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INVITED REVIEW

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Molecular properties of P2X receptors

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Abstract P2X receptors for adenosine tri-phosphate (ATP) are a distinct family of ligand-gated cation channels with two transmembrane domains, intracellular amino and carboxy termini and a large extracellular ligand binding loop. Seven genes ($P2X_{1\text{-}7}$) have been cloned and the channels form as either homo or heterotrimeric channels giving rise to a wide range of phenotypes. This review aims to give an account of recent work on the molecular basis of the properties of P2X receptors. In particular, to consider emerging information on the assembly of P2X receptor subunits, channel regulation and desensitisation, targeting, the molecular basis of drug action and the functional contribution of P2X receptors to physiological processes.

Keywords ATP · P2X receptors · Regulation · Transgenic mice · Mutagenesis

Introduction

P2X receptors for adenosine tri-phosphate (ATP) are ligand-gated cation channels. The receptors were initially classified based on their pharmacological properties and subsequently recordings of P2X receptor channel activity leading to depolarisation of neurons and smooth muscle were described. P2X receptors are expressed throughout the body and have been shown to be involved in a wide range of physiological processes from sensory perception to the control of smooth muscle. Genes encoding P2X receptors were first identified by expression cloning from rat vas deferens [1] and phaeochromocytoma PC12 cells [2], subsequent endeavours revealed a family of seven P2X receptor subunits ($P2X_{1\text{–}7}$) [3]. These subunits form multimeric channels and a range of P2X receptor phenotypes have been described resulting from the homo- and heteromeric assembly of channels, regulation by alternative splicing, phosphorylation and interaction with other proteins. There have been several reviews on the molecular properties of P2X receptors [4–9]. The aim of this article is to give an overview of the receptors and an update on recent work identifying the molecular basis of their properties.

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Stoichiometry and the emergence of structural information

The P2X receptor subunit has two transmembrane domains, intracellular amino and carboxy termini and a large extracellular ligand-binding loop [1, 2, 10, 11]. It seemed unlikely that the two transmembrane domains would be able to form an ion-conducting pore on their own and this suggested that subunits associate to form multimeric channels. The first direct support for the multi-meric assembly of P2X receptor subunits came from co-expression studies of $P2X_2$ and $P2X_3$ receptors that assembled to form $P2X_{2/3}$ heteromeric channels with a novel phenotype [12]. A major advance in our understanding of the number of P2X receptor subunits that are required to form a functional channel was shown in cross-linking studies, where the receptors were detected as trimers [13, 14]. Trimeric assembly has also been supported by work on concatenated $P2X_2$ receptor channels [15, 16] and is consistent with a model of three ATP molecules binding to describe channel opening at native bullfrog sensory neuron P2X receptors [17], and recombinant $P2X_2$ receptor channels [18].

High-resolution microscope studies and crystal structures of ligand-binding domains have been important in visualising 3D structural information and the subunit stoichiometry of many ligand-gated ion channels. For example, the extracellular ligand-binding domain of glutamate receptors has been crystallised and solved as a

tetramer (for a review, see [19]). When just the extracellular loop of $P2X_2$ receptors was expressed, it formed as a tetrameric structure [20]. This is at odds with cross-linking studies (and microscopy; see below) and suggests that the extracellular domain may not multimerise correctly without the transmembrane domains that act to anchor the extracellular loop. Recent studies using atomic force microscopy (AFM) and electron microscopy of purified $P2X_2$ receptors have given us the first pictures of the receptors. AFM allows single isolated uncrystallised proteins to be studied and Barrera et al. used this technique to estimate the dimensions of the $P2X_2$ receptor (volume 409 nm^3). In addition, by using antibodies against introduced epitopes at the amino termini, they determined the bond angle of ~ 120° between the subunits consistent with a trimer [21]. AFM has also been performed on recombinant $P2X_2$ channels isolated from insect cells and imaged in an aqueous environment [22]. Electron microscopy has been used to image nicotinic acetylcholine receptors and resolve their pentameric structure [23] and a similar approach was utilised by Mio et al. to look at recombinant $P2X_2$ receptors purified from insect cells [24]. They showed that the $P2X_2$ receptor forms as a crown-capped inverted three-sided pyramid.

Heteromeric channels

The initial $P2X_2$ and $P2X_3$ receptor co-expression studies demonstrated that the receptors can form as heteromeric channels [12] and, subsequently, the rules governing what channels can associate and their resultant properties have been revealed. An extensive study looking at the co-immunoprecipitation of epitope-tagged P2X receptor subunits, provided the initial benchmark of possible subunit combinations [25] and further studies have indicated that the cellular environment may also contribute to what combinations are possible [26]. $P2X_{1,2,3,4\ and\ 5}$ receptor subunits can form as part of either homo- or heteromeric assemblies [25] and the second transmembrane domain may contribute to the likelihood of subunit association [27]. In contrast, $P2X_6$ receptors seemed only to assemble in heteromeric channels and $P2X_7$ receptors did not heteropolymerise with other P2X receptor subunits. In the majority of functional studies, $P2X_6$ receptors fail to form functional channels and it appears that the inability to form functional homomeric channels may arise from the incorrect processing of the receptor in the ER or the absence of a particular auxillary subunit/protein required for efficient assembly and trafficking.

In recombinant systems, several novel P2X receptor phenotypes have been described that are composites of the constituent subunits: $P2X_{1/2}$ [28], $P2X_{1/4}$ [26], $P2X_{1/5}$ [29, 30], $P2X_{2/3}$ [12], $P2X_{2/6}$ [31], $P2X_{4/6}$ [32] (for properties, see Table 1). Co-expression studies indicate the production of a heteromeric channel but give no indication of the subunit stoichiometry. In a study using substituted cysteine residues in $P2X_2$ and $P2X_3$ receptor subunits, Jiang et al. showed that the $P2X_{2/3}$ receptor is likely to form with the stoichiometry of one $P2X_2$ receptor subunit and two $P2X_3$ subunits; he also gave insight into the proximity of the residues at the outer vestibule of the P2X receptor pore [33]. In addition, developments of the AFM technique should allow for the determination of the stoichiometry of heteromeric P2X receptor assemblies to further our understanding of the contribution of individual subunits to heteromeric channels. So far, heteromerisation has only been described when two different subunits associate, and it remains to be seen whether three different subunits can form a functional channel.

Given that several P2X receptor subunits can be expressed in some cells, this indicates that multiple P2X receptor sub-types may be produced. For example, in sensory neurons, phenotypes corresponding to homomeric $P2X_2$ and $P2X_3$ as well as heteromeric $P2X_{2/3}$ channels have been described in a single neuron [34–36]. How the

Table 1 A table summarising the properties of P2X receptors

	ATP EC_{50} (μM)	α,β-meATP sensitivity	Current decay	Effect of High Ca^{2+}	Effect of High pH	Effect of Zn^{2+} (10 μM)
$P2X_1$	1	Yes	Fast	No effect	No effect[†]	Decrease
$P2X_2$	10	No	Slow	Decrease	Decrease	Increase
$P2X_3$	1	Yes	Fast	No effect	No effect[†]	Increase
$P2X_4$	7	No	Slow	–	No effect[†]	Increase
$P2X_5$[a]	0.5	No	Slow	Decrease	No effect	Increase
$P2X_6$[b]	0.5	Yes	Slow	–	–	–
$P2X_7$	>100	No	Slow	Decrease	–	Decrease
$P2X_{1/2}$[c]	0.6	Yes	Fast / slow	–	Increase	–
$P2X_{1/4}$[d]	–	Yes	Slow	–	–	–
$P2X_{1/5}$	~ 5	Yes	Fast / slow	No effect	Decrease	–
$P2X_{2/3}$	1	Yes	Slow	Decrease	Decrease	Increase
$P2X_{2/6}$	32	No	Slow	–	Decrease	Increase
$P2X_{4/6}$	6	Yes	Slow	–	–	Increase[††]

A dash signifies that these conditions have not been studied for that receptor (data taken from [4, 163; [a]57; [b]147; [c]28; [d]26; [†]164; [††]32;]

association of the subunits is regulated and whether co-expression may result in differential targeting of the receptors remain to be determined.

Basic properties of the channels

The properties of P2X receptor are dependent on the subunit composition of the channel, and studies on recombinant receptors have characterised their profiles [4]. The different channels can be discriminated to some extent based on their time-course, agonist and antagonist profile and sensitivity to regulation by extracellular calcium, protons and zinc (see Table 1). In many cases, the properties of native P2X receptors correspond closely with those of recombinant channels, and these have been substantiated by studies with knockout mice. However, in native preparations multiple P2X receptor sub-types may be expressed complicating the attribution of the functional molecular subtypes.

Time-course of the response

P2X receptor channels open rapidly following ATP binding. The subsequent time-course of the response is dependent on the receptor in question and can be subject to regulation. In general, $P2X_1$ and $P2X_3$ receptors are classified as those showing rapid desensitisation with responses to sustained application of maximal agonist concentration decaying by >90% within 1–2 s. The rest of the homomeric and heteromeric $P2X_{2/3}$ receptors show relatively sustained responses to 1–2 s applications of agonist, and other heteromeric channels have responses with fast and slow components (see Table 1). There are some reported differences in the time-course between different studies and expression systems, suggesting that the time-course may be subject to regulation by expression level, cell type or local environment. The molecular basis of the time-course of P2X responses has been addressed in a range of studies. Initial work with chimaeric P2X receptors demonstrated that both the transmembrane domains could contribute to the time-course [37]. Subsequently, analysis of splice variants of $P2X_2$ receptors indicated that the intracellular C-terminal tail regulated the time-course [38–40]. Recent work has shown that part of the C-terminal tail is involved in the formation of the dilating pore of rat $P2X_2$ receptors [41], and the mutation Y378A in the $P2X_4$ receptor leads to instantaneous opening of a dilated state following activation by ATP [42]. The contribution of the intracellular amino terminus was shown following mutation of a protein kinase C site that is present in the amino terminus and conserved throughout the P2X receptor family. This mutation leads to a reduction in peak current amplitude and a speeding in the time-course of a range of P2X receptors [43–45]. Mutations in the extracellular domain of $P2X_1$ receptors can also significantly change the time-course of the response as well as ATP potency [46]. Chimaeras swapping portions of the extracellular domain have suggested that the amino terminal half of the extracellular domain contributes to desensitisation and the C terminal half to the stability of the open channel [7, 47]. Taken together, these studies show that regulation of the time-course of P2X receptor channels is a multi-component process involving interactions of several regions of the receptor.

Desensitisation and recovery

Desensitisation is a way of modifying the responsiveness to sustained/repeated applications of ATP and consists of a cycle of (1) transition into the desensitised state corresponding, most likely, to a ligand-bound closed form of the receptor and then (2) a process of recovery from desensitisation (agonist unbinding and, possibly, a recovery step from the agonist-free desensitised to recovered state) when the channel can be activated again. For example, at $P2X_1$ and $P2X_3$ receptors, desensitisation (decay of current in the continued presence of agonist) takes ~ 1–2 s and then following washout of agonist, there is a recovery period (≥5 min) before a subsequent equivalent response can be evoked.

In studies on recombinant rat $P2X_1$ receptors expressed in *Xenopus* oocytes, Rettinger and Schmalzing characterised the desensitisation and recovery of the receptor [48]. They showed that the channel needs to open before it can go into a desensitised state, and although the EC_{50} for the peak current response is ~ 0.7 μM, sustained stimulation with low nanomolar concentrations of agonist can desensitise the receptor. This would be expected from the cumulative activation of receptors and then passing into the desensitised state, making them unavailable for subsequent activation and the long time required for recovery from desensitisation [48]. Modelling of these effects gives an EC_{50} of steady state response of <100 nM and indicates that agonist unbinding from the receptor contributes to recovery from desensitisation. Support that the rate-limiting step for recovery from desensitisation is the unbinding of the agonist from the ligand-bound desensitised receptor comes from further studies on $P2X_{1/2}$ chimeras with the intracellular amino terminus and first transmembrane domain of $P2X_2$ and the extracellular, TM2 and intracellular carboxy terminal from the rat $P2X_1$ receptor [49]. In this chimera, the EC_{50} for ATP is ~ 3 nM (consistent with previous modelling studies). The rate of decay of the current on removal of the agonist is slow for ATP and for a range of agonists, there is an inverse relationship between the EC_{50} value and the wash-off rate of the response. But even under these conditions, the time constant for ATP unbinding of 63 s of the chimera is much faster than the time taken for the $P2X_1$ receptor to recover from desensitisation, suggesting that there is an additional regulatory process in recovery.

At $P2X_3$ receptors recovery from desensitisation seems to be dependent on the agonist used to activate the receptor [50, 51]. For example, at recombinant human $P2X_3$ receptors, the EC_{50} value and time-course of desensitisa-

tion are essentially the same for ATP and αβ-meATP; however, recovery from desensitisation of ATP responses is three times slower than for those to αβ-meATP [51]. This clearly shows that the recovery from desensitisation is a separate process from the inactivation/desensitisation in the continued presence of agonist. In radioligand binding studies, Pratt et al. [51] showed that the rate of unbinding of [^{32}P]-ATP was similar to that of recovery from desensitisation, this suggests that the agonist remains bound to the desensitised receptor and once the agonist is unbound the receptor can recover quickly and be available once again for activation. This seems different from $P2X_1$, where recovery from desensitisation is slower than the estimate of ATP unbinding from chimaeric receptors.

Pratt et al. went on to show that in the desensitised state low nanomolar concentrations of ATP had a marked desensitising effect; however, the same concentration, when applied to fully recovered receptors, was ineffective at desensitising subsequent responses [51]. These results suggested that the desensitised state of the receptor shows increased affinity for ATP and that the long-time taken/rate limiting step for recovery results from the requirement for the agonist to dissociate from the high affinity site on the desensitised receptor. The time-course of radiolabelled ATP unbinding and that of recovery from desensitisation are similar. This suggests that agonist unbinding is rate-limiting; however, there must also be an agonist-independent recovery step to allow re-binding of agonist. A similar change in drug affinity at the $P2X_3$ receptor following receptor desensitisation comes from studies using the $P2X_1$ and $P2X_3$ receptor antagonist P^1,P^5-di[inosine-5′]pentaphosphate (Ip_5I) [52]. Ford et al. showed that Ip_5I is only effective as an antagonist at rat dorsal root ganglion $P2X_3$ receptors when the receptor has been activated/passed through the desensitised state [53]. These results suggest that $P2X_3$ receptor desensitisation results not only in a closed form of the channel but also in a change in ligand binding. This indicates that the conformational change associated with closing of the channel in the presence of ligand involves not only closing of the ionic pore but also a change in the extracellular ligand-binding domain to account for the increased affinity of some ligands.

Recovery of $P2X_3$ receptors from desensitisation can also be regulated by the ionic composition. Calcium can play a role in speeding the recovery of native transient $P2X_3$-like responses [54–56]. Cook et al. reported that an increase in extracellular calcium speeds the time-course of recovery from desensitisation, and that barium (as effective as calcium), spermine and gadolinium are also effective (magnesium appears ineffective) [55]. One possibility coupled with the above studies is that calcium could be acting to increase the rate of unbinding [51]. Mutagenesis studies on the extracellular domain of the $P2X_3$ receptors suggest that some non-conserved negatively charged residues contribute to the regulatory role of calcium and the speed of recovery from desensitisation [56]. The faster rate of recovery for some of the mutants (of non-conserved negative charge) is associated with a decrease in agonist potency, and one explanation is that the speeding could result from a change in the rate of agonist unbinding from the receptor.

Calcium can also play a role in the responsiveness of recombinant $rP2X_5$ receptor-mediated responses [57]. Prolonged (~ 15 min) exposure to calcium resulted in a 50% run-down of responses; however, the amplitude of $rP2X_5$ receptor current was greater when calcium was present at the time of ATP application, indicating that calcium has a complex role in the regulation of responsiveness at this receptor [57]. In contrast, native rat $P2X_1$ receptors in mesenteric arteries appear to be insensitive to changes in calcium levels [58]. Taken together, these results suggest that there are no common unifying mechanism(s) for the desensitisation and recovery of P2X receptor subtypes.

Ionic pore

For a full account of ionic permeation through P2X receptors, see “Biophysics of P2X receptors” by Terrance Egan in this issue.

Regulation

The activity of P2X receptors can be regulated by a variety of signalling events, and early work by Ken Nakazawa showed the regulation of native neuronal P2X receptor channels by G-protein-coupled serotonin receptors [59] and nicotinic ion channels [60, 61]. In recombinant studies, $P2X_1$ receptors can be potentiated by mGluR1a, $P2Y_1$, $P2Y_2$ [62] and 5-HT2A receptors [63], and both $P2X_3$ and $P2X_{2/3}$ receptor currents are increased by stimulation of substance P and bradykinin [44]. One common feature of these recombinant studies is that they are all mediated by Gαq-coupled receptors; this signalling pathway is primarily associated with the release of calcium from intracellular stores and the activation of protein kinase C. In addition, G-protein-coupled potentiation of P2X responses can also be mimicked by phorbol ester stimulation and can be blocked by kinase inhibitors, e.g. [43], suggesting that direct phosphorylation of the channel by protein kinase C may regulate function.

The intracellular domains of the P2X receptor family show a high degree of variability in amino acid composition and length of the C-terminal tail. However, throughout the family, there is a conserved protein kinase C site in the amino terminal domain (preceded two residues by a conserved tyrosine residue). Mutation to remove the threonine residue or other disruptions of the conserved protein kinase C site led to a reduction in the peak amplitude of $P2X_1$ and $P2X_2$ receptor-mediated responses and a speeding in the time-course of the response [43, 45] or, in the case of $P2X_3$ receptors, abolished functional responses [44]. $P2X_1$ receptors are basally phosphorylated [62], and phosphorylation of T18 in the $P2X_2$ receptor can be detected with an anti-phosphothreonine antibody [43]. It is interesting that the effects of phorbol esters are

dependent on the length of the C-terminal tail of the $P2X_2$ receptor; with no effect on the full-length receptor that gives relatively sustained responses but, in contrast, prolongation of the time-course of the desensitising truncated receptor [43]. These results and those of chimeras of the $P2X_2$ and $P2X_3$ receptor suggest that the C terminal domain of the $P2X_2$ receptor can regulate the contribution of the protein kinase C (PKC) site [44]. At $P2X_2$ receptors, the mutant K20T that removes the consensus PKC site was not potentiated by phorbol 12-myristate 13-acetate (PMA) [43], indicating that PKC phosphorylation can regulate the channel. However, in contrast, at $P2X_1$ receptors, a series of mutants to disrupt either of the components of the PKC site were still potentiated by G-protein-coupled receptor stimulation or phorbol ester treatment [62]. These results suggest that the conserved PKC site plays different roles in the regulation of $P2X_1$ and $P2X_2$ receptors. The $P2X_1$ receptor is likely to be phosphorylated at a number of sites; however, any change in the phosphorylation status of the receptor in response to phorbol ester stimulation was below the limit of detection and suggests that the receptor may be regulated by phosphorylation of an accessory protein [62], as has been described for example for the $P2X_7$ receptor [64].

Phosphorylation at sites other than the conserved PKC site has also been shown to regulate the activity of P2X receptor channels. Protein kinase A activation inhibits $P2X_2$ receptor-mediated responses through phosphorylation at serine 431 [65] and calcium/calmodulin-dependent protein kinase II has been shown to potentiate ATP responses by increasing trafficking of the receptor [66]. Recent studies have suggested that phosphorylation may not be restricted only to the intracellular domains and that $P2X_3$ receptors can be regulated by an ecto-protein kinase C [67].

In addition to modulation by phosphorylation, P2X receptor channels can be regulated by interaction with other proteins. Cross-talk with other ion channels has been described for native $P2X_2$ like receptors. For example P2X receptor and nicotinic receptor-mediated currents were non-additive in sympathetic [61, 68] and enteric neurons [69, 70], and similar results were described for recombinant $P2X_2$ receptors and $\alpha_3\beta_4$ nicotinic receptors [71]. It is now clear that $P2X_2$ receptors can also interact with 5-HT_3 [72, 73] and GABA channels [74–76].

Cross-regulation of the channels appears to be a complex process with several regulatory factors having been described. Evidence that the channels are physically associated comes from co-immunoprecipitation studies that indicated a direct interaction of the $P2X_2$ receptor with the 5-HT_3 receptor, $\alpha_3\beta_4$ nicotinic channels [73] and $GABA_{A\&C}$ receptor channels [75, 76], and fluorescence resonance energy transfer (FRET) studies have shown that $P2X_2$ and $\alpha_4\beta_2$ nicotinic channels are ~ 80 Å apart [77]. Co-localisation and FRET between $P2X_2$ and $\alpha_4\beta_2$ nicotinic channels can be measured in hippocampal neurons but not in ventral midbrain somata [77], suggesting that some factor is required to facilitate the interaction of the subunits. However, the molecular basis of the association of the channels remains to be determined.

A molecular basis of the cross-talk has been determined in co-expression studies of $P2X_2$ and 5-HT_3 or GABA ionotropic receptors. Boue-Grabot et al. showed that $P2X_2$ receptors and either $\alpha_3\beta_4$ nicotinic, 5-HT_3 or ionotropic GABA receptors showed cross-inhibition; however, truncation of the C-terminal tail of the $P2X_2$ receptor abolished the cross-talk, but did not affect the co-assembly of the channels [73, 75, 76]. Similarly, cross-talk could be inhibited by expression of a mini-gene encoding the distal C-terminal portion of the $P2X_2$ receptor and intracellular portions of the interacting channels, e.g. the large intracellular loop between the third and fourth transmembrane domains of the 5-HT_3 receptor [73, 75, 76]. These results suggest that the intracellular domains of the channels contribute to the cross-talk between $P2X_2$ and other ligand-gated ion channels, but that their physical association, as shown by immunoprecipitation, is regulated by another mechanism. Studies on chimaeric P2X receptor subunits have shown that the second transmembrane domain is important for determining the heteromeric assembly of P2X receptor subunits [27], and it is possible that this region may also play a role in the association of $P2X_2$ receptors with other ligand-gated channels.

Most of the research has focused on the interactions of native and recombinant $P2X_2$ receptors, and the extent to which other P2X receptor subunits may show cross inhibition is unclear. Work on dorsal root ganglion neurons showed that fast ($P2X_3$), slow ($P2X_{2/3}$) and mixed desensitising P2X receptor currents showed occlusion with GABA currents [74]; this was dependent on calcium and chloride flow [74]. This contrasts with the recombinant studies of $P2X_2$ and GABA channels, where cross-talk was independent of ionic flow [75, 76]. Further studies will be required to determine the extent of interaction of different P2X receptor subunits with other ligand-gated ion channels.

In addition to cross-talk with other ligand gated channels, P2X receptor subunits may also interact with other regulatory proteins, and this is an emerging area. Affinity purification, mass spectroscopic analysis and yeast two-hybrid screens were used to analyse partner proteins for the $P2X_7$ receptors and showed that the $P2X_7$ receptor exists as a signalling complex that, by changes in phosphorylation, can regulate channel function [64, 78, 79]. Mass spectroscopic analysis of extracts of rat brain synaptosomes that bound to GST fusion proteins of the C terminal tails of $P2X_{2,5\text{ and }7}$ receptors have also been used to identify interacting proteins [80]. This showed that $P2X_{2,5\text{ and }7}$ receptors all bound to myelin basic protein, but that βIII tubulin bound selectively to a proline-rich region of the C-terminal tail of the $P2X_2$ receptor and suggests that microtubules may be involved in the localisation and possible regulation of $P2X_2$ receptors. Yeast two-hybrid screening of the C-terminal domain of the $P2X_2$ receptor identified an interaction between the channel and the β-amyloid precursor protein interacting with protein Fe65; this co-immunoprecipitates with the full-length $P2X_2$

receptor but not C-terminally truncated splice variant $P2X_{2(b)}$ [81]. In addition, $P2X_2$ receptors and Fe65 were co-localised at postsynaptic specialisations of excitatory synapses, and in recombinant studies, binding of Fe65 reduced the permeability change of the $P2X_2$ receptor associated with prolonged receptor activation [81]. $P2X_4$ receptors can also be regulated by protein interactions and the C-terminal domain (sequence YEQGL) of the receptor associates with the adaptor protein complex 2 (AP2) and this regulates $P2X_4$ receptor surface expression and internalisation [82, 83].

These studies show that, like other classes of ion channels, P2X receptors may be subject to regulation by a variety of mechanisms, including interactions with other ligand-gated ion channels, G-protein-coupled receptors and a range of other signalling proteins and pathways. This provides a variety of mechanisms for the dynamic regulation of P2X receptor channel function in response to a range of stimuli.

Targeting

The cell-surface expression of P2X receptors appears to be a regulated process, and recent studies have demonstrated conserved and subunit specific mechanisms associated with the C-terminal domain in the regulation of membrane targeting. Sequence analysis reveals a conserved C-terminal motif (YXXXK close to the second transmembrane domain) that is involved in P2X receptor trafficking and stabilisation of the receptor at the cell surface [84]. Mutation of this sequence decreases surface expression of a range of P2X receptors and is thought to be a general mechanism of regulation. In addition to this motif, other regions of P2X receptor subunits have been shown to regulate channel expression, and targeting. $P2X_4$ receptors may be selectively targeted for internalisation through clathrin-coated vesicles (see above) [82, 83]. This regulatory mechanism for $P2X_4$ receptors can be further modulated by the drug ivermectin that acts to increase the surface expression of $P2X_4$ receptors that can account, in part, for its action to potentiate ATP-evoked responses [42, 85, 86]. A C-terminal polymorphism (resulting in I568N) in the human $P2X_7$ receptor results in reduced cell-surface expression [87] and surface expression may also be regulated by a C-terminal dibasic lipid interaction motif [88].

Changes in the surface expression can regulate the responsiveness of cells to ATP. For example, the surface expression of $P2X_2$, $P2X_{2/6}$ and $P2X_5$ receptors and the peak amplitude of current were increased following co-expression with epithelial sodium channels [89]. In addition, changes in receptor density may not only change the number of available channels but also regulate their properties as shown for $P2X_2$ receptors, where ATP potency, rectification and pore-forming properties are modified by the expression level [90–92], suggesting that receptor clustering or interaction with other proteins can regulate their properties.

P2X receptors are unlikely to be randomly inserted into the cell membrane and can show clustering or may be targeted to different parts of the cell; for example, hippocampal interneuron $P2X_2$ receptor-mediated responses are recorded from pre-synaptic nerve terminals but not the cell body [93]. As the cell membrane is not homogenous, membrane proteins may be organised into signalling micro-domains. One mechanism for bringing proteins and signalling molecules together can be the inclusion in lipid rafts. These are rich in cholesterol and glycerosphingolipid and result in liquid-ordered micro-domains within the liquid-disordered glycerophospholipid membrane bilayer [94]. Recent studies indicate that $P2X_1$ and $P2X_3$ receptors can localise to lipid rafts [95, 96]. For the $P2X_1$ receptor, it appears that inclusion in lipid rafts is essential for the regulation of the receptor as cholesterol depletion and disruption of the lipid rafts inhibits $P2X_1$ receptor-mediated responses [95]. $P2X_{2,4\text{ and }7}$ receptors in cerebellar granular neurons were not localised to lipid rafts [96]. These results suggest that P2X receptor subtypes can be differentially targeted to lipid raft domains; however the molecular basis of these and other trafficking signals to particular regions of the cell membrane remain to be determined.

Drug action at P2X receptors

ATP binds to the large extracellular loop of P2X receptors and results in channel activation. An understanding of the molecular basis of binding sites on the receptor will be useful in the development of models of the receptor for rational drug design. ATP binding to a range of enzymes and other proteins has been studied extensively and amino acids involved in forming the binding sites have been identified at the structural level. P2X receptors, however, do not contain common consensus ATP binding motifs, e.g. the Walker motif [97], and a mutagenesis approach has been used to develop and test models of the ATP binding site. A model of the $P2X_4$ receptor has also been proposed based on the similarity of the second half of the extracellular domain of P2X receptors with the catalytic domain of class II aminoacyl-tRNA synthetases [98] and mutagenesis [99]. This model suggests that asparagine 280 coordinates binding of the magnesium ion complexed with ATP, the adenine ring binds to phenylalanine 230 and lysine 190, histidine 286 and arginine 278 coordinate the binding of the phosphate groups and this is supported by decreases in ATP potency for the mutants K190A, F230A and D280A [99]. The mutants equivalent to lysine 190 results in a decrease in ATP potency at $P2X_1$ and $P2X_2$ receptors supporting a role of this conserved amino acid in ATP action. However, there are also differences between the $P2X_4$ receptor model and the $P2X_1$ receptor, where the equivalent mutation to phenylalanine 230 was without effect on ATP action [100]. The contribution of the first half of the receptor to this model remains to be elucidated, and this region also includes a conserved lysine residue that has been shown to have a major effect on ATP potency at

$P2X_1$ and $P2X_2$ receptors [46]. In addition, residues corresponding to K309 in the $P2X_1$ receptor, when mutated in $P2X_2$ and $P2X_7$ receptors, produced a large decrease in ATP potency [46, 101, 102], and the nearby residue R307 in the $P2X_7$ receptor that has been shown to regulate ATP potency [103], are not predicted to interact directly with ATP in the class II aminoacyl-tRNA synthetase model. One possibility is that these discrepancies between the receptors may reflect the differences in pharmacology between the subtypes.

It seems likely that residues important for ATP action at the receptor would be conserved in the family and in the extracellular loop of the P2X receptors >90 of the ~280 amino acids identical in at least five of the seven P2X receptor subunits. Alanine-scanning-based mutagenesis has been used to look at the role of these conserved amino acids, and a picture has emerged of residues and regions that can play an important role in mediating the action of ATP and has led to the development of a model of the agonist binding site. The P2X receptor is a dynamic protein with changes in its structural conformation associated with ATP binding and subsequent opening of the receptor channel. Mutations could, thus, result in changes in ATP potency through an effect on the ATP binding site or conformational changes associated with the opening of the channel. Comparison with residues involved in ATP binding at solved crystal structures has been used to substantiate contributions to the binding site of $P2X_1$ receptors. Changes in gating of the channel may also regulate the potency of agonists at the receptor, and residues in the pore lining transmembrane domains can regulate ATP potency [104].

ATP is a charged molecule with negative charge on the phosphate groups and in physiological solutions positive charge associated with the magnesium complexed with ATP. Thus, it seemed likely that as for other ATP binding proteins, conserved charged residues in the P2X receptor could be important in coordinating the action of ATP. The contribution of conserved charged residues was determined in both human $P2X_1$ and rat $P2X_2$ receptor backgrounds [46, 101, 105]. These showed that mutation of positively charged lysine (K) and arginine (R) residues K68, K70, R292 and K309 ($P2X_1$ receptor numbering) resulted in a large decrease (up to a 1,000-fold decrease for K68A) in ATP potency at the receptor, suggesting that these residues play a role in the coordination of the negatively charged phosphate group of ATP.

In addition, mutation of K188 in the $P2X_2$ receptor resulted in an ~ 100-fold decrease in ATP potency (the equivalent mutation K190A had a small effect at the $P2X_1$ receptor, and nearby residues decreased potency at the $P2X_1$ receptor, indicating a contribution of this region to ATP action). At $P2X_7$ receptors, mutagenesis [102] and characterisation of polymorphisms in the receptor support the role of these charged amino acids in the ATP function at the receptor [102, 103]. Little or no change in ATP potency was seen at individual point mutants to replace negatively charged glutamate (E) or aspartate residues (D) at $P2X_1$ receptors; a similar pattern was seen on a $P2X_2$ receptor background with the exception of an ~ 100-fold decrease in ATP potency at the D259A mutant and no response were detected for E85A and D261A mutant $P2X_2$ receptors.

These results show that there is consensus within the P2X receptors of conserved core residues that make an important contribution to ATP action consistent with the likely evolution of the family of P2X receptor subunits from a common ancestor. In addition, these studies on the charged amino acids also demonstrate that there are some differences between subunits. This may reflect the different pharmacological properties of the channels and may indicate that the interaction of conserved and non-conserved amino acids may play a role in regulation or fine-tuning of the properties.

Uncharged polar amino acids can also contribute to ligand action at receptors either by hydrogen bonding with the agonist or stabilising structural changes. Alanine mutants of conserved uncharged polar amino acids demonstrated a contribution of T186 and N290 ($P2X_1$ receptor numbering) at both $P2X_1$ and $P2X_2$ receptors [101, 106] and associated with the decrease in ATP potency was a loss of function of the partial agonists BzATP and Ap_5A at the $P2X_1$ receptor [106]. Aromatic amino acids have also been shown to be involved in ATP binding at a range of proteins. Alanine replacement mutagenesis of conserved aromatic amino acids in a $P2X_1$ receptor background showed that phenylalanine (F) at positions 185 and 291 reduced ATP potency. These residues are adjacent to T186 and N290-R292 and suggest that the motifs F185T186 and N290F291R292 could be involved in coordinating the binding of the adenine ring of ATP [100]. Similar contributions of aromatic residues have been described for the dead-box helicases [107].

Conserved cysteine, proline and glycine residues are also of interest, as these may give some structural constraint to the receptor through the formation of disulphide bonds, introduction of kinks/tight turns in the protein or introduction of flexibility, respectively. The ten conserved cysteine residues in the extracellular domain of the P2X receptors are likely to form disulphide bonds constraining parts of the receptor [108, 109]. The introduction of cysteine residues and the formation of disulphide bonds has also proved useful in defining the proximity of residues that can be incorporated into models; at the $P2X_2$ receptor, close proximity between residues in adjacent subunits has been shown for residues V48-I328 and H120-H213 [16, 33, 110]. The unique properties of individual conserved proline and glycine residues in the extracellular loop do not appear to play an essential role in ATP action at the $P2X_1$ receptor [111, 112].

Mutation of the majority of conserved amino acids in the extracellular loop of the $P2X_1$ receptor has little or no effect on the response to ATP, indicating that they do not play an essential role, and this adds weight to mutants where a substantial change is seen (Fig. 1). The results from systematic alanine scanning of conserved residues in the extracellular loop give rise to a model of the site of ATP action at the $P2X_1$ receptor, with K68 and K309 binding to the phosphate tail and the motifs F185T186 and

N290F291R292 coordinating the binding of the adenine ring (Fig. 2). A similar binding environment has been described for the crystal structure of rat synapsin II (PDB code 1i7l) [113], and a molecular model of the $P2X_1$ receptor binding site based on this is shown in Fig. 3. At the receptor, three molecules of ATP appear to be required to open the channel; however, it remains to be determined whether these ATP binding sites are contained within individual receptor subunits or form at the interface between subunits.

Molecular manipulations define functional roles

P2X receptors are expressed widely throughout the body, and functional responses have been described in many cell types (for an extensive review, see [4]). ATP can be released from a variety of sources including neurons (co-released with other neurotransmitters, e.g. noradrenaline or acetylcholine), damaged cells, platelets, in response to an increase in pO_2 [114], and recent studies show that connexin gap junctions may be involved in ATP release [115, 116]. In many cases, the properties of recombinant channels correspond well with those of native receptors; however, often due to the expression of multiple P2X receptor subtypes within a cell, splice variants, hetero-merisation of subunits, and the lack of a full range of selective drugs, the attribution of the molecular basis of native P2X receptors is not always possible. Molecular approaches, such as knockout mice, anti-sense, interference RNA and dominant negative mini-genes, have been used to substantiate the role of particular P2X receptor subunits in native responses.

$P2X_1$ receptors The contribution of $P2X_1$ receptors to a range of physiological responses has been supported by studies on receptor deficient and transgenic mice. Native responses corresponding to homomeric $P2X_1$ receptors have been described in arterial, vas deferens and bladder smooth muscle [1, 117–119]. P2X-receptor-mediated currents and nerve-evoked contractions are abolished in $P2X_1$-receptor-deficient mice supporting a role for homomeric $P2X_1$ receptor channels in these tissues [120–122]. In addition, these mice have established a role for the $P2X_1$ receptor in the autoregulatory control of renal blood flow [123, 124]. $P2X_1$ receptors are also expressed in platelets and mediate changes in intracellular calcium [125]. Regulation of $P2X_1$ receptors, however, has a large effect on the thromboembolism with $P2X_1$ receptor deficiency providing protection [126] and overexpression of $P2X_1$ receptors promoting mortality [127]. It seems likely that the $P2X_1$ receptor can play a priming role in the responsiveness to thrombotic stimuli [126, 127]. In the nervous system, the $P2X_1$ receptor subunit contributes $\alpha\beta$-meATP sensitivity to neuronal P2X receptors in

Fig. 1 Contribution of conserved amino acids in the extracellular loop to ATP potency at $P2X_1$ receptors. **a** Human $P2X_1$ receptor amino acids that are conserved in at least five of the seven human P2X receptor subunits in *red* (those that have been mutated to alanine are in *bold*) and the two transmembrane domains (residues predicted to form the transmembrane domains TM1 and TM2 are in *black and underlined*). (**b**) Summary of the effects of individual alanine point mutants of conserved amino acids in the human $P2X_1$ receptor. Alanine point mutations that resulted in a decrease in ATP potency are shown in *red* [46, 100, 106, 111, 112]. Residues where individual alanine substitution resulted in either no change or less than a fourfold change in ATP potency are shown as *grey dash* (–) [46, 100, 106, 111, 112]. Conserved cysteine residues that are thought to form disulphide bonds are shown in *blue*; these correspond to the pairs C117-C165, C126-C149, C132-C159, C217-C227 and C261-C270 [108]. Residues where alanine point mutants were non-functional are shown in *purple*; phenylalanine 195 and tryptophan 259 were mutated to tyrosine to conserve the aromatic group. There was no effect on channel properties [100]: for proline 272 substitution with glycine, isoleucine or phenylalanine rescued channel function [111], and at glycine position 96, and 301 channel function was rescued by proline or cysteine substitution; at glycine 250 substitution by serine but not proline, cysteine, aspartic acid, phenylanine, isoleucine, lysine or asparagine was tolerated [112]

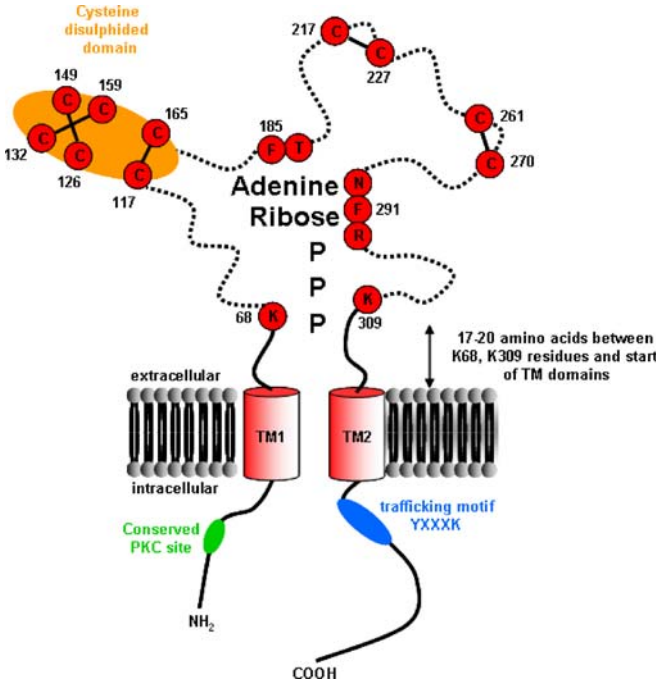

Fig. 2 Model of the $P2X_1$ receptor. The intracellular amino and carboxy termini are associated with conserved protein kinase C (PKC) and trafficking motifs, respectively. The channel has two transmembrane domains (TM1 and TM2). The large extracellular loop is the site of ATP action; residues shown highlighted in *red* correspond to conserved amino acids that when mutated to alanine resulted in a significant decrease in ATP potency. The conserved positively charged lysine (K) residues 68 and 309, as well as arginine (R) 292, may be involved in the regulation of binding of the negatively charged phosphate tail of ATP. Phenylalanines (F) 185 and 292 may coordinate the binding of the adenine ring. The proposed disulphide bonds are shown and include a highly disulphided domain between cysteine (C) residues 117–165. *Dotted lines* correspond to regions of the extracellular domain where their function remains to be determined

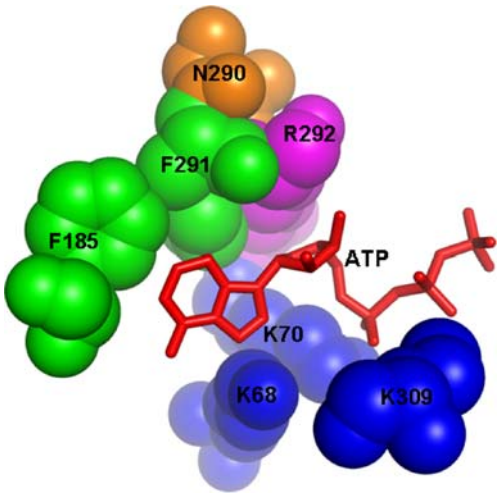

Fig. 3 A model of the $P2X_1$ receptor ATP-binding domain based on mutagenesis data and the crystal structure of rat synapsin II with ATP bound (PDB code 1i7l) [113]. Residues asparagine-290 (N290) [106], phenylalanine-291 (F291) [100] and arginine-292 (R292) [46] form a highly conserved motif across the P2X family, and mutagenesis studies confirm its role in ATP action. A similar motif (NWK) is found in rat synapsin II and also forms part of the ATP-binding environment. Positively charged lysine residues (K68, K70 and K309) are thought to interact with the negatively charged phosphates of ATP in the $P2X_1$ receptor [46]; they are also present in the ATP-binding environment of rat synapsin II. The aromatic residue phenylalanine-185 (F185) may be involved with coordinating the binding of the adenine ring of ATP, a corresponding phenylalanine residue was found in the ATP-binding site of rat synapsin II

sympathetic superior cervical ganglion neurons [128] and presynaptic receptors in the auditory brainstem [129].

$P2X_2$ receptors $P2X_2$ receptors were originally cloned from the rat PC12 phaeochromocytoma cell line [2] and, subsequently, have been shown to be expressed throughout the nervous system. $P2X_2$-receptor-deficient mice have shown the role of these receptor subunits in the presynaptic regulation of transmitter release in the hippocampus [93], carotid body function and the regulatory responses to hypoxia [130], fast synaptic excitation in myenteric neurons and peristalsis of the small intestine [131]. $P2X_2$ receptors also mediate responses of sympathetic neurons [132].

$P2X_3$ receptors These receptor subunits are found predominantly in sensory neurons and their role has been documented following molecular manipulation of receptor expression. These studies have shown a contribution of the $P2X_3$ receptor subunits to pain sensation, visceral mechanosensory transduction and gut peristalsis [133–138].

$P2X_{2/3}$ receptors $P2X_{2/3}$ receptors were the first described heteromeric P2X receptor channels [12] and are the first heteromeric channels to be studied following gene knockout [130, 132]. Generation of these mice by breeding of $P2X_2$ and $P2X_3$ knockouts generated fewer double knockouts than predicted and ~ 90% of double KO mice born died before weaning, suggesting that the heteromeric receptor plays an important physiological role. The 10% of $P2X_{2/3}$ knockout mice that survived to adulthood appeared normal. Using these mice $P2X_{2/3}$ receptors have been shown to be involved in mechanosensory transduction in the bladder, P2X receptor responses in nodose sensory neurons and may be involved in persistent inflammatory pain and contribute to sensitisation centrally [132]. $P2X_{2/3}$ receptors may also contribute to responses to hypoxia; following $P2X_3$-receptor-deletion sinus nerve activity to hypoxia is similar to WT; $P2X_2$ receptor deletion results in a decrease in responsiveness that is further reduced in $P2X_{2/3}$ knockout mice, indicating that these heteromeric channels contribute to sinus nerve discharge and the response to hypoxia [130]. Myenteric AH neurons are also likely to express functional $P2X_{2/3}$ receptors [131, 138]. $P2X_{2/3}$ receptor knockout mice have also been used to show that ATP is a transmitter involved in taste sensation [139]. Data from either $P2X_2$ or $P2X_3$ KO strains show only a modest loss in taste responses compared to abolition of taste-evoked activity in the $P2X_{2/3}$ knockout, suggesting that the heteromeric channel plays an important role in gustatory signalling.

$P2X_4$ receptors Nerve damage can lead to normally innocuous tactile stimuli becoming painful (allodynia), and these effects can be reversed by $P2X_4$ receptor antisense treatment [140]. Stimulation of $P2X_4$ receptors is an essential step in the induction, and maintenance of allodynia and spinal nerve injury results in an increase in $P2X_4$ receptor levels in the affected side of the spinal cord resulting from an increased expression of $P2X_4$ receptors in hyperactive microglia [140]. Subsequent work has shown that activated microglia release brain-derived neurotrophic factor. This modified anion gradients in spinal cord neurons, and changed GABAergic transmission from inhibitory to excitatory, and this removal of inhibitory signalling resulted in allodynia [141]. Recent work has also shown that $P2X_4$ receptors expressed on endothelial cells can contribute to the localised regulation of blood flow [142].

$P2X_5$ receptors Polymorphisms have been described for human $P2X_5$ receptors. These single nucleotide polymorphisms can lead to truncation of the receptor due to disruption of splicing [143–145] and it is estimated that only ~ 16% of individuals may be able to produce functional full-length channels [144].

$P2X_6$ receptors The $P2X_6$ receptor shows widespread expression in the central nervous system [146]; however, the functional roles of this receptor subunit remain to be determined. One interesting feature of the channel is that, in most cases, recombinant expression fails to form functional homo-trimeric channels [21, 25] possibly due to problems with glycosylation [147]. This raises the question of whether the $P2X_6$ receptor readily forms functional homomeric channels in vivo or if it normally functions as part of a heteromeric P2X receptor.

$P2X_7$ receptors The $P2X_7$ receptor is expressed predominantly by cells of immune origin, where its activation leads the release of the cytokine interleukin-1β (IL-1β) and is involved in inflammatory responses [148], models of arthritis [149] and cell death [150]. In a partial nerve ligation model of neuropathic hypersensitivity, thermal and mechanical hyperalgesia was absent in $P2X_7$ receptor KO mice implicating this receptor, and the subsequent release of IL-1β, in the induction and maintenance of pain states [151]. This suggests that $P2X_7$ receptor antagonists may be effective analgesics. Knockout mice have also identified a role for the $P2X_7$ receptor in osteoclasts and bone formation showing reduced bone thickening and increased resorption [152–154] possibly as a result of reduced skeletal mechanotransduction [155]. The contribution of $P2X_7$ receptors in mice may also have been underestimated as in some mouse strains, in which a mutation in the cytoplasmic domain decreases sensitivity to activation [156]. The function of human $P2X_7$ receptors can also be affected by a range of polymorphisms that have proved useful in demonstrating the contribution of these receptors [87, 103, 157–159]. The $P2X_7$ receptor KO mice have also proved to be useful in the assessment of antibody selectivity and have shown that a number of commonly used antibodies that are useful in peripheral tissues show non-selective activity in CNS tissues. This is consistent with in situ hybridisation work and reporter studies with the LacZ transgene, which suggest that $P2X_7$ receptor expression is absent or below the limit of detection in neuronal tissues [160, 161]. These studies show that it is important, wherever possible, to verify the selectivity of antibodies in KO models and, for example, studies with anti-$P2X_1$ receptor antibodies, have shown them to be effective in a range of peripheral cell types, including smooth muscle, but are non-selective in neuronal tissues [162].

Concluding remarks

In the 12 years since the cloning of the first P2X receptors, our understanding of the molecular basis of their properties and their physiological roles has come a long way. The future challenges include understanding (1) how cells that express multiple P2X receptor subunits regulate the assembly and trafficking of different functional trimeric receptors, (2) the contribution of P2X receptors to localised signalling domains/complexes that can be involved in cross-talk and regulation and (3) the sites of drug action at P2X receptors.

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