



## Review

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# P2X receptors

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Extracellular adenosine 5'-triphosphate (ATP) activates cell surface P2X and P2Y receptors. P2X receptors are membrane ion channels preferably permeable to sodium, potassium and calcium that open within milliseconds of the binding of ATP. In molecular architecture, they form a unique structural family. The receptor is a trimer, the binding of ATP between subunits causes them to flex together within the ectodomain and separate in the membrane-spanning region so as to open a central channel. P2X receptors have a widespread tissue distribution. On some smooth muscle cells, P2X receptors mediate the fast excitatory junction potential that leads to depolarization and contraction. In the central nervous system, activation of P2X receptors allows calcium to enter neurons and this can evoke slower neuromodulatory responses such as the trafficking of receptors for the neurotransmitter glutamate. In primary afferent nerves, P2X receptors are critical for the initiation of action potentials when they respond to ATP released from sensory cells such as taste buds, chemoreceptors or urothelium. In immune cells, activation of P2X receptors triggers the release of pro-inflammatory cytokines such as interleukin 1 $\beta$ . The development of selective blockers of different P2X receptors has led to clinical trials of their effectiveness in the management of cough, pain, inflammation and certain neurodegenerative diseases.

This article is part of the themed issue 'Evolution brings Ca<sup>2+</sup> and ATP together to control life and death'.

## 1. Beginnings of the field

The study of the proteins that came to be known as P2X receptors has three distinct origins. The earliest was the description by Burnstock & Holman [1,2] of the junction potentials (ejps) recorded with glass microelectrodes from smooth muscle cells of the guinea pig vas deferens. By analogy with the similar depolarizations observed in skeletal muscle (endplate potentials), the inference was that the transmitter released from the nerve briefly increased the permeability of the smooth muscle membrane to cations. Noradrenalin was thought to be the transmitter released from the sympathetic nerves, a conclusion buttressed by the finding in 1970 that pretreatment with 6-hydroxydopamine to destroy sympathetic nerves abolished the ejp [3].

By 1978, evidence was becoming compelling that adenosine 5'-triphosphate (ATP) was the sympathetic transmitter in the bladder [4] and the use of the analogue  $\alpha\beta$ -methylene-ATP ( $\alpha\beta$ meATP) as a desensitizing blocker extended this conclusion to the vas deferens [5]. In 1985, Burnstock & Kennedy [6] characterized such actions of ATP, which were mimicked and/or blocked by  $\alpha\beta$ meATP, as involving P2X receptors. These were distinguished from P2Y receptors, at which 2-methyl-thio-ATP was a more effective agonist and activation of which typically led to smooth muscle relaxation. It is important to recognize that these studies on the vas deferens and bladder had an intrinsically *physiological* context, in the sense that they were directed at understanding the effects of ATP released from nerve cells, specifically post-ganglionic sympathetic nerves.

The other two origins of P2X receptors were more *pharmacological*, in the sense that they involved studies of the action of exogenous ATP. One was the observation that ATP caused the release of histamine from mast cells and that this was associated with an increase in permeability of the mast cell membrane [7]. The most effective form of ATP appeared to be ATP<sup>4-</sup>. This tetrabasic form of

the molecule forms only a small fraction of the total ATP in physiological solutions, most being complexed as MgATP or CaATP. The permeability increase was monitored as the release of  $^{32}\text{P}$ -labelled intermediary metabolites such as phosphatidyl inositol following pre-loading of the cells with  $^{32}\text{P}$  inorganic phosphate, but it could also be followed as the uptake of fluorescent dyes such as ethidium or propidium [8]. Several other cells types were found that responded to  $\text{ATP}^{4-}$ , including macrophages, neutrophils, gland cells and endothelium, and the receptor involved became called P2Z (reviewed in [9]).

The third original approach was electrophysiological: in 1983, three separate groups studied the action of exogenous ATP on membrane potential or currents. Recording from sensory neurons cultured from rat dorsal root ganglia, Krishtal and co-workers [10] showed that ATP (1–100  $\mu\text{M}$ ) evoked an inward membrane current within milliseconds of its application. This resulted from an increase in conductance to cations. Kolb & Wakelam [11] recorded the single channel currents elicited by application of ATP (10  $\mu\text{M}$ ) to chicken muscle cells, which had properties indicating an increase in conductance to sodium and potassium ions. Later that year, Jahr & Jessell [12] confirmed the findings of Krishtal *et al.* and further distinguished the rapidly desensitizing depolarization of rat dorsal root ganglion cells induced by ATP from the more sustained depolarization observed in neurons cultured from the dorsal horn of the spinal cord.

More detailed biophysical characterization of ATP-induced currents followed, for smooth muscle [13], sensory neurons [14,15], pheochromocytoma cells [16] and locus coeruleus neurons [17], and three papers presented additional evidence for synaptic transmission mediated directly by ATP [18–20]. On the other hand, the lack of selective and potent antagonists hampered any conclusive demonstration of the physiological function of ATP-operated P2X receptors.

## 2. Following cDNA cloning

The decade beginning in 1983 was a period in which cDNAs were isolated for almost all membrane ion channels [21,22] and P2X receptors joined this group in 1994. A team at the Glaxo Institute for Molecular Biology in Geneva [23] identified a cDNA encoding the P2X1 receptor by injecting oocytes with progressive fractions of a cDNA expression library made from RNA extracted from the rat vas deferens. A similar approach starting with the RNA from PC12 cells was used by Brake *et al.* [24] to clone the P2X2 receptor. The five further members of the family were identified by homology-based approaches from a wide range of tissues [25] and the seven mammalian genes were subsequently identified. The properties of the P2X7 receptor, most notably its relative low sensitivity to ATP and the permeability to larger molecular weight dyes, indicated that it corresponded to the P2Z receptor named by Gordon in 1986 [9]. It was found that P2X receptor genes were widely expressed throughout vertebrates and lower eukaryotic organisms, but unlike several other ion channels they have not been found in prokaryotes. The genes can be identified in green algae, which represent the earliest separation of animals from plants ([26], see also [27]). In the slime mould *Dictyostelium discoides*, P2X receptors have a predominantly intracellular distribution and function [28,29].

Many inferences about molecular structure became possible as a result of the expression of cDNAs, particularly when

biophysical measurements were combined with mutagenesis. It was immediately clear that each P2X receptor subunit had two membrane-spanning domains (TM1 and TM2), with intracellular N- and C-terminus, and that most of the protein was located as a large ectodomain. Several lines of evidence indicated that the functional protein was a trimer: indeed, three ATP-binding sites had been suggested by Bean [14] on the basis of his ATP dose–response curves from bullfrog sensory neurons. This evidence included biochemical approaches using blue native polyacrylamide gel electrophoresis [30] and functional approaches with co-expression and concatenation of subunits carrying reporter mutations [31–35]. The demonstration that P2X receptors were trimers set them in clear distinction from the tetrameric glutamate-gated ion channels and the pentameric nicotinic superfamily (which also includes channels gated by glycine,  $\gamma$ -aminobutyric acid and 5-hydroxytryptamine; [22]). These approaches also showed that functional channels could form as hetero- or homo-trimers (e.g. P2X2/3 and P2X1/5 receptors; [25]).

The P2X receptor genes contain 10–12 introns and are found on five chromosomes [25]. The genes for P2X1 and P2X5 receptors, and for P2X4 and P2X7 receptors, are adjacent: this presumably reflects relatively recent duplication. The only channelopathy described is a loss-of-function mutation in the P2X2 receptor that results in hearing loss in Chinese [36] and Italian [37] families. Other efforts to associate single nucleotide polymorphisms with disease propensity in humans have revealed several but rather weak associations: these have been comprehensively reviewed for the P2X7 receptor [37,38].

Determination of the distribution of the P2X receptors also followed the molecular cloning, at either the RNA or protein level ([39–42], see also [25]). These studies indicated that P2X receptors were much more widely expressed through vertebrate tissues than had been previously anticipated on the basis of functional studies. Notable examples were the predominant expression of P2X2, P2X4 and P2X6 subunits in the central nervous system, the abundance of P2X4 and P2X7 receptors in glandular tissue and immune cells and the very limited distribution of P2X3 subunits in a subset of sensory neurons involved in taste, bladder filling, baroreception and certain modalities of pain [25,43].

It has become clear that ATP does not have any widespread direct role as a fast neurotransmitter in the central nervous system [43,44]. P2X receptors are found on glia and neurons. On astrocytes in mouse cortex, the predominant form is a P2X1/P2X5 heteromer [45], whereas microglia express mostly the P2X7 receptor [40,46]. On neurons in the hippocampus, P2X4 receptors are located at the periphery of the post-synaptic density [47]. Recent evidence suggests that ATP released from astrocytes can activate these receptors and lead to a reduction in the trafficking of  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors to the synapse [48]. The P2X4 receptor has a relatively high calcium permeability [49] which, unlike the case of the NMDA receptor, allows calcium entry at both hyperpolarized and depolarized membrane potentials. This calcium entry appears to inhibit AMPA receptor trafficking by a mechanism involving calmodulin-dependent kinase II and/or a calcium-dependent phosphatase [48]. A wider role for P2X receptors in the operation or trafficking of other ion channels has long been suspected [50–52] and is now beginning to be worked out in molecular detail [53–55].

In the peripheral nervous system, important new roles for P2X receptors in afferent signalling have been established.

ATP is released from chemosensing cells to activate P2X receptors in terminals of the carotid sinus nerve [56]. ATP release by taste buds is the first step in all modalities of taste sensation: the ATP activates P2X2/P2X3-subunit-containing receptors on the gustatory nerve [57]. An analogous signalling role from urothelium to primary afferent nerves has been inferred for sensing bladder distension [58].

Release of inflammatory cytokines by ATP was well known before the cDNA cloning, but the expression of P2X7 receptors on a wide range of immune cells has prompted an intensive study of their role in inflammation [59,60]. The availability of mice lacking P2X7 receptors, as well as the development of a range of antagonists, is now documenting roles for ATP signalling in skin [61], bone [62,63], glandular epithelium [64] and cancer cells [65].

### 3. Molecular *modus operandi*

The determination of the structure of a truncated zebrafish P2X4 receptor by X-ray crystallography (closed state [66]; open state and closed states [67]) essentially confirmed the inferences based on 15 years of work of mutagenesis combined with functional expression [68,69]. Of course, it achieved much more than that. It provided an immediate structural view of a novel family of membrane proteins, in which the pore-forming region extends to include the epithelial sodium channel (ENaC) and acid-sensing ion channel (ASIC) [70]. It indicated a new type of gated pore formation in biological membranes. It explained how ATP was a selective agonist without the requirement for hydrolysis [71]. It provided the atomic template for drug development (see reviews [68,72,73]).

Each protomer of the truncated P2X receptor resembles a dolphin [66]. The tail flukes are missing but would be positioned inside the cell, with the posterior part of the body (the peduncle) traversing the membrane, and the bulk of the body with dorsal fin, left and right flippers, head and beak protruding into the extracellular solution. The contacts with ATP are provided by eleven amino acids, four from one subunit and seven from another [67] (figure 1). The binding of ATP results in the head domain of one dolphin subunit being pulled downwards toward the left flipper of the adjacent subunit (figure 1). This torsions the body region outwards so as to increase the separation between the three subunits, opening three lateral portals through which ions can enter the central vestibule. It also pulls apart the six transmembrane domains, of which the three TM2 line the central axis of the permeation pathway (figure 1).

These movements can be readily deduced by a comparison of the crystal structures of the closed [66] and open (ATP-bound) [67] states, and they are strongly supported by several functional approaches. For example, disulfide locking of pairs of engineered cysteine residues has provided direct evidence for the approximation of amino acid residues [32,77–84]. The open–closed transition of the receptor can also be driven by light in the complete absence of ATP, when a light-sensitive azobenzene molecule is incorporated into the receptor by attachment to cysteine residues in two different subunits [85,86]. The *cis*–*trans* isomerization of the azobenzene by 440 nm irradiation pushes apart residues (P329C) in two adjacent subunits to open the channel, and the *trans*–*cis* conformational change at 360 nm conversely closes the channel [86]. A third application of cysteine substitution has been made by attaching lipophilic

side chains at a position likely to move through the outer lipid leaflet of the membrane. Thus, in the P2X2[I328C] receptor, the addition of a propyl-methanethiosulfonate is as effective to open the channel as ATP itself [87].

From the structural point of view, the zebrafish P2X4 receptor that was used for X-ray crystallography lacks both the intracellular N- and C-terminus. Although such truncated molecules do form functional channels [66], it is known that the parts of these termini that are close to the transmembrane domains contain highly conserved short amino acid sequences that are important determinants of normal expression and function. These are YXTX[K/R] before TM1, and YXXXK after TM2. It is not difficult to imagine the folding of six YXXXK domains (two from each subunit) so as to form a stable ‘hanging basket’ in the cytoplasm internal to the inner opening of the permeation pathway, stabilized perhaps by interactions between the positive lysine side chains and the  $\pi$  electron clouds of the Tyr residues. Currents evoked by ATP at P2X1 receptors show rapid desensitization (within tens of milliseconds), whereas currents at P2X2 receptors are sustained for several seconds. Transfer of the protein segments immediately before TM1 has profound effects on this desensitization [88]. Similarly, deletion of a cysteine-rich region that is found after TM2 only in the P2X7 receptor has effects on the time course of the ATP-induced current as well as on the passage of large fluorescent dyes through the P2X7 channel [89]. There will be much more to learn from a crystal structure of the holoprotein.

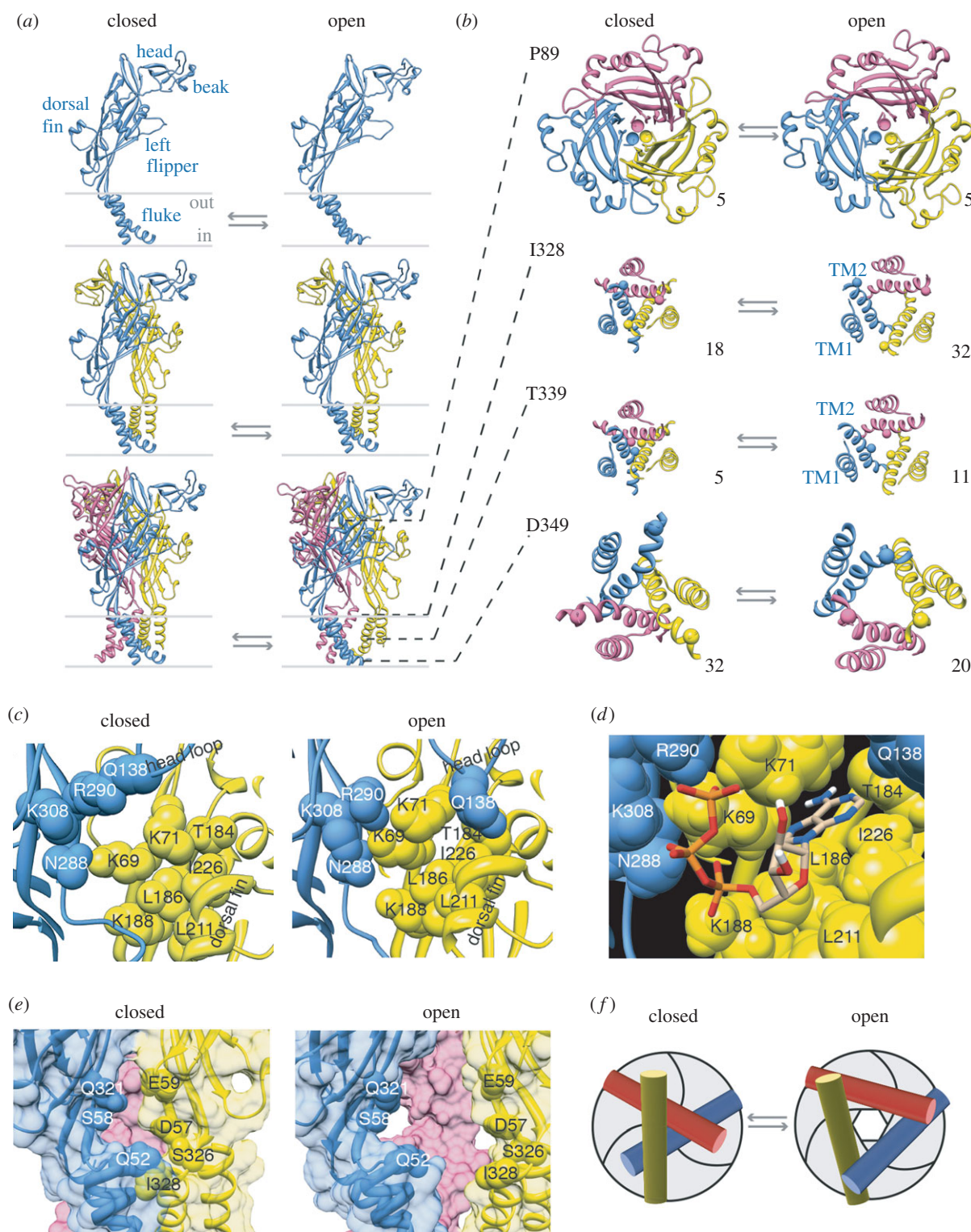
The present open channel structure provides a pathway through the membrane for small cations. In the zebrafish P2X4 receptor the C $\alpha$  atoms of the alanine (A347) residues lie on a circle with diameter of 1.2 nm [67], and the same is true for S342, which lies at the narrowest part of the pore in models of the rat P2X7 receptor [90]. However, the activated rat P2X7 receptor allows the permeation of dyes as large as sulforhodamine methane thiosulfonate, which has dimensions of  $0.90 \times 1.40 \times 1.65$  nm [90]. This implies that some P2X receptors can also adopt conformations with a wider permeation pathway. The ASIC also forms a permeation pathway by obliquely intersecting TM2 segments. It has been shown to exhibit two open states, one with a wider permeation pathway, depending on the extracellular pH [91].

### 4. Therapeutic exploitation

P2X7 receptors have been the most intensively investigated and many pharmaceutical companies have synthesized small molecules that are potent and selective blockers of the human receptor [92]. These include Abbott, Actelion, Affectis, AstraZeneca, Evotec, GlaxoSmithKline, Janssen, Johnson and Johnson, Merck, Neurogen, Nissan Chemical, Pfizer, Roche and Schering [92,93]. Because the activation of P2X7 receptors by ATP is a key step in the release of inflammatory cytokines from microglia primed with bacterial lipopolysaccharide [94], P2X receptors have long been considered as possible therapeutic targets in inflammatory pain [95,96]. Initial hopes that they may have efficacy were disappointed in Phase IIb trials in rheumatoid arthritis [97,98]. Ongoing studies include preclinical work using animal models of neuropsychiatric and neurodegenerative disease [38].

P2X3 receptors have a very limited distribution on primary afferent fibres and they have been targeted for visceral pain [99] and, more recently, for the treatment of chronic cough. A P2X3





**Figure 1.** P2X receptor structure, the ATP-binding site and the permeation pathway. (a) Assembly of P2X2 receptors from three subunits, depicted in closed (*left*) and open (*right*) states. Upper, middle and lower panels show one, two and three subunits, respectively. (b) Cross-sections of open and closed rat P2X2 receptors at the level of the residues indicated, the C $\beta$  atoms of which are shown as spheres. Number in lower right of each panel is the diameter of the circle (in Å), passing through the three C $\beta$  atoms. D349 viewed from below, others viewed from above. (c) Key residues in the ATP-binding pocket, in open and closed configurations. Two subunits depicted. (d) ATP molecule positioned in binding pocket. The U-shape of the triphosphate chain curls around the nitrogen atom of K69. (e) The lateral portal between two subunits (blue and yellow; pink subunit visible through the portal). Key residues on the edge of the portal are indicated. (f) Schematic to illustrate iris-like movement of TM2 domains. Molecular models created in Modeller 9v7 [74] using 4DW0 (closed) and 4DW1 (open) as templates, energy minimized using MolProbity [75] and displayed in Chimera 1.6 [76].

receptor antagonist from Afferent Pharmaceuticals (AF-219) was an effective antitussive in a randomized, double-blind, placebo-controlled phase 2 study [100,101]. One confounding factor with such trials of P2X3 receptor antagonists will be the difficulty in conducting 'blind' trials. ATP is a transmitter released by taste buds and it activates P2X3-subunit-containing

receptors on gustatory nerves [57], and all the patients in the trial who took AF-219 reported disturbance of taste [99].

P2X1 receptors were first identified in the vas deferens and mice lacking the receptor have no ejps and impaired ejaculation [102]. Noradrenalin that is also released from sympathetic nerves contracts the vas deferens by activating

$\alpha$ 1A adrenoceptors, but without membrane depolarization. The possibility of further exploiting these effects in the development of a male contraceptive has recently been boosted by the demonstration that mice lacking both P2X1 receptors and  $\alpha$ 1A receptors are completely infertile [103].

**Competing interests.** I declare I do not have any competing interest.

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