α -pregnan-20-one potentiates the ATP-gated currents, but less effectively. In contrast, 0.3 to 10 μ M pregnanolone, but not its sulfated derivative, inhibits the ATP-gated currents by approximately 40% in both cell types. Gonadal steroids 17 β -estradiol and progesterone were inactive, revealing explicit structural requirements for steroid action on receptor function. Alfaxalone or 3 α ,21-dihydroxy-5 α -pregnan-20-one gated P2X4R at concentrations 30- to 100-fold larger than those required to modulate the receptor, eliciting suramin and brilliant blue-sensitive currents, and potentiated the P2X4R more than 10-fold over the effect of 10 μ M zinc (Codocedo et al., 2009).

Adrenal steroid hormones also rapidly influence P2XR function. In mouse HT4 neuroblastoma cells, the glucocorticoids corticosterone and cortisol and the synthetic glucocorticoid hormone dexamethasone inhibit P2XR-mediated calcium influx through a membrane-initiated, nongenomic pathway (Han et al., 2005). A rapid nongenomic effect of dexamethasone on the ATP-induced changes in cytosolic calcium was also observed in cochlear spiral ganglion neurons (Yukawa et al., 2005). Rapid inhibition of the ATP-induced currents and calcium signals by corticosterone was also observed in rat DRG neurons (Liu et al., 2008b, 2010).

H. Other Modulators

1. Reactive Oxygen Species. Reactive oxygen species (ROS), including superoxide and other oxygen ions, free radicals and peroxides are chemically unstable, highly reactive molecules known to induce cell damage via oxidative stress. Hydrogen peroxide is a prominent endogenous ROS that can significantly increase as a result of several pathophysiological conditions. In a recent report, the modulation of P2X2R function by hydrogen peroxide, mercury, and the mitochondrial stress inducers myxothiazol and rotenone was described. Intracellular Cys⁴³⁰ was identified as a redox sensor for this receptor. It is noteworthy that the ATP-evoked currents in P2X4R were slightly inhibited by hydrogen peroxide, suggesting an opposite redox modulation that remains to be identified (Codocedo et al., 2009). These novel findings indicate that P2XRs can modify their activity depending on the redox state of the cell. Future experiments are required to determine whether other P2XRs are also modulated by ROS.

2. Carbon Monoxide. Carbon monoxide (CO), the metabolic product of heme oxygenases, is a potent modulator of a wide variety of physiological processes. It has

been reported that CO also potentiates ATP-evoked currents in cells that express the P2X2R but was ineffective in cells expressing either the P2X3R or the heteromeric P2X2/3R. Moreover, these authors found a small but significant inhibition of the ATP-evoked currents in P2X4R-expressing cells (Wilkinson et al., 2009). These results suggest that P2X2R and P2X4R may have an allosteric site for CO or that this gas exerts its actions through indirect mechanisms. In this regard, the effects of CO in P2X2R and P2X4R are similar to those of ROS and mitochondrial stress inducers (Coddou et al., 2009). Therefore, it is plausible that CO can bind to mitochondrial complexes and stimulate ROS production, a mechanism that has been shown to modulate L-type Ca_v channels (Peers et al., 2009).

3. Cibacron Blue 3GA. The putative nonselective P2R antagonist Cibacron blue (the purified *ortho*-isomer) (Ralevic and Burnstock, 1998) acts as an allosteric regulator of P2XRs at concentrations lower than that required for P2R antagonism. One study described its potentiating activity on rP2X4R. Pretreatment with Cibacron blue mediated a 4-fold increase in the potency of ATP (EC₅₀, 3.3 μ M) without affecting the maximum response (Miller et al., 1998). Electrophysiological and calcium influx data also suggest that Cibacron blue functions as a positive allosteric modulator of hP2X3R activity and may also contribute to the increasing the rate of receptor recovery from agonist-induced desensitization. These effects were independent of the agonist used to activate receptor and were concentration-dependent with an EC₅₀ of 1.4 μ M (Alexander et al., 1999). Cibacron blue also potentiates BzATP-induced nociception, probably acting through P2X3R and P2X2/3R (Jarvis et al., 2001). However, the effects of Cibacron blue on recombinant P2X2/3R have not been tested.

4. Propofol, Ketamine, and Toluene. Propofol, an intravenous anesthetic that is widely used clinically, potentiates the activity of P2X4Rs expressed in HEK293 cells and has no effect on P2X2Rs or P2X2/3Rs (Tomioka et al., 2000; Davies et al., 2005b). Propofol also potentiates native P2X7Rs expressed in microglia, an effect that was mimicked by two other clinically relevant intravenous anesthetics, thiopental and ketamine (Nakanishi et al., 2007). In another study, neither ketamine nor propofol affected P2X2Rs (Furuya et al., 1999). Toluene, an abused organic solvent, potentiates the ATP-evoked P2X2R and P2X4R currents, as well as P2X4/6R and P2X2/3R currents, and inhibits P2X3R currents (Woodward et al., 2004). There is no information regarding the residues involved in the actions of these compounds.

5. Tetramethylpyrasine. This is an alkaloid used in traditional Chinese medicine as an analgesic for injury and dysmenorrhea (Liang et al., 2005). It inhibits the effects of nucleotides at native P2X3Rs responsible for the primary afferent transmission of neuropathic pain states, presumably acting as an allosteric modulator by

binding at an unidentified extracellular domain (Gao et al., 2008).

V. Future Directions

Although it took 7 years to get the zP2X4.1R crystal (Silberberg and Swartz, 2009), it is still reasonable to suggest that one of the P2XRs will be crystallized in the presence of agonist, which will provide a major advance in understanding the binding domain and gating mechanisms of these receptors. In the absence of such data, additional structural based modeling could provide a better understanding of the possible organization of orthosteric binding sites and development of subunit specific antagonists to be used in further studies and therapeutics and the gating of the channel pore. Further studies should also help in understanding how changes in intracellular receptor domains are transmitted to the rest of the channel, including changes caused by the association of P2XRs with numerous interacting proteins. Future studies could also clarify how activation of numerous signaling pathways by calcium influx and the cross-talk with G protein-coupled receptors affects P2XR function. In addition to structural and mathematical modeling, voltage-clamp fluorometry and other techniques used to examine the changes in accessibility of the protein domains could provide some temporal resolution of the conformational changes caused by the binding of an agonist in the presence and absence of allosteric modulators.

Allosteric modulators constitute a valuable alternative for future drug design, especially for receptor-specific drugs. The close topological location of the trace metal coordination amino acid residues to the orthosteric ATP sites also provides clues for further structural modeling and investigation into the actions of metals in the receptor ectodomain. Trace metals can be released to the synaptic cleft and exert effects on signaling, altering neuronal excitability, but further studies are needed to clarify the physiological conditions for the action of these metals on P2XR function *in vivo*. The finding that other endogenous compounds, such as protons, ROS, or neurosteroids, modulate P2XR activity also places allosteric modulation in a more physiological/pathophysiological context. For example, changes in cellular pH caused by ischemia could affect P2X3R activity. Furthermore, an intracellular redox sensor for the P2X2R that modulates channel activity could play an important role in oxidative stress or the response to abundant ROS production by either pathophysiological conditions or drugs. Likewise, the discovery that P2XRs are modulated by neurosteroids in a positive and negative manner raises the possibility that P2XR signaling and brain excitability could change with variations in steroid levels caused by physiological and pathophysiological conditions as reproductive cycles and emotional and/or endocrine state of the body. These observations

provide a basis for further studies regarding the dependence of the activity of a particular receptor on the metabolic state of cells, tissues, and organs.

VI. Conclusions

The recent 3.5-Å crystallography of zP2X4.1R in a closed state is a remarkable breakthrough in the purinergic field. Not only has it provided a detailed description of P2XR tertiary and quaternary structure but also it confirmed the assumption of the trimeric nature of P2XRs raised from pioneer biochemical, functional and microscopy studies. The identity of the 10 highly conserved ectodomain cysteines, plus the correct assignment of the five possible sulfhydryl bonds and their role in the tertiary receptor folding, was established beyond a doubt. The structure of zP2X4.1R was solved in the absence of ATP, which limits our understanding of the residues involved in the formation of orthosteric binding sites and the positive and negative cooperativity with allosteric binding sites. However, the cloning of P2X sequences that are distantly related through phylogeny has demonstrated that relatively few ectodomain residues are conserved across all subunits, and their importance in receptor functions has been shown in mutagenesis studies. As with other ligand-gated channels, P2XRs have numerous regulatory sites in both the extra- and intracellular domains. Therefore, channel activity is multiregulated by a complex set of synapse- and/or paracrine-derived native agents, as well as intracellular metabolites. In contrast to the ATP binding sites, the nonconserved residues seem to play a critical role in allosteric modulation of these channels by trace metals, divalent cations, and protons, which explains the receptor specificity of the actions of these molecules. P2XRs are also modulated by membrane lipids, kinases, and ROS acting intracellularly. Drugs also modify ATP signaling. A prominent example is the positive and potent modulator role of IVM on P2X4R that probably occurs within the TM receptor domain and is currently used as a pharmacological criterion to characterize the involvement of this particular receptor in cellular functions. Thus, the P2XR affinity for ATP and/or the changes in the maximal currents is not only determined by the ligand concentration but also depends on the availability of these regulators in the receptor milieu. The current information regarding the residues involved in orthosteric and allosteric modulation combined with the channel structure provided by crystallization will lead to a better understanding of the structure-activity relationships that govern these processes.

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