MINIREVIEW—SPECIAL ISSUE IN MEMORY OF AVRAM GOLDSTEIN

P2X Receptors as Drug Targets

R. Alan North and Michael F. Jarvis

Faculty of Medical and Human Sciences, University of Manchester, Manchester, United Kingdom (R.A.N.); and Early Discovery, AbbVie, Inc., North Chicago, Illinois (M.F.J.)

Received November 16, 2012; accepted December 19, 2012

ABSTRACT

The study of P2X receptors has long been handicapped by a poverty of small-molecule tools that serve as selective agonists and antagonists. There has been progress, particularly in the past 10 years, as cell-based high-throughput screening methods were applied, together with large chemical libraries. This has delivered some drug-like molecules in several chemical classes that selectively target P2X₁, P2X₃, or P2X₇ receptors. Some of these are, or have been, in clinical trials for rheumatoid arthritis, pain, and cough. Current preclinical research programs are studying P2X receptor involvement in pain, inflammation,

osteoporosis, multiple sclerosis, spinal cord injury, and bladder dysfunction. The determination of the atomic structure of P2X receptors in closed and open (ATP-bound) states by X-ray crystallography is now allowing new approaches by molecular modeling. This is supported by a large body of previous work using mutagenesis and functional expression, and is now being supplemented by molecular dynamic simulations and *in silico* ligand docking. These approaches should lead to P2X receptors soon taking their place alongside other ion channel proteins as therapeutically important drug targets.

Introduction

Drugs acting on ion channels are among the oldest therapeutics, with quinine, procaine, and curare among the best known. They also include some of the most widely prescribed drugs, such as benzodiazepines, calcium channel blockers, and sulfonylurea antidiabetic agents. As ion channel families have been identified over the past 30 years and their genes cloned (Alexander et al., 2011), there has been a major effort to develop small-molecule agonists and antagonists that are specific, selective, and safe (Wickenden et al., 2012).

Three main classes of ligand-gated channels are now recognized (Fig. 1). The first is the pentameric nicotinic superfamily: these channels may show selectivity for cations or anions, and include members gated by acetylcholine, 5-hydroxytryptamine, γ -aminobutyric acid, glycine, and (in invertebrates) glutamate. Tetramers make up the second family, and include cation-selective channels gated by extracellular glutamate as well as others gated by intracellular cyclic nucleotides. This family also has potassium-selective members, such as the inward rectifiers which are gated by intracellular ATP by virtue of an associated sulfonylurea binding protein. The development of these receptors as drug targets has been reviewed (Bowie, 2008; Hurst et al., 2012). The third family includes two small

This work was supported by the Wellcome Trust [Grant 093140/Z/10/Z].
dx.doi.org/10.1124/mol.112.083758.

ABBREVIATIONS: A-317491, 5-[[[(3-phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid; A-438079, 3-[[[5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl]methyl]pyridine]; A-740003, *N*-[1-[[[(cyanoamino)(5-quinolinylamino)methylene]amino]-2,2-dimethylpropyl]-3,4-dimethoxybenzeneacetamide]; AACBA, *N*-(adamantan-1-ylmethyl)-5-[(3*R*-amino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide; ADP β S, adenosine 5'-(β -thiodiphosphate); BBG, Brilliant Blue G; GSK314181A, (adamantan-1-ylmethyl)-5-[(3*R*-amino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide; IL-1 β , interleukin-1 β ; Iso-PPADS, pyridoxal phosphate-6-azophenyl-2',5'-disulfonic acid; KN-62, 4-[(2*S*)-2-[(5-isoquinolylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenylisoquinoline sulfonic acid; α meATP, α βmethylene-adenosine 5'-triphosphate; β meATP, β γmethylene-adenosine 5'-triphosphate; MK-3901, *N*-[1(*R*)-(5-fluoropyridin-2-yl)ethyl]-3-(5-methylpyridin-2-yl)-5-[5(*S*)-(2-pyridyl)-4,5-dihydroisoxazol-3-yl]benzamide; MRS 2220, cyclic pyridoxine-R^{4,5}-monophosphate-6-azophenyl-2',5'-disulfonic acid; NF023, 8,8'-[carbonylbis(imino-3,1-phenylene carbonylimino)]bis-1,3,5-naphthalene-trisulphonic acid; NF279, 8,8'-[carbonylbis(imino-4,1-phenylene carbonylimino-4,1-phenylene carbonylimino)]bis-1,3,5-naphthalene trisulfonic acid; NF449, 4,4',4'',4'''-[carbonylbis(imino-5,1,3-benzene-triyl-bis(carbonylimino))]tetakis-1,3-benzene disulfonic acid; NF770, 7,7'-(carbonylbis(imino-3,1-phenylene carbonylimino-3,1-(4-methyl-phenylene)carbonylimino)]bis(1-methoxy-naphthalene-3,6-disulfonic acid); NF778, 6,6'-(carbonylbis(imino-3,1-phenylene carbonylimino-3,1-(4-methyl-phenylene)carbonylimino)]bis(1-methoxy-naphthalene-3,5-disulfonic acid); PPADS, pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid; PSB-1011, disodium 1-amino-4-[3-(4,6-dichloro[1,3,5]triazine-2-ylamino)-4-sulphophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate; PSB-12062, *N*-(*p*-methylphenylsulfonyl)phenoxazine; RO3, 5-(2-isopropyl-4,5-dimethoxy-benzyl)-pyrimidine-2,4-diamine; RO4, 5-(5-iodo-2-isopropyl-4-methoxy-phenoxy)-pyrimidine-2,4-diamine; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)adenosine-5'-triphosphate; YO-PRO-1, 4-((3-methyl-2(3*H*)-benzoxazolylidene)methyl)-1-(3-(trimethylammonio)propyl)quinolinium.

Fig. 1. Trimeric P2X receptor compared with tetrameric glutamate and pentameric nicotinic (Cys-loop) receptor. Zebrafish P2X4 receptor (modified from PDB ID [4DW1](#)) (left). Rat GluA2 receptor (modified from PDB ID [3KG2](#)) (center). *Caenorhabditis elegans* glutamate-gated chloride channel (modified from PDB ID [3RHW](#)) (center). Each is shown from the outside of the cell.

groups, which are similar in overall membrane topology, but unrelated in primary amino acid sequence. One group includes the four subunits that form acid-sensing ion channels and the four subunits that form epithelial sodium channels. The other group includes the seven P2X receptor subunits. In either group of this third family, channels are formed by assembly of three subunits, either as homotrimers or heterotrimers.

P1 and P2 receptors were first distinguished by Burnstock (1978), on the basis of whether adenosine or ATP was the more effective ligand. The P1 classification was later replaced by A, as it was realized that the preferred agonist is adenosine rather than ATP (A₁, A_{2A}, A_{2B}, and A₃; Alexander et al., 2011). Burnstock and Kennedy (1985) introduced the designators P2X and P2Y, on the basis of agonist and antagonist selectivity in a range of tissues. ATP analogs in which methylene groups replaced oxygen atoms in the phosphate chain [$\alpha\beta$ methyleneadenosine 5'-triphosphate ($\alpha\beta$ meATP) and $\beta\gamma$ methyleneadenosine 5'-triphosphate ($\beta\gamma$ meATP)] tended to activate P2X receptors, whereas an adenosine 5'-diphosphate analog with a β -sulfur atom [adenosine 5'-(β -thiodiphosphate) (ADP β S)] was more selective for P2Y receptors. Also at this time, Gordon (1986) named the purine receptors on platelets and mast cells P2T and P2Z, respectively. At the time of the first receptor cloning, it became unambiguously clear that P2X receptors were ligand-gated ion channels (Brake et al., 1994; Valera et al., 1994), and P2Y receptors were G protein-coupled receptors (Webb et al., 1993). Receptor classification based on agonist rank orders of agonist potency (Ahlquist, 1948) and on receptor dissociation equilibrium constants (Arunlakshana and Schild, 1959) was, at this time, rapidly giving way to a classification system based on protein primary structure. It was soon realized that P2Z receptors corresponded to P2X₇ (Surprenant et al., 1996), and that P2T receptors were the same as P2Y₁₂ (Hollopeter et al., 2001).

As receptors, P2X receptors are selectively activated by ATP, much less activated by ADP, and not activated at all by AMP or adenosine or other purines (e.g., GTP) or pyrimidines (e.g., UTP and CTP). As channels, the conductive pathway is selective for cations over anions: the channel opens within a few milliseconds of ATP application, and closes within tens of milliseconds when the application is discontinued (deactivation). Ionic currents through homomeric P2X₁ and P2X₃ receptors decline during the application of ATP (desensitization) within tens or hundreds of milliseconds; for P2X₄ and P2X₂ receptors, this decline occurs in seconds or tens of seconds; and for P2X₇ receptors, there is little decrease in the current even over several minutes. P2X receptors, most markedly P2X₇ receptors, show complex gating behavior in which the permeation pathway dilates during several seconds

of ATP application from a pore that only allows the passage of small cations (Na⁺, K⁺, Ca²⁺) to one that allows permeation of larger cations (*N*-methyl-D-glucamine) and dyes such as ethidium and 4-((3-methyl-2(3H)-benzoxazolydene)methyl)-1-(3-(trimethylammonio)propyl)quinolinium (YO-PRO-1).

P2X Antagonists

One of the first reported antagonists of ATP receptor-mediated effects on smooth muscle contractility was the alkaloid adrenergic receptor antagonist quinidine (Burnstock, 1972). Many early nonselective ATP antagonists were high-molecular-weight organic molecules that included derivatives of the antiparasitic agent suramin, and various histochemical dyes (Jacobson et al., 2002). Suramin is a large polysulfonated molecule (Fig. 2) that is active at multiple targets including human immunodeficiency virus reverse transcriptase, vasoactive intestinal peptide receptors, G protein-coupled receptors, and multiple P2 receptor subtypes (Jacobson et al., 2002). A number of truncated forms of suramin have been identified that have increased potency and selectivity for P2X receptors (Fig. 2). For example, 8,8'-[carbonylbis(imino-3,1-phenylenecarbonylimino)]bis-1,3,5-naphthalene-trisulphonic acid (NF023) is approximately 10- to 20-fold more selective for P2X versus the P2Y receptors. Other suramin derivatives, including 8,8'-[carbonylbis(imino-4,1-phenylenecarbonylimino)]bis-1,3,5-naphthalene trisulphonic acid (NF279) and 4,4',4'''-[carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))]tetrakis-1,3-benzene disulfonic acid (NF449), are potent P2X₁ receptor antagonists (Ziyal et al., 1997; Damer et al., 1998) (Fig. 2). However, NF279 also effectively blocks slowly desensitizing P2X₂ and P2X₇ receptors (Donnelly-Roberts et al., 2009). Brilliant Blue G (BBG; Fig. 3) is a potent antagonist at P2X₇ receptors with an IC₅₀ value of approximately 400 nM (Bultmann et al., 1994; Jiang et al., 2000). However, BBG was recently reported to block voltage-gated sodium channels at low micromolar concentrations (Jo and Bean, 2011).

Another structural class of P2X receptor antagonists includes derivatives of the coenzyme pyridoxal-5'-phosphate such as pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (Jacobson et al., 2002). PPADS is most potent at human P2X₁, P2X₇, and P2Y₁ receptors, and is approximately 10–200-fold less potent at other P2 receptor subtypes (Donnelly-Roberts et al., 2009). Numerous P2X receptor antagonists based on the PPADS template (Fig. 2) containing pyridoxal and phenyl moieties have been described that show improved potency at some P2X subtypes (e.g., P2X₁ and P2X₃) (Jacobson et al., 2002).

Nonselective P2X Agonists

Nonselective P2X Antagonists

P2X Modulators

P2X1 Antagonists

Fig. 2. Chemical structures of nonselective P2X receptor agonists, antagonists, and modulators, and antagonists at P2X1 receptors. Nucleotide-based agonists are active at multiple P2X receptor subunits. For example, 2',3'-O-(benzoyl-4-benzoyl)-5'-ATP is a nanomolar agonist at P2X1 and P2X3 receptors, and has an approximately 10,000 lower affinity for P2X7 receptors. Suramin and PPADS have been historically used as P2X receptor antagonists, but have many off-target pharmacological actions. NF449 has higher selectivity for P2X1 receptors compared with NF279 and NF023. The P2X receptor modulators (Cibacron Blue and MRS 2220) likely bind allosteric recognition sites on their respective receptors.

Nucleotide derivatives have also served as useful ligands to negatively modulate the effects of ATP. The agonist αβmeATP (Fig. 2) can functionally block P2X receptor responses via rapid desensitization of the receptor (Bland-Ward and Humphrey, 1997; Burnstock, 2007). Trinitrophenyl-ATP (TNP-ATP; Fig. 2) and the corresponding di- and monophosphate derivatives represent the first nanomolar antagonists at P2X1, P2X3, and P2X2/3 subtypes (Virginio et al., 1998). TNP-ATP is several orders of magnitude less potent at P2X4, P2X5, and P2X7 (Virginio et al., 1998; Jacobson et al., 2006; Jarvis, 2010).

TNP-ATP is rapidly metabolized, which limits its utility as a functional P2X3 receptor antagonist (Lewis et al., 1998; Donnelly-Roberts et al., 2008). Oxidized ATP is an irreversible antagonist (Jacobson et al., 2002) for the P2X₇ receptor at high concentrations (>100 μM) (Donnelly-Roberts et al., 2009). Diinosine polyphosphates [e.g., P¹P⁵-di-(inosine-5')pentaphosphate] are highly selective antagonists for fast-desensitizing P2X receptors (i.e., P2X1 and P2X3) compared with their activity at more slowly desensitizing P2X receptors (e.g., P2X2) (Jacobson et al., 2002).

Fig. 3. Receptor-selective P2X receptor antagonists. IP5I (diinosine pentaphosphate), RO3, and RO4 likely bind allosteric recognition sites on their respective receptors. Competitive receptor antagonism has been demonstrated for TNP-ATP, A-317491, A-438079, and A-804598. Several new receptor-selective antagonists for P2X2 (PSB-10211, NF770, NF778) and for P2X4 (PSB-12062) have been recently reported. AACBA is also known as (adamantan-1-ylmethyl)-5-[(3R-amino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide (GSK314181A). See text for further details.

The isoquinoline derivative 4-[(2*S*)-2-[(5-isoquinoliny)sulfonyl]methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenylisoquinoline sulfonic acid (KN-62; Fig. 3) is an antagonist of Ca^{2+} /calmodulin-dependent protein kinase II and a non-competitive antagonist at P2X₇ receptors at low micromolar concentrations (Gargett and Wiley, 1997; Donnelly-Roberts et al., 2009). KN-62 was originally reported to be a selective antagonist of human versus rodent P2X₇ receptors (Humphreys et al., 1998); subsequent studies have shown that it is active at mouse P2X₇ receptors, although with slower on-rates compared with human P2X₇ receptors (Donnelly-Roberts et al., 2009).

Potentiators of the action of ATP at P2X receptors have also been identified. Coomassie Blue, reactive blue 2, and the pyridoxal phosphate derivative cyclic pyridoxine-R^{4,5}-monophosphate-6-azophenyl-2',5'-disulfonic acid (MRS 2220; Fig. 2) selectively enhance the effects of ATP at fast-desensitizing P2X (P2X₁ and P2X₃) receptors (Soltoff et al., 1989; Jarvis et al., 2001; Jacobson et al., 2002). Trace metals (e.g., zinc and copper) also modulate the activity of multiple P2X receptors via allosteric mechanisms (Coddou et al., 2011).

The generation of receptor-selective antagonists for individual P2X receptors has been challenging given the wide diversity of recognition sites and actions for which ATP is a crucial ligand (North, 2002; Burnstock, 2007; Jarvis and Khakh, 2009). The molecular cloning and characterization of the two superfamilies of P2 receptors has enabled discrete interrogations of ligand-receptor interactions at individual receptor subtypes (Jacobson et al., 2002; Müller, 2010; Gum et al., 2012). Outlined in the following sections is an overview of the currently available antagonists that have potent and selective actions at P2X receptors. Their structures are shown in Figs. 2 and 3.

P2X₁. The aromatic sulfonates, NF449 and NF279, are potent antagonists of P2X₁ receptors (Rettinger et al., 2005). However, these suramin analogs likely have differences in equilibrium kinetics that can modulate their apparent pharmacology (Donnelly-Roberts et al., 2009). Although NF449 selectively blocks only P2X₁ receptors, NF279 blocks P2X₁, P2X₂, and P2X₇ receptors with similar affinities under conditions of extended (30–60 minutes) incubations (Donnelly-Roberts et al., 2009).

P2X₂. Antagonists that have potent and selective activity for P2X₂ receptors have been lacking. PPADS, pyridoxalphosphate-6-azophenyl-2',5'-disulfonic acid (iso-PPADS), and TNP-ATP are active in the low micromolar range (Jacobson et al., 2006); however, TNP-ATP has nanomolar affinity for fast-desensitizing P2X₁ and P2X₃ receptors (Virginio et al., 1998; Jacobson et al., 2002; Jarvis, 2010). Suramin and the dihydropyridine nifedipine are also weak antagonists of the P2X₂ receptor (Jacobson et al., 2002). Using the anthraquinone scaffold exemplified by reactive blue 2, Müller and colleagues (Baqi et al., 2011) generated 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-1011), a P2X₂ antagonist with high selectivity versus P2X₄ and P2X₇ receptors and somewhat lower (~5-fold) selectivity versus P2X₁ and P2X₃ receptors (Baqi et al., 2011). Several suramin derivatives [e.g., 7,7'-(carbonylbis(imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene)carbonylimino))bis(1-methoxy-naphthalene-3,6-disulfonic acid) (NF770) and 6,6'-(carbonylbis(imino-3,1-phenylene)carbonylimino-3,

1-(4-methyl-phenylene)carbonylimino))bis(1-methoxy-naphthalene-3,5-disulfonic acid) (NF778)] that are potent P2X₂ antagonists have been generated based, in part, on a structural analysis of the P2X₄ receptor (Wolf et al., 2011).

P2X₃. A-317491 (5-[[[(3-phenoxyphenyl)methyl][(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid) was the first competitive, selective, small antagonist that was both potent and selective for P2X₃ receptors (Jarvis et al., 2002). Subsequently, other potent P2X₃ antagonists have been reported, including 5-(2-isopropyl-4,5-dimethoxybenzyl)-pyrimidine-2,4-diamine (RO3) and 5-(5-iodo-2-isopropyl-4-methoxy-phenoxy)-pyrimidine-2,4-diamine (RO4), that are at least 100-fold less active across a wide range of kinases, receptors, and ion channels (Gever et al., 2006; Carter et al., 2009). The diaminopyrimidine class of P2X₃ antagonists has improved physicochemical properties relative to earlier P2X₃ antagonists, and is likely to bind to an allosteric site on the channel (Gever et al., 2010; Ballini et al., 2011). Other recently described P2X₃ antagonists include *N*-[1(R)-(5-fluoropyridin-2-yl)ethyl]-3-(5-methylpyridin-2-yl)-5-[5(S)-(2-pyridyl)-4,5-dihydroisoxazol-3-yl]benzamide (MK-3901) and AF-219 (structure undisclosed). The latter has advanced into clinical studies (Gum et al., 2012).

P2X₄. Despite the successful crystallization of the P2X₄ receptor (Kawate et al., 2009), the discovery of potent antagonists for P2X₄ receptors is at an early stage. Non-selective antagonists include TNP-ATP, BBG, and close analogs of the monoamine uptake blocker paroxetine (Gum et al., 2012). All of these antagonists have low affinity for the P2X₄ receptor (IC_{50} ~2–15 μM), and are significantly more potent at other targets (Gum et al., 2012). Recently, a phenoxazine derivative, *N*-(*p*-methylphenylsulfonyl)phenoxazine (PSB-12062), was identified as a potent (IC_{50} ~1–2 μM), selective (~35-fold versus P2X₁, P2X₂, P2X₃, and P2X₇), and noncompetitive blocker of P2X₄ receptors (Hernandez-Olmos et al., 2012).

P2X₇. There is now a large diversity of chemotypes that are potent and selective antagonists of P2X₇ receptor antagonists (Guile et al., 2009; Gum et al., 2012). Many of the new antagonist pharmacophores exemplified by 3-[[5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl]methyl]pyridine (A-438079), *N*-[1-[[[(cyanoamino)(5-quinolinylamino)methylene]amino]-2,2-dimethylpropyl]-3,4-dimethoxybenzeneacetamide (A-740003), and *N*-(adamantan-1-ylmethyl)-5-[(3*R*-amino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide (AACBA) provide tool compounds with suitable drug-like properties to enable in vivo studies (Gum et al., 2012; Keystone et al., 2012). Early P2X₇ antagonists, such as KN-62 and BBG, show preferential activity at human and rat P2X₇ receptors, respectively (Donnelly-Roberts et al., 2009). However, the apparent species differences for P2X₇ antagonists diminish as a function of increased potency for the receptor (Donnelly-Roberts et al., 2009). BBG has recently been shown to potently block sodium channels and P2X₄ receptors (Jo and Bean, 2011).

Therapeutic Indications

Pain. Chronic pain results from a neurochemical and phenotypic sensitization of peripheral and central sensory nerves that is characterized by increased sensitivity to painful stimuli (hyperalgesia) and the perception of pain in response to normally innocuous stimuli (allodynia) (Perl, 2007; Woolf, 2011). This sensitization process can result from acute or persistent tissue trauma, and is mediated by many pronociceptive

neurotransmitters and neurotrophic factors including ATP, glutamate, substance P, calcitonin gene-related peptide, and proinflammatory cytokines (Basbaum et al., 2009). Exogenous ATP elicits pain in both humans and laboratory animals, and enhances pain sensations associated with inflammation (Bleehen and Keele, 1977; Hamilton and McMahon, 2000). The ability of ATP to modulate pain-associated neuronal excitability associated with nociception is mediated directly by activation of homomeric and heteromeric P2X3 receptors on central and distal processes of peripheral nerves, and indirectly by activation of P2X and P2Y receptors on glial cells (astrocytes and microglia) in the periphery and spinal cord (Jarvis, 2010).

PPADS and suramin have been used to attenuate nociceptive responses in a variety of experimental pain models (Inoue et al., 2007; Jarvis, 2010). However, attribution to specific interactions with individual P2X receptors is necessarily limited by the nonselective pharmacology of these relatively weak antagonists (Jacobson et al., 2002; Donnelly-Roberts and Jarvis, 2007, 2008).

Local administration of the potent but nonselective P2X3 antagonist TNP-ATP into relevant sites has been shown to block the pronociceptive effects of P2 receptor agonists such as $\alpha\beta$ meATP (Jarvis et al., 2001). Similarly, systemic administration of the selective P2X3 antagonist A-317491 produces dose-dependent effects across multiple pain modalities, including neuropathic, inflammatory, and nociceptive pain states (Jarvis et al., 2002). The role of homomeric and heteromeric P2X3 receptors in mediating chronic pain states is further corroborated by the ability of other structurally different and potent P2X2/3 and P2X3 antagonists (e.g., RO4 and MK-3901) to reduce nocifensive behavior in chronic pain models (Gum et al., 2012). Interestingly, AZ-2, an antagonist that is selective for only the homomeric form of the P2X3 receptor, effectively reversed mechanical allodynia induced by complete Freund's adjuvant following systemic and intraplantar dosing, but was ineffective when dosed intrathecally (Cantin et al., 2010, 2012).

In addition to a direct role of P2X3 receptors to modulate nociceptive sensory processing, P2X4 and P2X7 receptors on activated microglia also influence noxious sensation. P2X7(−/−) and P2X4(−/−) mice show reduced nociceptive sensitivity in neuropathic pain states compared with wild-type controls, and this phenotype is consistent with the antinociceptive phenotype of mice lacking both isoforms of interleukin-1 (IL-1 α and β) (Chessell et al., 2005; Honore et al., 2009; Jarvis, 2010). Selective P2X7 receptor antagonists A-740003 and A-438079 reduce inflammatory hyperalgesia in rodents that are not secondary to P2X7-mediated anti-inflammatory effects (see the following section) (Honore et al., 2006; Donnelly-Roberts et al., 2008). P2X7 receptor-selective antagonists also reduce nociception in some experimental models of neuropathic pain, an effect that is mediated, at least in part, by spinal and/or supraspinal sites of action (Inoue, 2006; McGaraughty et al., 2007).

Arthritis. Agonist stimulation of P2X7 receptors that are localized on macrophages and microglia opens a nonselective cation channel, and leads to the rapid maturation and release of interleukin-1 β (IL-1 β) (Perregaux and Gabel, 1994; MacKenzie et al., 2001; Ferrari et al., 2006; Di Virgilio, 2006). These actions serve as a mechanistic basis for the role of the P2X7 receptor in inflammation. P2X7(−/−) mice show attenuated

cytokine signaling and ATP-evoked processing of pro-IL-1 β , as well as decreased signs and symptoms of arthritis in experimental models (Labasi et al., 2002). Pharmacological studies using selective antagonists also support a role for P2X7 receptors in inflammatory conditions. Pre-emptive dosing of AACBA has been shown to attenuate collagen-induced arthritis in rats (Broom et al., 2008). Further, an adamantine-based P2X7 receptor antagonist, AZD9056 (structure undisclosed), provided preliminary signals of clinical efficacy in reducing the symptoms of rheumatoid arthritis in humans; however, this effect was not maintained in a longer-duration phase II study (Keystone et al., 2012).

Neuroprotection. In addition to its actions as a fast neurotransmitter, ATP can also function as a danger signal in response to tissue trauma (Di Virgilio, 2006; Ferrari et al., 2006; Miller et al., 2011; Trang and Salter, 2012). Within the central nervous system, ATP acting at P2X receptors on both neurons and a variety of glial cells (astrocytes, oligodendrocytes, and microglia) participates in the communications between neuron and glia (Tozaki-Saitoh et al., 2011). ATP can also serve as an activator of microglia cells associated with conditions of neuronal hyperexcitability and/or injury (Inoue et al., 2007; Inoue, 2006; Tozaki-Saitoh et al., 2011). Several P2 receptors, including P2X4, P2X7, P2X12, and P2Y14, have been shown to mediate various aspects of microglial cell signaling in the context of chronic pain, spinal cord injury, multiple sclerosis, and stroke (Jarvis, 2010; Tozaki-Saitoh et al., 2011). Phenotypic characterization of mice lacking some of these receptors (e.g., P2X4 and P2X7) provides evidence for a beneficial role of blocking these receptors in neuropathological conditions (Khakh and North, 2012); however, rigorous pharmacological support for these roles is not yet available (Jarvis, 2010). Activation of P2X7 receptors leads to the rapid maturation and release of the inflammatory cytokine IL-1 β (MacKenzie et al., 2001), which, in turn, can exacerbate neuronal degeneration associated with infection, multiple sclerosis, and stroke (Di Virgilio, 2006; Miller et al., 2011). A role for P2X7 receptors in chronic disease is further supported by the positive association of several discrete C-terminal single-nucleotide polymorphisms in the P2X7 receptor with diseases such as infection, leukemia, depression, bipolar disorder, and neuropathic pain (Miller et al., 2011; Sorge et al., 2012).

Bladder. ATP plays a key role in the mechanosensation of hollow organs (Burnstock, 2007). In this regard, homomeric and heteromeric P2X3 receptors are highly localized on lumbosacral and splanchnic nerve terminals that conduct sensations of stretch, pressure, and irritation (Ford, 2012). The phenotypes of gene-disrupted mice lacking P2X2, P2X3, or both receptors are characterized by reduced bladder reflexes and elevation of bladder volume thresholds (Ford, 2012). Selective P2X3 receptor antagonists, including A-317491 and RO4, reduce bladder hyperactivity in experimental models (Lu et al., 2007; Brederson and Jarvis, 2008; Ford, 2012). These data support further investigation of the roles of P2X3 receptors in overactive/unstable bladder disorders, interstitial cystitis, and benign prostatic hyperplasia (Burnstock, 2007; Ford, 2012).

Airway Hyperactivity. The neurophysiological role of P2X3 receptors in sensory nerve function in airway passages is similar to that mediating somatic nociception (Undem and Nassenstein, 2009). This similarity has driven hypotheses

concerning the involvement of P2X3 receptors in the symptoms of airway dysfunction including cough and bronchial hyper-reactivity (Ford, 2012). ATP enhances citric acid-evoked and histamine-evoked cough in preclinical models, effects that can be attenuated by P2X3 selective antagonists (Kamei and Takahashi, 2006; Ford, 2012). In humans, local delivery of ATP initiates cough and bronchospasm (Basoglu et al., 2005). In addition to an involvement of P2X3 receptors in neurogenic cough, activation of P2X7 receptors may contribute to inflammation associated with airway dysfunction (Ford, 2012).

Structure-Based Approaches

P2X receptors are relatively small, trimeric membrane proteins (Figs. 1 and 4C). Each subunit (40–70 kDa) has two membrane-spanning domains. The intracellular N terminus is short (about 30 amino acids); the intracellular C terminus is longer and quite variable among the seven different subunits. The bulk of the protein (some 270 amino acids, about 30 kDa) thus lies in the extracellular space. There are no known ancillary subunits that are required for function, although

several associated proteins have been identified (Kim et al., 2001; Wilson et al., 2002; Masin et al., 2006; Chaumont et al., 2008). Our present understanding of receptor structure is based on the X-ray crystallography of closed (PDB ID 4DW0) and open (PDB ID 4DW1) zebrafish P2X4 receptors solved at better than 3 Å (Kawate et al., 2009; Hattori and Gouaux, 2012) (Fig. 4A). The proteins purified for crystallization, although functional when expressed in oocytes, were truncated close to where they reach the inner membrane surface. They therefore lack the intracellular domains, and in particular the membrane-proximal 15 amino acids which contain a highly conserved motif (YXXXX) in both the N- and the C-terminal regions. It seems likely that this immediately cytoplasmic region has a highly ordered structure, not only because of its conservation but also because it has been shown to be critically involved in channel desensitization (Werner et al., 1996; Allsopp and Evans, 2011). This makes it possible that the absence of this region might alter the disposition of transmembrane domains from that adopted in the crystals studied to date.

Interpretation of the atomic detail provided by these structures is complemented by 15 years of functional studies on receptors that have been mutated and expressed. The ATP

Fig. 4. P2X receptor structure. (A) Two dolphin-shaped subunits are depicted. ATP binding is associated with an upward movement of the dorsal fin (chain A, at back), a downward movement of the head region (chain B, at front), and a retraction of the left flipper (chain A, at front) in the directions shown by the arrows. The lower body (light blue) is flexed outward, carrying with it transmembrane domains (green). Reproduced (with permission) from Hattori and Gouaux (2012). (B) ATP binding pocket. Residues involved in direct interactions with ATP are shown from homology model of the rat P2X2 receptor, based on PDB ID 4DW1. Atoms identified are as follows: chain A (blue) N288 ND2, K308 NZ, R290 NH1; chain B (pink) K71 NZ, K69 NZ, T184 OG1, I226 CG2, L186 CD2, L211 CB, K188 NZ. (C) Lateral portals that form the ion entry pathway into extracellular vestibule. The lateral portal between two subunits (blue and pink) is outlined by a green oval. The third subunit (yellow) is visible through the open portal. Arrowhead marks level of section shown in (D). Rat P2X2 receptor. (D) Dilatation of outer part of transmembrane domains. Positions of TM1 (filled gray circle) and TM2 (open gray circle) are indicated. Residues shown as space fill are I328 at outer end of TM2: dashed circle passes through Cα atoms. Rat P2X2 homology model.

binding pocket is formed by regions of two different subunits which provide an electropositive environment to the approaching ATP molecule (R290 and K308 on A chain; K69 and K71 on B chain; P2X2 numbering). In the open-channel structure, these four positive charges interact directly with oxygen atoms on the phosphate chain (Fig. 4B). In the case of R290, an existing intrasubunit salt bridge (with E167) is replaced by a salt bridge with a γ oxygen of ATP (Hausmann et al., 2013). Six further conserved residues contact ATP, several through hydrophobic interactions with the ribose and adenine moiety (L186, L211, I226). Base specificity is provided by direct hydrogen bonds between $-\text{NH}_2$ and N^1 nitrogen atoms of adenine and the backbone and side-chain oxygens of T184 (Hattori and Gouaux, 2012) (Fig. 4B). The overall movements underlying the channel opening are illustrated in Fig. 4A, in which key sections of each subunit of the P2X receptor are analogized to the body parts of a dolphin.

As the binding pocket closes around the bound ATP, the head domain descends, the left flipper retracts, and the dorsal fin rises. These movements of the left flipper and dorsal fin exert a lateral distortion on the lower-body domain, flexing the walls outward through intersubunit separation of its component β -sheets. This markedly enlarges the lateral portals between each pair of subunits (Fig. 4C), increases the diameter of the extracellular vestibule, and thus separates the outer ends of the transmembrane domains (Fig. 4D). The pulling apart of the outer ends of the transmembrane domains increases the diameter at the narrowest part of the permeation pathway by an iris-like movement of all six transmembrane domains. This involves significant breakage of interactions between the transmembrane domains, and must be associated with the entry of the membrane lipid into the large interstices that form between their ends (Hattori and Gouaux, 2012).

The approximation of the head domain and the dorsal fin can also be observed by normal mode analysis and molecular dynamics (Du et al., 2012; Jiang et al., 2012), and can be detected by changes in the fluorescence from suitably attached tetramethylrhodamine (Lörinczi et al., 2012). In the P2X2 receptor, it allows the approach of H120 (on the underside of the head) to H123 (on the upper aspect of the dorsal fin) such that these two residues can form a zinc binding site; this was originally shown to be an intersubunit by Nagaya et al. (2005) and Tittle et al. (2007). Jiang et al. (2012) have recently shown that extracellular zinc, binding at this site but in the absence of ATP, can directly promote opening of a P2X receptor which has been biased toward the open state by a minor mutation in the TM2 [P2X2(T339S); Cao et al., 2007]. We note that in the P2X7 receptor, three amino acid residues of the dorsal fin are missing, corresponding perhaps to one turn of the short α -helix. These include L211 in P2X2 (L217 in zebrafish P2X4) that contributes directly to the ATP binding site (Fig. 4B); this might account for the very low affinity shown by P2X7 receptors for ATP.

ATP Binding Pocket. Nucleotide analogs represent challenging starting points for drug development, given their likely poor stability and multiplicity of recognition sites (Jacobson and Boeynaems, 2010; Gum et al., 2012). That said, two dinucleoside tetraphosphate-containing molecules (diquafosol and denufosol) have sufficient drug-like properties to enable advancement into human clinical trials (Jacobson

and Boeynaems, 2010). The orientation of ATP within its binding site allows attachments to be made at the γ -phosphate, and several such compounds retain their agonist activity [for example, P^1P^5 -di(adenosine-5')pentaphosphate (Ap5A); Cinkilic et al., 2001]. The 2' and/or 3'-oxygen atoms of the ribose are also exposed to solvent, and the best-studied ligands with attachments at these positions are 2',3'-O-(benzoyl-4-benzoyl)-ATP and TNP-ATP. In the former case, the molecule remains an agonist activity; however, except at P2X7 receptors, it is less efficacious than ATP. On the other hand, TNP-ATP is a competitive antagonist with nanomolar affinity at P2X1 and P2X3 receptors (Virginio et al., 1998). It seems likely that the protruding trinitrophenyl moiety simply prevents jaw closure (Lörinczi et al., 2012), and it will be instructive to determine by molecular docking and mutagenesis why this is more effective in the case of the P2X1 and P2X3 receptors than P2X2 and P2X4, at which it is 1000 times less effective.

The first efforts at developing novel antagonists based on atomic structure are now appearing. Wolf et al. (2011) showed that the suramin analog NF770 was a competitive antagonist at P2X2 receptors (IC_{50} 19 nM): it was 4, 50, >10,000, and >10,000 times less potent at blocking P2X3, P2X1, P2X4, and P2X7 receptors, respectively. The high potency resulted in large part from replacing one of the three sulfonates on the naphthalene moiety with a methoxy group. Molecular docking indicated that the oxygen atom of this $\text{CH}_3\text{O}-$ group hydrogen bonded directly with R290 (chain B), whereas one of the other two sulfonates interacted closely with G72 (chain A). R290 is critical for ATP binding (as described earlier), and NF770 would therefore serve as a competitive antagonist. The analog NF778 has the methoxy substitution in a different position (1-methoxy-3,5-disulfonic as distinct from 1-methoxy-3,6-disulfonic acid), and shows a much weaker interaction due to the loss of this direct hydrogen bond.

There are other intersubunit clefts in the ectodomain that might offer attractive drug binding sites, and where conservation among P2X subtypes is less than that found at the ATP binding pocket. The lateral portal itself might be considered as a potential antagonist binding, although the fact that it opens to such a large size (Fig. 3C) in the open channel might make complete blockade problematic.

Transmembrane Region. Ivermectin is a macrocyclic lactone that has clinical use for onchocerciasis (river blindness) (Fig. 2). It has actions on a range of ion channels (Cully et al., 1994; Krusek and Zemkova, 1994; Krause et al., 1998), but has been particularly useful in the study of P2X receptors because it potentiates currents selectively at P2X4 receptors (Khakh et al., 1999a). Given the potential role for P2X4 receptors on microglia as therapeutic targets (Inoue et al., 2007), it is also relevant that these actions of ivermectin have been reported not only on heterologously expressed receptors but also on cultured cerebellar microglia (C8-B4 cells) (Samways et al., 2012).

Ivermectin binding involves amino acid residues on both transmembrane domains, but particularly around the outer half of TM1 (Priel and Silberberg, 2004; Silberberg et al., 2007; Jelinkova et al., 2008). Opening of the P2X receptor involves iris-like movements of the transmembrane helices, and this involves considerable rearrangements of contacts between them (Hattori and Gouaux, 2012). For example, in the closed channel, a conserved aromatic side chain in the

outer half of TM1 (Y42: P2X4 numbering) interacts with residues at the outer end of TM2 residues in the adjacent subunit (I332, I333, and M336 in the rat P2X4 receptor); this interaction breaks in the open channel as lipid enters the intervening space (Hattori and Gouaux, 2012). Somehow, bound ivermectin favors this separation of the outer ends of the TM helices, thus increasing current amplitude by retarding channel closing (Silberberg et al., 2007). Residues at the very outer end of TM1 contribute to the lower edge of the lateral “portal” through which ions enter the extracellular vestibule and thence the pore itself. These include E51 (rat P2X4: Q52 in rat P2X2, N54 in zebrafish P2X4), which is required for the relatively high fractional calcium entry shown by P2X4 receptors (Samways and Egan, 2007; Samways et al., 2011), and substitution of N54 by E in the zebrafish P2X4 receptor confers sensitivity to ivermectin (prolonged deactivation). Moreover, in addition to increasing and prolonging the current evoked by a brief application of ATP at human P2X4 receptors, ivermectin also reduces the fraction of the current carried by calcium ions (from about 14 to 8%) (Samways et al., 2012). Taken together, this implies that the ivermectin binding site likely extends along the surface of TM1 from Y42 to E51; when bound, it facilitates opening, impairs closing, and screens any effect of the electro-negativity at E51 on the entry of calcium ions. The distance along the surface of the helix from Y42 C β to E51 C β , taken from a homology model of the rat P2X4 receptor, is 10.4 Å, which is only about half the length of ivermectin in an extended conformation (Albers-Schönberg et al., 1981).

Permeation Pathway. Many ion channels can be directly blocked by agents that enter and bind in the permeation pathway; local anesthetics such as lidocaine and dihydropyridine calcium channel blockers are good examples. Do such blockers exist for P2X receptors? Given that two open states are described, particularly for P2X7 receptors, it might be asked whether drugs could be developed that block one and not the other. Certainly, mutations in the permeation pathway can have this effect. In the P2X4 receptor, a fast sodium-selective current (I₁) and a slower current through an N-methyl-D-glucamine-permeable pathway (I₂) can be distinguished by their time courses and reversal potentials (Khakh et al., 1999b). I₁ is abolished by the substitution G347R, whereas I₂ is abolished by the substitution G347Y (G347 is situated one helical turn internal to the narrowest part of the channel: T339 in P2X2, A347 in zebrafish P2X4). One such candidate might be calmidazolium, which, at 10 μ M, blocks the sodium current but not YO-PRO-1 uptake in human embryonic kidney cells expressing P2X7 receptors (Virginio et al., 1998). Calmidazolium is a monovalent cation which blocks several cation-permeable channels (Klockner and Isenberg, 1987; Kleene, 1994). With dimensions of 1.4 $\times$ 1.4 $\times$ 1.2 nm, it may permeate P2X7 receptors in their I₂ dilated state, but not pass in the undilated state (Hattori and Gouaux, 2012). The determination of the structure of a P2X receptor in a further open state, offering a wider permeation pathway, will be important. A structural basis for a similar dynamic selectivity has recently been demonstrated for the related acid-sensing ion channels, which adopts a large (1.0 nm), nonselective, open state at pH 7.25 and a smaller (0.5 $\times$ 0.7 nm) sodium-selective state at pH 5.5 (Bacconis and Gouaux, 2012).

In the case of the P2X7 receptor, it is possible that the C terminus might itself be targeted for drug discovery. In

the case of P2X2, entry into the I₂ state is associated with substantial movements of the C terminus (Eickhorst et al., 2002). There is extensive evidence that an associated intracellular signaling molecule is engaged by the activated P2X7 receptor, and that this is important for downstream signaling such as activation of caspase and release of IL-1 β (North, 2002). A recent approach to pain control targeted such an interaction (Sorge et al., 2012). A small peptide corresponding to residues in the cytoplasmic tail of the mouse P2X7 receptor (SLHDSPTGQGGGYKKRRQR: underline is 445 to 455 of the receptor, and the remainder is part of a cell-penetrating trans-activating transcriptional activator peptide) was found to attenuate the mechanical allodynia induced by sciatic nerve injury (Sorge et al., 2012). This is an attractive avenue for drug development but, in this case, presumably reflects an interaction between the peptide and the interacting protein (perhaps a Src tyrosine kinase: Iglesias et al., 2008) rather than the P2X7 receptor itself. Such approaches have the advantage of providing selectivity among P2X receptors, given that there is no relatedness among the sequence of amino acid sequences in their C termini (except for the membrane proximal region). But they offer the relative disadvantage that we currently know nothing about the structure of any P2X receptor C terminus.

Summary and Conclusions

It is almost 20 years since interest in these receptors accelerated sharply with the cloning of their cDNAs. There remain positives and negatives with regard to P2X receptors as drug targets. The positives include 1) subunit diversity (human subunits are about 40% identical at the amino acid level); 2) further diversity through channel formation as heterotrimers (e.g., P2X2/3 and P2X1/5); 3) a very restricted tissue distribution for some subunits (e.g., P2X3 and P2X7); 4) a large extracellular domain that undergoes substantial conformational rearrangement on channel opening; and 5) crystal structures available for closed and open (ATP-bound) states. On the negative side, we have limited information as to the key physiological roles for P2X receptors in most physiological and pathological processes. These roles are now beginning to be worked out, with the aid of knockout mice and small-molecule tools. Although caution is required in the use of available knockout mice (see Kaczmarek-Hajek et al., 2012), there has been good progress in the development of small-molecule tools. As this review illustrates, several such tools are now available for P2X1, P2X2/3, P2X3, and P2X7 receptors, and are beginning to appear for P2X2 and P2X4 receptors. We now look forward to seeing more of these moving into animal models of disease and, of course, into trials of clinical efficacy in humans.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: North, Jarvis.

References

- Ahlquist RP (1948) A study of the adrenotropic receptors. *Am J Physiol* **153**: 586–600.
- Albers-Schönberg G, Arson BH, Chabala JC, Douglas AW, Eskola P, Fisher MH, Lusi A, Mrozik H, Smith JL, and Tolman RL (1981) Avermectins: structure determination. *J Am Chem Soc* **103**:4216–4221.
- Alexander SPH, Mathie A, and Peters JA (2011) Guide to receptors and channels (GRAC), 5th edition. *Br J Pharmacol* **164**:S1–S324.

- Allsopp RC and Evans RJ (2011) The intracellular amino terminus plays a dominant role in desensitization of ATP-gated P2X receptor ion channels. *J Biol Chem* **286**: 44691–44701.
- Arunlakshana O and Schild HO (1959) Some quantitative uses of drug antagonists. *Br Pharmacol Chemother* **14**:48–58.
- Baconguis I and Gouaux E (2012) Structural plasticity and dynamic selectivity of acid-sensing ion channel-spider toxin complexes. *Nature* **489**:400–405.
- Ballini E, Virginio C, Medhurst SJ, Summerfield SG, Aldegheri L, Buson A, Carignani C, Chen YH, Giacometti A, and Lago I et al. (2011) Characterization of three diaminopyrimidines as potent and selective antagonists of P2X3 and P2X2/3 receptors with in vivo efficacy in a pain model. *Br J Pharmacol* **163**:1315–1325.
- Baqi Y, Hausmann R, Rosefort C, Rettinger J, Schmalzing G, and Müller CE (2011) Discovery of potent competitive antagonists and positive modulators of the P2X2 receptor. *J Med Chem* **54**:817–830.
- Basbaum AI, Bautista DM, Scherrer G, and Julius D (2009) Cellular and molecular mechanisms of pain. *Cell* **139**:267–284.
- Basoglu OK, Pelleg A, Essilfie-Quaye S, Brindicci C, Barnes PJ, and Kharitonov SA (2005) Effects of aerosolized adenosine 5'-triphosphate vs adenosine 5'-monophosphate on dyspnea and airway caliber in healthy nonsmokers and patients with asthma. *Chest* **128**:1905–1909.
- Bland-Ward PA and Humphrey PPA (1997) Acute nociception mediated by hindpaw P2X receptor activation in the rat. *Br J Pharmacol* **122**:365–371.
- Bleehen T and Keele CA (1977) Observations on the algogenic actions of adenosine compounds on the human blister base preparation. *Pain* **3**:367–377.
- Bowie D (2008) Ionotropic glutamate receptors & CNS disorders. *CNS Neurol Disord Drug Targets* **7**:129–143.
- Brake AJ, Wagenbach MJ, and Julius D (1994) New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* **371**:519–523.
- Brederson JD and Jarvis MF (2008) Homomeric and heteromeric P2X3 receptors in peripheral sensory neurons. *Curr Opin Invest Drugs* **9**:716–725.
- Broom DC, Matson DJ, Bradshaw E, Buck ME, Meade R, Coombs S, Matchett M, Ford KK, Yu W, and Yuan J et al. (2008) Characterization of N-(adamantan-1-ylmethyl)-5-[(3R-amino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide, a P2X7 antagonist in animal models of pain and inflammation. *J Pharmacol Exp Ther* **327**: 620–633.
- Bültmann R, Trendelenburg M, and Starke K (1994) Blockade of P2X-purinoreceptors by trypan blue in rat vas deferens. *Br J Pharmacol* **113**:349–354.
- Burnstock G (1972) Purinergic nerves. *Pharmacol Rev* **24**:509–581.
- Burnstock G (1978) A basis for distinguishing two types of purinergic receptor, in *Cell membrane receptors for drugs and hormones: A multidisciplinary approach* (Straub RW and Boils L eds) pp 107–118, Raven Press, New York.
- Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* **87**:659–797.
- Burnstock G and Kennedy C (1985) Is there a basis for distinguishing two types of P2-purinoreceptor? *Gen Pharmacol* **16**:433–440.
- Cantin L, Bayraktarian M, Buon C, Hu Y, Kennedy V, Laplante M, Leung C, Luo X, Popovic N, Rene O et al. (2010) Pyrrolopyrimidine-based P2X3 antagonists for the treatment of pain. *ACS National Meeting*, Boston, MA; August 24–26, MEDI-474.
- Cantin LD, Bayraktarian M, Buon C, Grazzini E, Hu YJ, Labrecque J, Leung C, Luo X, Martino G, and Paré M et al. (2012) Discovery of P2X3 selective antagonists for the treatment of chronic pain. *Bioorg Med Chem Lett* **22**:2565–2571.
- Cao L, Young MT, Broomhead HE, Fountain SJ, and North RA (2007) Thr339-to-serine substitution in rat P2X2 receptor second transmembrane domain causes constitutive opening and indicates a gating role for Lys308. *J Neurosci* **27**: 12916–12923.
- Carter DS, Alam M, Cai H, Dillon MP, Ford APDW, Gever JR, Jahangir A, Lin C, Moore AG, and Wagner PJ et al. (2009) Identification and SAR of novel diaminopyrimidines. Part 1: The discovery of RO-4, a dual P2X3/P2X2/3 antagonist for the treatment of pain. *Bioorg Med Chem Lett* **19**:1628–1631.
- Chaumont S, Compan V, Toulme E, Richler E, Housley GD, Rassendren F, and Khakh BS (2008) Regulation of P2X2 receptors by the neuronal calcium sensor VILIP1. *Sci Signal* **1**:ra8.
- Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, and Peck WL et al. (2005) Disruption of the P2X7 purinoreceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain* **114**:386–396.
- Cinkilic O, King BF, van der Giet M, Schlüter H, Zidek W, and Burnstock G (2001) Selective agonism of group I P2X receptors by dinucleotides dependent on a single adenine moiety. *J Pharmacol Exp Ther* **299**:131–136.
- Coddou C, Stojilkovic SS, and Huidobro-Toro JP (2011) Allosteric modulation of ATP-gated P2X receptor channels. *Rev Neurosci* **22**:335–354.
- Cully DF, Vassilatis DK, Liu KK, Paress PS, Van der Ploeg LH, Schaeffer JM, and Arena JP (1994) Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. *Nature* **371**:707–711.
- Damer S, Niebel B, Czeche S, Nickel P, Ardanuy U, Schmalzing G, Rettinger J, Mutschler E, and Lambrecht G (1998) NF279: a novel potent and selective antagonist of P2X receptor-mediated responses. *Eur J Pharmacol* **350**:R5–R6.
- Di Virgilio F (2006) Purinergic signalling between axons and microglia, in *Purinergic signalling in neuron-glia interactions*. No. 276 (Chadwick DJ and Goode J eds) pp 253–262, Wiley, Chichester.
- Donnelly-Roberts DL and Jarvis MF (2007) Discovery of P2X7 receptor-selective antagonists offers new insights into P2X7 receptor function and indicates a role in chronic pain states. *Br J Pharmacol* **151**:571–579.
- Donnelly-Roberts D, McGaraughty S, Shieh C-C, Honore P, and Jarvis MF (2008) Painful purinergic receptors. *J Pharmacol Exp Ther* **324**:409–415.
- Donnelly-Roberts DL, Namovic MT, Han P, and Jarvis MF (2009) Mammalian P2X7 receptor pharmacology: comparison of recombinant mouse, rat and human P2X7 receptors. *Br J Pharmacol* **157**:1203–1214.
- Du J, Dong H, and Zhou HX (2012) Gating mechanism of a P2X4 receptor developed from normal mode analysis and molecular dynamics simulations. *Proc Natl Acad Sci USA* **109**:4140–4145.
- Eickhorst AN, Berson A, Cockayne D, Lester HA, and Khakh BS (2002) Control of P2X(2) channel permeability by the cytosolic domain. *J Gen Physiol* **120**:119–131.
- Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, and DiVirgilio F (2006) The P2X7 receptor: a key player in IL-1 processing and release. *J Immunol* **176**:3877–3883.
- Ford AP (2012) In pursuit of P2X3 antagonists: novel therapeutics for chronic pain and afferent sensitization. *Purinergic Signal* **8** (Suppl 1):3–26.
- Gargett CE and Wiley JS (1997) The isoquinoline derivative KN-62 a potent antagonist of the P2Z-receptor of human lymphocytes. *Br J Pharmacol* **120**:1483–1490.
- Gever JR, Cockayne DA, Dillon MP, Burnstock G, and Ford APDW (2006) Pharmacology of P2X channels. *Pflügers Arch* **452**:513–537.
- Gever JR, Soto R, Henningsen RA, Martin RS, Hackos DH, Panicker S, Rubas W, Oglesby IB, Dillon MP, and Milla ME et al. (2010) AF-353, a novel, potent and orally bioavailable P2X3/P2X2/3 receptor antagonist. *Br J Pharmacol* **160**: 1387–1398.
- Gordon JL (1986) Extracellular ATP: effects, sources and fate. *Biochem J* **233**: 309–319.
- Guile SD, Alcaraz L, Birkinshaw TN, Bowers KC, Ebdon MR, Furber M, and Stocks MJ (2009) Antagonists of the P2X(7) receptor. From lead identification to drug development. *J Med Chem* **52**:3123–3141.
- Gum RJ, Wakefield B, and Jarvis MF (2012) P2X receptor antagonists for pain management: examination of binding and physicochemical properties. *Purinergic Signal* **8** (Suppl 1):41–56.
- Hattori M and Gouaux E (2012) Molecular mechanism of ATP binding and ion channel activation in P2X receptors. *Nature* **485**:207–212.
- Hamilton SG and McMahon SB (2000) ATP as a peripheral mediator of pain. *J Auton Nerv Syst* **81**:187–194.
- Hausmann R, Günther J, Kless A, Kuhlmann D, Kassack MU, Bahrenberg G, Markwardt F, and Schmalzing G (2013) Salt bridge switching from Arg290/Glu167 to Arg290/ATP promotes the closed-to-open transition of the P2X2 receptors. *Mol Pharmacol* **83**:73–84.
- Hernandez-Olmos V, Abdelrahman A, El-Tayeb A, Freudendahl D, Weinhausen S, and Müller CE (2012) N-substituted phenoxazine and acridone derivatives: structure-activity relationships of potent P2X4 receptor antagonists. *J Med Chem* **55**:9576–9588.
- Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurdin P, Nurdin A, and Julius D et al. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* **409**:202–207.
- Honore P, Donnelly-Roberts D, Namovic MT, Hsieh G, Zhu CZ, Mikusa JP, Hernandez G, Zhong C, Gauvin DM, and Chandran P et al. (2006) A-740003 [N-(1-((cyanoimino)(5-quinolinylamino) methyl)amino-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide], a novel and selective P2X7 receptor antagonist, dose-dependently reduces neuropathic pain in the rat. *J Pharmacol Exp Ther* **319**:1376–1385.
- Honore P, Donnelly-Roberts D, Namovic M, Zhong C, Wade C, Chandran P, Zhu C, Carroll W, Perez-Medrano A, and Iwakura Y et al. (2009) The antihyperalgesic activity of a selective P2X7 receptor antagonist, A-839977, is lost in IL-1 α phosphatase knockout mice. *Behav Brain Res* **204**:77–81.
- Humphreys BD, Virginio C, Surprenant A, Rice J, and Dubyak GR (1998) Isoquinolines as antagonists of the P2X7 nucleotide receptor: high selectivity for the human versus rat receptor homologues. *Mol Pharmacol* **54**:22–32.
- Hurst R, Rolfe H, and Bertrand D (2012) Nicotinic acetylcholine receptors: from basic science to therapeutics. *Pharmacol Ther* DOI: 10.1016/j.pharmthera.2012.08.012.
- Iglesias R, Locovei S, Roque A, Alberto AP, Dahl G, Spray DC, and Scemes E (2008) P2X7 receptor-Pannexin1 complex: pharmacology and signaling. *Am J Physiol Cell Physiol* **295**:C752–C760.
- Inoue K (2006) The function of microglia through purinergic receptors: neuropathic pain and cytokine release. *Pharmacol Ther* **109**:210–226.
- Inoue K, Tsuda M, and Tozaki-Saitoh H (2007) Modification of neuropathic pain sensation through microglial ATP receptors. *Purinergic Signal* **3**:311–316.
- Jacobson KA and Boeynaems J-M (2010) P2Y nucleotide receptors: promise of therapeutic applications. *Drug Discov Today* **15**:570–578.
- Jacobson KA, Costanzi S, Joshi BV, Besada P, Shin DH, Ko H, Ivanov AA, and Mamedova L (2006) Agonists and antagonists for P2 receptors. *Novartis Found Symp* **276**:58–68, discussion 68–72, 107–112, 275–281.
- Jacobson KA, Jarvis MF, and Williams M (2002) Purine and pyrimidine (P2) receptors as drug targets. *J Med Chem* **45**:4057–4093.
- Jarvis MF (2010) The neural-glial purinergic receptor ensemble in chronic pain states. *Trends Neurosci* **33**:48–57.
- Jarvis MF, Burgard EC, McGaraughty S, Honore P, Lynch K, Brennan TJ, Subieta A, Van Biesen T, Cartmell J, and Bianchi B et al. (2002) A-317491, a novel potent and selective non-nucleotide antagonist of P2X3 and P2X2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat. *Proc Natl Acad Sci USA* **99**: 17179–17184.
- Jarvis MF and Khakh BS (2009) ATP-gated P2X cation-channels. *Neuropharmacology* **56**:208–215.
- Jarvis MF, Wismer CT, Schweitzer E, Yu H, van Biesen T, Lynch KJ, Burgard EC, Kowaluk EA, and Kowaluk EA (2001) Modulation of BzATP and formalin induced nociception: attenuation by the P2X receptor antagonist, TNP-ATP and enhancement by the P2X(3) allosteric modulator, cibacron blue. *Br J Pharmacol* **132**:259–269.
- Jelínková I, Vávra V, Jindřichová M, Obsil T, Zemkova HW, Zemkova H, and Stojilkovic SS (2008) Identification of P2X(4) receptor transmembrane residues contributing to channel gating and interaction with ivermectin. *Pflügers Arch* **456**: 939–950.
- Jiang L-H, Mackenzie AB, North RA, and Surprenant A (2000) Brilliant blue G selectively blocks ATP-gated rat P2X(7) receptors. *Mol Pharmacol* **58**:82–88.
- Jiang R, Taly A, Lemoine D, Martz A, Cunrath O, and Grutter T (2012) Tightening of the ATP-binding sites induces the opening of P2X receptor channels. *EMBO J* **31**: 2134–2143.

- Jo S and Bean BP (2011) Inhibition of neuronal voltage-gated sodium channels by brilliant blue G. *Mol Pharmacol* **80**:247–257.
- Kaczmarek-Hájek K, Lörinczi E, Hausmann R, and Nicke A (2012) Molecular and functional properties of P2X receptors—recent progress and persisting challenges. *Purinergic Signal* **8**:375–417.
- Kamei J and Takahashi Y (2006) Involvement of ionotropic purinergic receptors in the histamine-induced enhancement of the cough reflex sensitivity in guinea pigs. *Eur J Pharmacol* **547**:160–164.
- Krause RM, Buisson B, Bertrand S, Corringer PJ, Galzi JL, Changeux JP, and Bertrand D (1998) Ivermectin: a positive allosteric effector of the $\alpha 7$ neuronal nicotinic acetylcholine receptor. *Mol Pharmacol* **53**:283–294.
- Kawate T, Michel JC, Birdsong WT, and Gouaux E (2009) Crystal structure of the ATP-gated P2X(4) ion channel in the closed state. *Nature* **460**:592–598.
- Keystone EC, Wang MM, Layton M, Hollis S, and McInnes IB; D1520C00001 Study Team (2012) Clinical evaluation of the efficacy of the P2X7 purinergic receptor antagonist AZD9056 on the signs and symptoms of rheumatoid arthritis in patients with active disease despite treatment with methotrexate or sulphasalazine. *Ann Rheum Dis* **71**:1630–1635.
- Khakh BS, Bao XR, Labarca C, and Lester HA (1999a) Neuronal P2X transmitter-gated cation channels change their ion selectivity in seconds. *Nat Neurosci* **2**:322–330.
- Khakh BS and North RA (2012) Neuromodulation by extracellular ATP and P2X receptors in the CNS. *Neuron* **76**:51–69.
- Khakh BS, Proctor WR, Dunwiddie TV, Labarca C, and Lester HA (1999b) Allosteric control of gating and kinetics at P2X(4) receptor channels. *J Neurosci* **19**:7289–7299.
- Kim M, Jiang LH, Wilson HL, North RA, and Surprenant A (2001) Proteomic and functional evidence for a P2X7 receptor signalling complex. *EMBO J* **20**:6347–6358.
- Kleene SJ (1994) Inhibition of olfactory cyclic nucleotide-activated current by calmodulin antagonists. *Br J Pharmacol* **111**:469–472.
- Glöckner U and Isenberg G (1987) Calmodulin antagonists depress calcium and potassium currents in ventricular and vascular myocytes. *Am J Physiol* **253**:H1601–H1611.
- Krúšek J and Zemková H (1994) Effect of ivermectin on gamma-aminobutyric acid-induced chloride currents in mouse hippocampal embryonic neurones. *Eur J Pharmacol* **259**:121–128.
- Labasi JM, Petrushova N, Donovan C, McCurdy S, Lira P, Payette MM, Brissette W, Wicks JR, Audoly L, and Gabel CA (2002) Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol* **168**:6436–6445.
- Lewis CJ, Surprenant A, and Evans RJ (1998) 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP)—a nanomolar affinity antagonist at rat mesenteric artery P2X receptor ion channels. *Br J Pharmacol* **124**:1463–1466.
- Lörinczi E, Bhargava Y, Marino SF, Taly A, Kaczmarek-Hájek K, Barrantes-Freer A, Dutertre S, Grutter T, Rettinger J, and Nicke A (2012) Involvement of the cysteine-rich head domain in activation and desensitization of the P2X1 receptor. *Proc Natl Acad Sci USA* **109**:11396–11401.
- Lu SH, Groat WC, Lin AT, Chen KK, and Chang LS (2007) Evaluation of purinergic mechanism for the treatment of voiding dysfunction: a study in conscious spinal cord-injured rats. *J Chin Med Assoc* **70**:439–444.
- MacKenzie A, Wilson HL, Kiss-Toth E, Dower SK, North RA, and Surprenant A (2001) Rapid secretion of interleukin-1 β by microvesicle shedding. *Immunity* **15**:825–835.
- Masin M, Kerschensteiner D, Dümke K, Rubio ME, and Soto F (2006) Fe65 interacts with P2X2 subunits at excitatory synapses and modulates receptor function. *J Biol Chem* **281**:4100–4108.
- McGaraughty S, Chu KL, Namovic MT, Donnelly-Roberts DL, Harris RR, Zhang X-F, Shieh C-C, Wismer CT, Zhu CZ, and Gauvin DM et al. (2007) P2X7-related modulation of pathological nociception in rats. *Neuroscience* **146**:1817–1828.
- Miller CM, Boulter NR, Fuller SJ, Zakrzewski AM, Lees MP, Saunders BM, Wiley JS, and Smith NC (2011) The role of the P2X₇ receptor in infectious diseases. *PLoS Pathog* **7**:e1002212.
- Müller CE (2010) Emerging structures and ligands for P2X3 and P2X4 receptors—towards novel treatments of neuropathic pain. *Purinergic Signal* **6**:145–148.
- Nagaya N, Tittle RK, Saar N, Dellal SS, and Hume RI (2005) An intersubunit zinc binding site in rat P2X2 receptors. *J Biol Chem* **280**:25982–25993.
- North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* **82**:1013–1067.
- Perl ER (2007) Ideas about pain, a historical view. *Nat Rev Neurosci* **8**:71–80.
- Perregaux DG and Gabel CA (1994) Interleukin-1 β maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J Biol Chem* **269**:15195–15203.
- Priel A and Silberberg SD (2004) Mechanism of ivermectin facilitation of human P2X4 receptor channels. *J Gen Physiol* **123**:281–293.
- Rettinger J, Braun K, Hochmann H, Kassack MU, Ullmann H, Nickel P, Schmalzing G, and Lambrecht G (2005) Profiling at recombinant homomeric and heteromeric rat P2X receptors identifies the suramin analogue NF449 as a highly potent P2X1 receptor antagonist. *Neuropharmacology* **48**:461–468.
- Samways DS and Egan TM (2007) Acidic amino acids impart enhanced Ca²⁺ permeability and flux in two members of the ATP-gated P2X receptor family. *J Gen Physiol* **129**:245–256.
- Samways DS, Khakh BS, Dutertre S, and Egan TM (2011) Preferential use of unobstructed lateral portals as the access route to the pore of human ATP-gated ion channels (P2X receptors). *Proc Natl Acad Sci USA* **108**:13800–13805.
- Samways DS, Khakh BS, and Egan TM (2012) Allosteric modulation of Ca²⁺ flux in ligand-gated cation channel (P2X4) by actions on lateral portals. *J Biol Chem* **287**:7594–7602.
- Silberberg SD, Li M, and Swartz KJ (2007) Ivermectin Interaction with trans-membrane helices reveals widespread rearrangements during opening of P2X receptor channels. *Neuron* **54**:263–274.
- Soltoff SP, McMillian MK, and Talamo BR (1989) Coomassie Brilliant Blue G is a more potent antagonist of P2 purinergic responses than Reactive Blue 2 (Cibacron Blue 3GA) in rat parotid acinar cells. *Biochem Biophys Res Commun* **165**:1279–1285.
- Sorge RE, Trang T, Dorfman R, Smith SB, Beggs S, Ritchie J, Austin JS, Zaykin DV, Vander Meulen H, and Costigan M et al. (2012) Genetically determined P2X7 receptor pore formation regulates variability in chronic pain sensitivity. *Nat Med* **18**:595–599.
- Surprenant A, Rassendren F, Kawashima E, North RA, and Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science* **272**:735–738.
- Tittle RK, Power JM, and Hume RI (2007) A histidine scan to probe the flexibility of the rat P2X2 receptor zinc-binding site. *J Biol Chem* **282**:19526–19533.
- Tozaki-Saitoh H, Tsuda M, and Inoue K (2011) Role of purinergic receptors in CNS function and neuroprotection. *Adv Pharmacol* **61**:495–528.
- Trang T and Salter MW (2012) P2X4 purinoceptor signaling in chronic pain. *Purinergic Signal* **8**:621–628.
- Udem BJ and Nassenstein C (2009) Airway nerves and dyspnea associated with inflammatory airway disease. *Respir Physiol Neurobiol* **167**:36–44.
- Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, and Buell G (1994) A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. *Nature* **371**:516–519.
- Virginio C, Robertson G, Surprenant A, and North RA (1998) Trinitrophenyl-substituted nucleotides are potent antagonists selective for P2X1, P2X3, and heteromeric P2X2/3 receptors. *Mol Pharmacol* **53**:969–973.
- Webb TE, Simon J, Krishek BJ, Bateson AN, Smart TG, King BF, Burnstock G, and Barnard EA (1993) Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett* **324**:219–225.
- Werner P, Seward EP, Buell GN, and North RA (1996) Domains of P2X receptors involved in desensitization. *Proc Natl Acad Sci USA* **93**:15485–15490.
- Wickenden A, Priest B, and Erdemli G (2012) Ion channel drug discovery: challenges and future directions. *Future Med Chem* **4**:661–679.
- Wilson HL, Wilson SA, Surprenant A, and North RA (2002) Epithelial membrane proteins induce membrane blebbing and interact with the P2X7 receptor C terminus. *J Biol Chem* **277**:34017–34023.
- Wolf C, Rosefort C, Fallah G, Kassack MU, Hamacher A, Bodnar M, Wang H, Illes P, Kless A, and Bahrenberg G et al. (2011) Molecular determinants of potent P2X2 antagonism identified by functional analysis, mutagenesis, and homology docking. *Mol Pharmacol* **79**:649–661.
- Woolf CJ (2011) Central sensitization: implications for the diagnosis and treatment of pain. *Pain* **152**(3, Suppl):S2–S15.
- Ziyal R, Ziganshin AU, Nickel P, Ardanuy U, Mutschler E, Lambrecht G, and Burnstock G (1997) Vasoconstrictor responses via P2X-receptors are selectively antagonized by NF023 in rabbit isolated aorta and saphenous artery. *Br J Pharmacol* **120**:954–960.

Address correspondence to: R. Alan North, Michael Smith Building, Faculty of Medical and Human Sciences, University of Manchester, Oxford Road, Manchester M13 9PL, UK. E-mail: r.a.north@manchester.ac.uk