

REVIEW

Structural interpretation of P2X receptor mutagenesis studies on drug action

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P2X receptors for ATP are ligand gated cation channels that form from the trimeric assembly of subunits with two transmembrane segments, a large extracellular ligand binding loop, and intracellular amino and carboxy termini. The receptors are expressed throughout the body, involved in functions ranging from blood clotting to inflammation, and may provide important targets for novel therapeutics. Mutagenesis based studies have been used to develop an understanding of the molecular basis of their pharmacology with the aim of developing models of the ligand binding site. A crystal structure for the zebra fish P2X4 receptor in the closed agonist unbound state has been published recently, which provides a major advance in our understanding of the receptors. This review gives an overview of mutagenesis studies that have led to the development of a model of the ATP binding site, as well as identifying residues contributing to allosteric regulation and antagonism. These studies are discussed with reference to the crystal to provide a structural interpretation of the molecular basis of drug action. *British Journal of Pharmacology* (2010) **161**, 961–971; doi:10.1111/j.1476-5381.2010.00728.x

Keywords: ATP; P2X; ion channel; structure-function; mutagenesis

Abbreviations: Ap5A, diadenosine pentaphosphate; ASIC, acid sensing ion channel; MTS, methanethiosulphonate; rP2X4, rat P2X4; TM, transmembrane; zP2X4, zebra fish P2X4

Perspective and functional roles of P2X receptors

ATP is more than just an intracellular energy carrier, it is an extracellular transmitter binding to, and activating ligand gated P2X receptors and G-protein coupled P2Y receptors (Burnstock and Kennedy, 1985; Dubyak, 1991; Abbracchio and Burnstock, 1994). P2 purinergic receptors were first proposed in the 1970s by Professor Geoffrey Burnstock (for a historical review, see Burnstock, 2006a), have subsequently been shown to be expressed in almost all cell types, and mediate functions from taste sensation to the recruitment of phagocytes to apoptotic cells (Finger *et al.*, 2005; Elliott *et al.*, 2009). The seven mammalian P2X subunits (P2X1-7) assemble as homomeric and heteromeric receptors with a range of phenotypes (North, 2002; Egan *et al.*, 2004; Roberts *et al.*, 2006). Pharmacological profiling, expression analysis, as well as knockout and knock-down approaches have defined the contributions of P2X receptors in native systems and suggested that P2X receptor selective drugs will be useful in the treatment of a variety of conditions including

thromboembolism, bladder instability, pain and inflammation (Roberts *et al.*, 2006; Burnstock, 2006b; Surprenant and North, 2009). The recent publication of a crystal structure of the zebra fish P2X4 (zP2X4) receptor in a closed agonist-unbound state (Kawate *et al.*, 2009) is a major advance for the understanding of P2X receptors. This review focuses on the molecular basis of P2X receptor pharmacological properties and how the crystal structure expands our understanding of the channel.

Pre-crystalization structural insight

P2X receptors were originally identified at the molecular level by expression cloning from the vas deferens and pheochromocytoma (PC12) cells (Brake *et al.*, 1994; Valera *et al.*, 1994). These receptors had no sequence homology with other ion channels, so bio-informatic and molecular/biochemical studies were first used to develop an understanding of the receptor structure. Sequence and hydrophobicity analysis suggested the P2X receptor subunit had two transmembrane (TM) segments, intracellular amino and carboxy termini and a large extracellular ligand binding loop (Brake *et al.*, 1994; Valera *et al.*, 1994). This topology was supported by immunohistochemical (Vulchanova *et al.*, 1996), mutagenesis (Buell *et al.*, 1996), and glycosylation studies (Newbolt *et al.*, 1998; Torres *et al.*, 1998) (for review, see North, 2002). Like the other families of ligand gated ion channels (LGICs), P2X receptors

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Received 21 December 2009; revised 19 January 2010; accepted 28 January 2010

form as multimers. Evidence for this came initially from studies co-expressing slowly desensitizing α,β -meATP-insensitive P2X2 receptors, and rapidly desensitizing α,β -meATP-sensitive P2X3 receptor subunits. This gave rise to a novel P2X2/3 heteromeric receptor that had the slowly desensitizing time course of the P2X2 receptor and the α,β -meATP sensitivity of the P2X3 receptor subunit (Lewis *et al.*, 1995). The subunit stoichiometry was subsequently shown to be a trimer by Nicke *et al.* (Nicke *et al.* 1998) based on the size of P2X receptors in Western blot studies using native blue gels or of cross-linked receptors. This agreed well with early predictions that three molecules of ATP were required to activate the channel (Bean, 1990), and also with subsequent models of opening based on single channel analysis (Ding and Sachs, 1999). These studies showed the stoichiometry and topology of P2X receptors was distinct from the pentameric nicotinic/cys loop and the tetrameric glutamate families of ionotropic receptors, which possess four and three membrane spanning segments, respectively, per subunit (Surprenant *et al.*, 1995). P2X receptors are therefore a third structural class of LGIC that form as trimers from subunits with two transmembrane segments, intracellular amino and carboxy termini, and a large extracellular loop.

Prior to a high-resolution crystal structure of the P2X receptor, predictions about tertiary structure came from cysteine mutagenesis studies. There are 10 conserved cysteine residues in the extracellular domain of mammalian P2X receptors that could form disulphide bonds and constrain the receptor structure. Cysteine reactive (2-aminoethyl) methanethiosulfonate hydrobromide-biotin was unable to bind to wild-type P2X1 receptors suggesting that no free cysteine residues were accessible (Ennion and Evans, 2002). However, biotinylation was detected when individual cysteine residues were mutated, as predicted if a cysteine to alanine mutation disrupted a disulphide bond pairing and exposed the free cysteine binding partner (Ennion and Evans, 2002). Single cysteine mutants also changed P2X receptor pharmacological properties, with similar effects for pairs of cysteine mutants (Clyne *et al.*, 2002b; Ennion and Evans, 2002). These findings suggested that disulphide bonds formed between cysteines 1–6, 2–4, 3–5, 7–8 and 9–10 in the extracellular domain (Ennion and Evans, 2002; Clyne *et al.*, 2002b). The disulphide bonding of the first six cysteine residues in the extracellular domain predicted the formation of a finger-like motif. Interestingly, mutation of several residues within this region reduced the allosteric effects of divalent cations and protons on ATP-evoked currents (for review, see Evans, 2009), and mutation of the cysteines abolished regulation by zinc at P2X2 receptors (Clyne *et al.*, 2002b). This suggests that the cysteine-bonded finger forms an allosteric regulatory domain. The co-expression of mutations that introduced additional cysteine residues into the extracellular domain can result in disulphide bond formation between adjacent subunits when the introduced residues are sufficiently close and in the correct orientation. This method provided information on the proximity of residues in adjacent subunits (Jiang *et al.*, 2003) and suggested that the ATP binding and regulatory allosteric sites are formed at the interface between P2X receptor subunits (Nagaya *et al.*, 2005; Marquez-Klaka *et al.*, 2007) (see below for details). Cysteine mutagenesis studies have therefore been useful in providing

the first insight into P2X receptor structural constraints and molecular distances between residues.

The first glimpses of the P2X receptor 3D structure came following receptor purification, atomic force microscopy and cryo-EM. These studies support the trimeric assembly of the receptor (Barrera *et al.*, 2005; Mio *et al.*, 2005; Nakazawa *et al.*, 2005; Young *et al.*, 2008). Recently, a structure at 15 Å resolution for the P2X2 receptor has been published (Mio *et al.*, 2009). These structures showed that the receptor has three-fold symmetry, a globular extracellular domain, and identified interactions between the intracellular amino and carboxy termini.

Crystalization of the zebra fish P2X4 receptor

The crystallization of membrane proteins is difficult and often requires modifications of the receptor to be made. The zP2X4 receptor took 7 years to solve (Silberberg and Swartz, 2009) requiring truncation, mutation and considerable patience (the Δ P2X4-B mutant alone took 9 months for the crystals to grow (Kawate *et al.*, 2009). This crystal structure confirms the receptor topology, that it is a trimer with threefold symmetry, and also shows that the extracellular domain projects 70 Å above the cell surface. The TM regions are shaped like an hourglass and are similar to those of the two TM segment trimeric acid sensing ion channel (ASIC) (Gonzales *et al.*, 2009). In the extracellular domain, the three P2X receptor subunits are entwined with a right-handed twist (Figure 1A), and there are three vestibules in the centre of the extracellular domain. This crystal structure also confirms the predicted disulphide bonding pattern of the receptor and the existence of a cysteine-rich finger domain. An excellent review of the structural information and comparison with other P2X receptor structures has been published recently (Young, 2009). The zP2X4 receptor was crystallized in the absence of ligand, and no confirmation was given that the purified protein could bind agonists or antagonists. The structure therefore provides no direct information on ATP binding, and this should be borne in mind as a caveat for interpretation. However, a possible agonist binding site was proposed based on previous mutagenesis studies. The remainder of the review summarizes the information available on ligand action and regulation from mutagenesis studies and discusses its interpretation in light of the zP2X4 receptor crystal structure.

Mutagenesis based predictions of the ATP binding site

Analysis of a wide range of proteins has identified several consensus motifs for ATP binding. For example, the Walker GXXXXGKT/S motif (X denotes any residue) can coordinate binding of the phosphate of ATP (Walker *et al.*, 1982), and a Q-motif comprising a conserved glutamine and an aromatic amino acid 17 amino acids upstream can contribute to adenine binding (Tanner *et al.*, 2003). However, such consensus motifs for ATP binding are not present in the P2X receptor family. Consequently a mutagenesis-based approach was used to determine the site of agonist action. Alanine is widely used

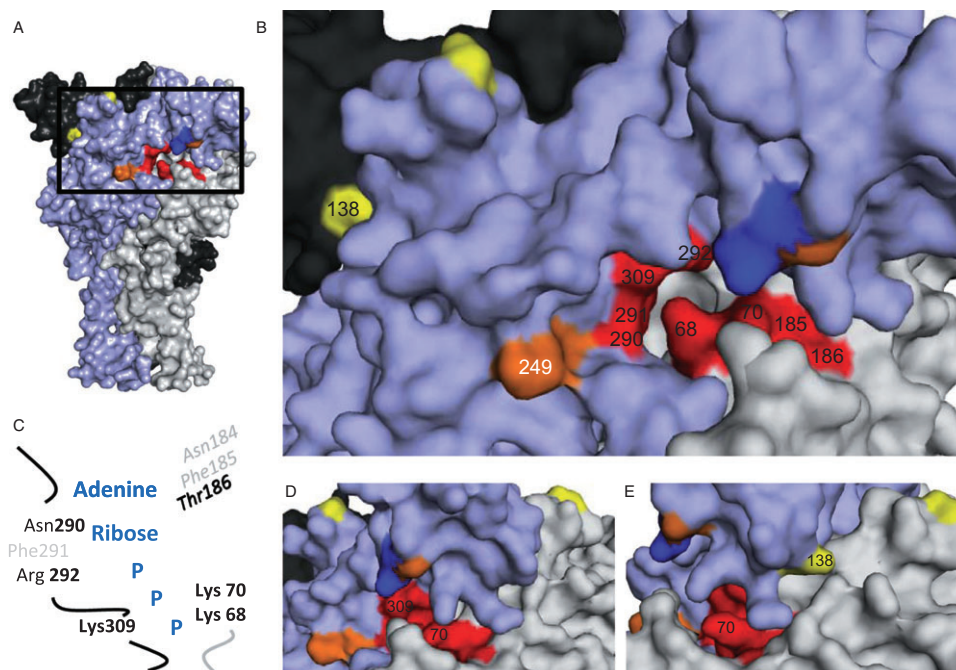


Figure 1 Crystal structure of the zebra fish P2X4 (zP2X4) receptor and the proposed sites for agonist and antagonist binding. (A) Homology model of the human P2X1 receptor based on the structure of the zP2X4 receptor (Kawate *et al.*, 2009) showing the three entwined subunits in grey, black and slate blue; the black box indicates the region incorporating residues involved in agonist and antagonist action and in more detail in panels B–D. (B) Residues predicted to form the ATP binding pocket are shown in red (P2X1 receptor numbering), the blue residue corresponds to an Arg residue in P2X7 that can be ADP ribosylated and activate the channel. Mutations that affect sensitivity to PPADs and suramin are shown in orange and yellow, respectively. (C) Most recent model of ATP binding at the P2X1 receptor based on mutagenesis studies (adapted from Roberts *et al.*, 2009); light grey for Phe185 indicates that the cysteine mutant at this position had no effect on ATP potency, and although this residue was accessible to (2-aminoethyl) methanethiosulfonate hydrobromide-biotin, MTS reagents did not affect ATP-evoked responses, suggesting that this residue does not directly into the agonist binding pocket. Similarly, Phe291 is shown in grey as MTS reagents did not modify ATP evoked responses at P2X2 and P2X4 receptors (Roberts *et al.*, 2008). (D and E) Rotations clockwise of the structure highlighted in (B) give an indication of the 3D proximity of the residues showing that Lys residue 138 shown to regulate suramin sensitivity is close to the proposed ATP binding pocket, but the side chain faces away from it on the other side of the cysteine rich head group. The figures were generated and re-coloured using PyMOL (DeLano Scientific, Palo Alto, CA, USA) and the structure 3H9V as a template for the hP2X1 receptor homology model. PPADs, pyridoxal 5-phosphate 6-azophenyl-2',4'-disulphonic acid.

in site directed mutagenesis studies, as this simple amino acid is well tolerated as a substitution. Initial studies focused on determining the effects of alanine mutations of conserved basic, aromatic and polar amino acids in the extracellular domain (Figure 2). The assumption being that as all the P2X receptors are activated by ATP, it is likely that they share a common core ATP binding site.

Positively charged amino acids are involved in coordinating the binding of the negatively charged phosphates of ATP. Mutants generated on P2X1 and P2X2 receptor backgrounds showed that residues Lys68, Lys70, Arg292 and Lys309 contribute to ATP potency (P2X1 receptor numbering)(Ennion *et al.*, 2000; Jiang *et al.*, 2000); with the greatest, >1000-fold, decreases in ATP sensitivity for Lys68Ala and Lys309Ala mutants at the P2X1 receptor (Ennion *et al.*, 2000). Aromatic amino acids bind the adenine ring of ATP in DEAD box helicases (Tanner *et al.*, 2003) and the ribose of ATP in UvrB DNA helicase (Theis *et al.*, 1999). Mutagenesis of conserved aromatic amino acids in the P2X1 receptor demonstrated a contribution of Phe185 and Phe291 in agonist action (Roberts and Evans, 2004), with ~10 and 140-fold reduction in ATP potency following alanine replacement mutagenesis respectively. Polar amino acids can also contribute to agonist binding, and mutagenesis studies on P2X1 and P2X2 recep-

tors highlighted a role of Thr186 and Asn290 (P2X1 receptor numbering) in ATP sensitivity (Jiang *et al.*, 2000; Roberts and Evans, 2006).

To date, more than 120 of the ~280 amino acids in the extracellular domain of the human P2X1 receptor, including over 60 conserved residues, have been mutated to alanine and/or cysteine (Evans, 2009; Roberts *et al.*, 2009) (Figure 2). The majority of these mutants had little or no effect on ATP potency; only eight of the mutants made substantial contributions to ATP sensitivity. Based on these studies, a model of how ATP binds to the receptor was proposed (Figure 1C), with lysine residues 68, 70 and 309 coordinating the binding of the negatively charged phosphate tail of ATP. Binding of the adenine ring may be coordinated by the aromatic region Asn290Phe291Arg292, with a regulatory role of Phe185Thr186 possibly by hydrogen bonding (all P2X1 receptor numbering) (Roberts *et al.*, 2006; Roberts *et al.*, 2009). The amino acids proposed in the model are conserved across mammalian P2X receptors, with similar effects of mutations of these residues reported for P2X2 and P2X4 receptors (Jiang *et al.*, 2000; Zemkova *et al.*, 2007; Roberts *et al.*, 2008), suggesting a common core ATP binding pocket.

The predicted ATP binding site is formed from four discrete clusters of amino acids (corresponding to residues 68, 70, 185,

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mutants  flye-g--tss-glissvsvKlK-lavtqlpglqpqv--va--Vf-aq-dnsfvvm-nfi
hp2X1 49  FLYEKGYQTSS-GLISSVSVKLKGLAVTQLPGLGPQVWDVADYVFPAGQDNSFVVMTNFTI 107
hp2X2 61  FIVQKSYQESETGPESSITKVKGITTSE-----HKVWDVEYVKPPEGGSVFSITTRVE 115
hp2X4 48  FVWEKGYQETD-SVSSVTKVKGVAVTNTSKLGFRIWDVADYVIPAQEENSLFVMTNVI 106

mutants  v-pk-tq-y-a-hp-eg-gi-ke-sg-tp-kakrkaq-ir--k-vaf-ndtvk---ifg-
hp2X1 108 VTPKQTQGYCAEHP-EG-GICKEDSGCTPGKAKRKAQGIRTGKCVAF-NDTVKTCEIFGW 164
hp2X2 116 ATHSQTQGYCPESIRVHNATCLSDALCVAGELDMLGNLRTGRCVPPYQGPSKTCEIFGW 175
hp2X4 107 LTMNQTQGYLCPEIP-DATTVCKSDASCTAGSAGTHSGNVSTGRCVAF-NGSVKTCEVAAW 164

mutant   --v-vdddiprpall-----FT-----n-r-lveevnaahm-t-lfhkth
hp2X1 165 CPVEDDDDIPRPALLREAENFTLFLKNSISFPRFKVNRRNLVEEVNAAHMKTCLFHKTLH 224
hp2X2 176 CPVEDGASVSQ-FLGTMANFTILLKNSISYPKFHFSKGNIADR-TDGYLKCTFHEASD 233
hp2X4 165 CPVEDDTHVPQPAFLKAAENFTLLVKNIWYPKFNFSKRNILPNITTTYLKSCIDAKTD 224

mutants  -l--v-ql-yvvqes-qn-stlaek--vv-itid-h--l-whvrh-r-i-e-hglye---
hp2X1 225 PLCPVFQLGYVVQESGQNFSTLAEKGGVVGITIDWCDLDWHVRHCRPIYEFHGLYE--- 281
hp2X2 234 LYCPIFKLGFIVEKAGESFTELAHKGGVIGVIINWDCDLDLPASECNPKYSFRRLDPKH- 292
hp2X4 225 PFCPIFRLGKIVENAGHSFQDMAVEGGIMGIQVNWCNLDRAASLCLPRYSFRRLDTRDV 284

mutants  ekn1---NFR-----K-----
hp2X1 282 EKNLSPGFNFRFARHFVE-NGTNYRHLFKVFGIRFDILVDGKAGKFD 327
hp2X2 293 -VPASSGYNFRFAKYKI-NGTTRTLIKAYGIRIDVIVHGQAGKFS 337
hp2X4 285 EHNVSPGYNFRFAKYRDLAGNEQRTLIKAYGIRFDIIVFGKAGKFD 331

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Figure 2 Conserved amino acids in the extracellular domain of mammalian P2X receptors and the effects of P2X1 receptor mutants on ATP potency. Amino acid sequences of the extracellular domain of human P2X1, 2 and 4 receptors are shown. Residues that are conserved in at least five mammalian P2X receptor subunits are shown for basic (red), aromatic (purple), polar (green), glycine/proline (yellow), and cysteine, valine, alanine, aspartate and glutamate (black). The top lines (mutants) show in bold and colour the conserved residues, which, when mutated, have a substantial effect on ATP potency and have been incorporated into the binding site model. In addition, alanine or cysteine point mutations that have no or a less than 10-fold effect on ATP potency are shown as dashes; non-conserved residues are in lower case.

186, 290, 291, 292, and 309 detailed above) that are separated by 115, 104 and 17 residues. This implies that the receptor must be folded/assembled in such a way as to bring these regions close together. The proximity of two of these clusters has been confirmed by cysteine replacement mutagenesis in the P2X1 receptor, which showed cysteine mutants of Lys68 and Phe 291 were sufficiently close to form a disulphide bond. This bond formed between adjacent subunits, and was inhibited in the presence of ATP (Marquez-klaka *et al.*, 2007). These results, in addition to supporting the model, also provide evidence that the ATP binding site forms at the interface between adjacent subunits, as had been suggested from studies on lysine mutants in P2X2/3 receptor heteromers (Wilkinson *et al.*, 2006).

Compatibility of mutagenesis based model for the ATP binding site and structural data

Although the zP2X4 receptor was crystallized in the absence of ATP (Kawate *et al.*, 2009), it does allow mutagenesis studies to be interpreted in a structural context and highlights a region that incorporates several residues important for determining P2X receptor pharmacological properties. The zP2X4 receptor crystal structure shows that the eight conserved residues predicted to contribute to the ATP binding site are indeed in close proximity (Kawate *et al.*, 2009), and this has also been found for a homology model of the P2X1 receptor (Lys68, Lys70, Phe185, Thr 186 from one subunit, and Asn290, Phe291, Arg292 and Lys309 in the adjacent one; P2X1 receptor numbering, Figure 1) and P2X2 receptors (Keceli and Kubo, 2009; Kubo *et al.*, 2009). Further support

that this region forms the agonist binding site comes from (i) the substantial effects of mutations of only these residues on ATP potency (Jiang *et al.*, 2000; Zemkova *et al.*, 2007; Roberts *et al.*, 2008) and radioligand binding (Roberts and Evans, 2004; Roberts *et al.*, 2009); (ii) the presence of the only conserved basic residues that are involved in determining agonist sensitivity (Lys 68, 70, 309, and Arg292 P2X1 numbering); it would be extraordinary if the ATP binding pocket did not include positively charged amino acids to coordinate the binding of the negatively charged phosphates; and (iii) residues involved in both allosteric regulation and antagonist action form a halo around this region (Figure 3, see later). These lines of evidence, though compelling, are circumstantial and do not directly prove the location of the ATP binding site. Studies on the P2X7 receptor, however, provide direct evidence to substantiate the location of agonist binding site. ADP-ribosyltransferases transfer ADP-ribose from NAD onto proteins and ADP-ribosylation at Arg125 activates P2X7 receptors (Adriouch *et al.*, 2007). Furthermore, in the zP2X4 receptor structure, the equivalent residue to Arg 125 is adjacent to the predicted ATP binding pocket. This suggests that ADP tethered by ribosylation to Arg125 on the P2X7 receptor can access that adjacent ATP binding pocket and activate the receptor (Adriouch *et al.*, 2007; Schwarz *et al.*, 2009).

If the region predicted to be the ATP binding site is correct, what insight does the zP2X4 receptor structure give to interpretation of mutagenesis studies and development of the model? In the model, it seems most likely that the conserved lysine residues contribute to binding of the phosphate of ATP as cysteine mutants of these residues show that ATP potency can be increased by positively charged MTS reagents and reduced by negatively charged MTS reagents (Roberts and

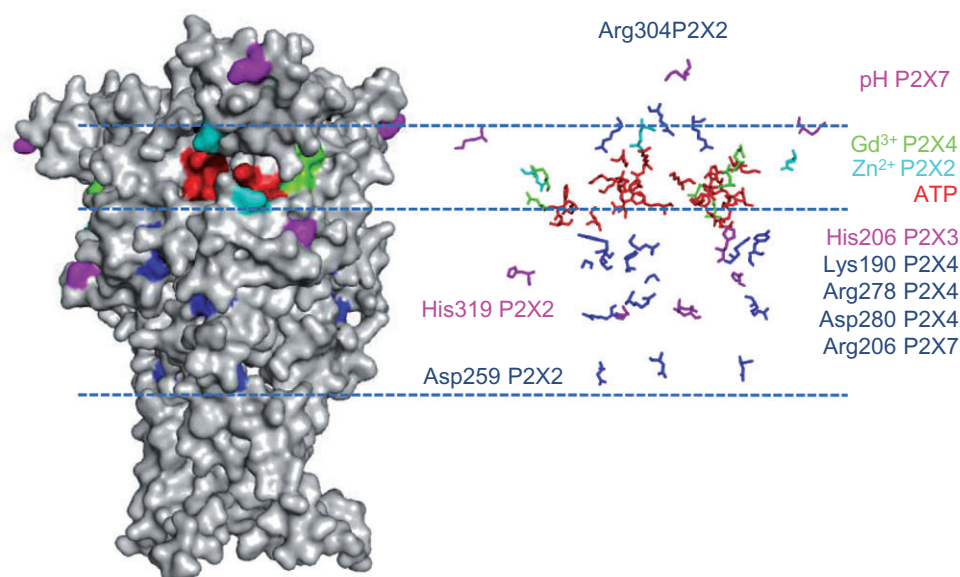


Figure 3 Mapping of residues involved in gating and ionic regulation to the P2X receptor structure. An homology model of the hP2X1 receptor built using the zebra fish P2X4 (zP2X4) receptor structure. Left-hand panel shows a surface rendered representation of the P2X receptor, and right panel shows a stick diagram of the receptor for residues shown to be involved in the binding of ATP (red), gadolinium (green), regulation by zinc (cyan) and pH (magenta). Residues where mutation decreased ATP potency, but the effects were not conserved across subunits, suggesting that the effects result from disruption of interaction with a variant amino acids, are shown in blue. The residues associated with gadolinium and zinc action are clustered around the ATP binding site, and those with pH and gating regulation either side of the ATP binding region with a concentration in a halo just below the ATP binding site.

Evans, 2007; Roberts *et al.*, 2008). From the structure of the zP2X4 receptor, it is clear that the conserved lysine residues are not buried within the protein, but are exposed on the surface within the predicted binding groove, raising the possibility that substitutions at the phosphate end of the ATP molecule could easily be accommodated. Support that the region around the terminal phosphate is open at the protein surface and comes from ADP ribosylation (see above) (Adriouch *et al.*, 2007). In addition, the ATP analogues adenosine tetraphosphate and diadenosine pentaphosphate (Ap5A) are agonists at some, but not all P2X receptors, showing that bulky substitutions, such as the addition of an ADP to ATP to make Ap5A, can be accommodated at the P2X1 receptor binding pocket (Ralevic *et al.*, 1995; Lewis *et al.*, 2000). These results suggest that the terminal phosphate of ATP faces out of the binding pocket, and the adenine and ribose components of ATP are bound deeper within the protein or further along the binding groove.

Phenylalanines can form part of a motif for adenine binding (Tanner *et al.*, 2003). Phe291 (P2X1 receptor numbering) was suggested to bind the adenine ring of ATP as mutation decreased agonist potency at P2X1, 2 and 4 receptors (Roberts and Evans, 2004; Roberts *et al.*, 2008). Interestingly, CTP is also a weak agonist at P2X receptors (Chen *et al.*, 1995; Soto *et al.*, 1996; Roberts and Evans, 2004), although whether the pyrimidine ring binds in the same way as adenine remains to be determined. The zP2X4 receptor shows that the conserved phenylalanine predicted to be involved in agonist recognition is the residue in the model that is farthest from the outer surface of the receptor (Kawate *et al.*, 2009). This supports results from mutagenesis studies at P2X2 and P2X4 receptors, where cysteine mutants of these residues were not

affected by MTS reagents and were considered inaccessible to these compounds (Roberts *et al.*, 2008). However, at P2X1 receptors, Phe291Cys was inhibited by both positive and negatively charged MTS reagents demonstrating accessibility and suggesting that it contributes to the ATP binding pocket. These differences between P2X receptors suggest that there are subtle variations in geometry between receptor subtypes. At P2X1 receptors, the accessibility of Phe291Cys is consistent with the disulphide bond that can form between Phe291Cys and Lys68Cys in an adjacent subunit (Marquez-Klaka *et al.*, 2007). However, disulphide bond formation for equivalent mutations was less efficient for P2X2 receptors, and a bi-functional cysteine reactive cross-linker was required to form disulphided receptor dimers for P2X3 and P2X4 receptors (Marquez-klaka *et al.*, 2009). Such differences in structure between receptors may be important in determining the subunit dependent pharmacological properties.

The predicted roles for the lysines 68, 70 and 309 in phosphate binding and phenylalanine 291 in binding the adenine ring suggests that Asn290 and Arg292 could be involved coordinating the binding of the ribose of ATP. The original model developed from P2X1 receptor mutagenesis studies also incorporated Phe185 Thr186, since alanine mutants of these residues decreased ATP potency (Roberts and Evans, 2004; Roberts & Evans, 2006). The 10-fold decrease in ATP potency for Phe185 with alanine substitution was modest (Roberts and Evans, 2004), and there was no change in sensitivity for the cysteine mutant (Roberts *et al.*, 2009). In addition for the Phe185Cys mutant ATP potency was not changed by application of cysteine reactive MTS reagents even though they are able to bind to the receptor (Roberts *et al.*, 2009). Similarly, at P2X2 and P2X4 receptors, the equivalent cysteine mutants

did not modify ATP sensitivity (Roberts *et al.*, 2008). These results suggest that the conserved residue Phe185 (P2X1 receptor numbering) does not directly interact with ATP at the binding pocket. At the adjacent conserved threonine residue, cysteine mutants had modest effects on ATP potency, and were sensitive to MTS reagents at P2X1, 2 and 4 receptors (Roberts *et al.*, 2008; Roberts *et al.*, 2009). This suggests that the conserved threonine contributes to ATP action. From the zP2X receptor structure, the PheThr doublet is adjacent to a conserved lysine residues, and Thr186 may therefore play a role in fine tuning the interaction of the phosphate tail of ATP with Lys70.

The binding of ATP to the P2X receptor results in conformational changes that lead to the opening of the channel pore (gating of the channel). The zP2X4 receptor structure shows that the proposed ATP binding site is ~45 Å from the channel pore, and it is unclear how agonist binding leads to the conformational changes that underlie opening/gating of the channel. Changes in ATP potency at mutant P2X receptors can arise from an effect on agonist binding and/or gating of the receptor. A radiolabelled 2-azido ATP binding assay was used to give insight into the interpretation of mutagenesis studies (Roberts and Evans, 2007). Binding of 2-azido ATP was reduced for cysteine mutants of Asn290, Phe291, Arg292 and Lys309 at the P2X1 receptor. However, the extent of the reduction did not mirror exactly the changes in ATP potency. Binding was reduced by ~50% for Asn290Cys and Phe291Cys, which showed ~50-fold reduction in ATP potency, while binding was reduced by >80% for Arg292Cys and Lys309Cys, which had 15- and 180-fold reductions in potency respectively (Roberts and Evans, 2007). These discrepancies between the changes in potency and binding can be explained by an effect on gating. The reduced potency at Arg292Cys may have resulted predominantly from an effect on binding (Roberts and Evans, 2007). In contrast, for the cysteine mutants Asn290 and Phe292, an effect on binding and also a reduction in gating seemed likely (Roberts and Evans, 2007). For Lys309Cys, the level of binding was on the limit of detection, and, compared with the smaller potency change of Arg292Cys with equivalent reduction in binding, suggested that a gating effect was also involved (Roberts and Evans, 2007). This was supported by single channel studies suggesting a gating role of the equivalent lysine residue in the P2X2 receptor (Cao *et al.*, 2007). These results indicate that residues within the binding pocket play a central role in transforming agonist binding into opening of the P2X receptor channel.

Interpretation of mutations that have an effect on ATP potency, but do not fit the proposed model for agonist binding

The effects of some conserved amino acid mutations do not fit the proposed ATP binding site model (described above), and can vary markedly between different P2X receptor subunits. At P2X2 receptors, the Lys188Ala, Asp259Ala and Arg304Ala mutations showed >300-fold decreases in ATP potency (Jiang *et al.*, 2000), whereas equivalent mutations at the P2X1 receptor had no effect or a <10-fold decrease (Ennion *et al.*, 2000);

Ennion *et al.*, 2001). The P2X4 receptor mutants Lys190Ala and Phe230Ala were essentially insensitive to ATP (Yan *et al.*, 2005), while the equivalent alanine mutants had little or no effect at P2X1 receptors (Ennion *et al.*, 2000; Roberts and Evans, 2004). At the mouse P2X7 receptor, mutation of the conserved Arg206 to Lys resulted in an increase in ATP potency (Schwarz *et al.*, 2009), while the equivalent alanine mutation at the P2X1 receptor R202A had no effect (Ennion *et al.*, 2000). These results highlight the importance of these residues in some subunits. Why then if the residues are conserved do they not show similar effects when mutated across P2X receptor subunits? P2X receptors show a range of properties dependent on the subtype, and these differences result from variations in amino acid sequence between the receptors. This suggests that the subtype dependent effect of a mutation of a conserved amino acid could also arise from an important regulatory role of a non-conserved residue. For example, at the P2X7 receptor, differences in amino acids at residues 127 and 284 contribute to the variation in agonist sensitivity between the rat and mouse isoforms (Young *et al.*, 2006). In addition, amino acid variations are likely to lead to subtle differences in the structures of P2X receptor subunits (as described above for the ability of cysteine mutants to form disulphide bonds in different P2X receptor subunits (Marquez-Klaka *et al.*, 2009), which can contribute to the heterogeneity in properties. Thus structural variations/non-conserved residues are likely to account for the subunit dependent differences in effects of conserved mutations and mediate the heterogeneity in pharmacological properties between receptor subtypes.

But how can these mutants result in a change in ATP sensitivity when they are not part of the proposed ATP binding site? One explanation is that the residues are involved in conformational changes following agonist binding that lead to the opening/gating of the P2X receptor ionic pore. Interestingly, mapping these residues onto the P2X receptor structure shows the majority are located in a ring below the proposed ATP binding pocket (Figure 3). This region provides the link between the ATP binding pocket and the channel pore and changes in properties could affect gating of the channel and ligand sensitivity. As the residues are in different stretches of the protein, it also suggests that interactions between portions are important and the whole region may undergo structural rearrangement following agonist binding and, significant movement in this region may provide the transition to open the channel gate.

Allosteric modulatory sites

The binding of ATP to the P2X receptor orthosteric site can be regulated by a number of allosteric agents, including divalent cations and protons. For example, the sensitivity of ATP at neuronal P2X receptors was increased by low concentrations of zinc (Cloues *et al.*, 1993; Li *et al.*, 1993). Mutagenesis studies have shown that histidine residues His120 and His213 underlie zinc potentiation at rat P2X2 receptors (Clyne *et al.*, 2002a; Lorca *et al.*, 2005). Subsequent elegant studies showed that cysteine mutants of these residues formed an inter-subunit disulphide bond, demonstrating the close proximity

of the histidine residues in adjacent subunits (Nagaya *et al.*, 2005). Earlier work suggested that mutation of cysteine residues in the cysteine rich domain (incorporating cysteines 1-6) disrupted zinc regulation, suggesting that disulphide bonds in the cysteine rich finger domain stabilize the zinc binding site (Clyne *et al.*, 2002b; Coddou *et al.*, 2007). The importance of the cysteine rich region has also been shown for regulation by copper (Coddou *et al.*, 2007). It is tempting to speculate that zinc could act to stabilize a conformational change associated with ATP binding and thus increase agonist potency. The P2X2 receptor double mutant His120Cys/His213Cys showed barely detectable responses to ATP; however, on treatment with the reducing agent, DTT currents were increased (Nagaya *et al.*, 2005). This suggests that the disulphide bond between the introduced cysteines either restricted access of ATP to the binding site or a conformational change associated with binding, and/or gating. Interestingly, His120 in the P2X2 receptor is equivalent to Arg125 in the P2X7 receptor, which is ADP ribosylated and can lead to receptor activation (Adriouch *et al.*, 2007), once again highlighting that this residue is close to the ATP binding site. The zP2X4 receptor crystal structure confirms these predictions, and shows that the residues are in close proximity to the predicted ATP binding site (Figure 1).

Trivalent cations can also have a regulatory role inhibiting P2X receptor function (Nakazawa *et al.*, 1997). Lanthanum and gadolinium blocked P2X1 and P2X2 currents in oocytes; this inhibition appeared competitive and was reversed by increasing the ATP concentration (Nakazawa *et al.*, 1997). The crystal of the zP2X4 receptor was solved in the presence of gadolinium, with residues Asp184 and Asn187 on the upper outer region of the receptor, and Glu98 at the core vestibule, coordinating the binding of the trivalent ion (Kawate *et al.*, 2009). Asn187 is just adjacent to Phe188Thr189, which are thought to contribute to the ATP binding pocket, suggesting that gadolinium binding at Asp 184 and Asn187 reduces ATP affinity. Interestingly, gadolinium increases recovery from desensitisation at transient sensory P2X and rat P2X3 receptors (Cook *et al.*, 1998). At these receptors, recovery from desensitisation and agonist unbinding were strongly linked (Pratt *et al.*, 2005). This indicates that gadolinium speeds the unbinding of agonist from P2X3 receptors and is consistent with an effect on reducing ATP affinity. These results suggest that the high (1 mM) gadolinium used in the receptor crystallization may make it difficult to solve the structure in an ATP bound form.

Protons can also regulate P2X receptors with the effect dependent on the channel subtype (Stoop *et al.*, 1997). Histidine residues mediate proton sensitivity at P2X2, 3, 4 and 7 receptors, although different residues are involved in each of these subunits. At P2X2 receptors, the His319Ala mutation reduced the potentiating effects of protons on ATP sensitivity (Clyne *et al.*, 2002a), while mimicking protonation of His319 with the His319Lys mutation increased ATP potency 40-fold. Analysis of the structure for the zP2X4 receptor predicts that His319 lines the vestibule formed at the centre of the receptor (Figure 3) and could therefore contribute to channel gating. In contrast, at P2X3 receptors, protons decrease ATP responses (Stoop *et al.*, 1997), and His206 contributes to proton sensitivity, as well as recovery from desensitisation (Gerevich *et al.*,

2007). P2X4 receptors were also inhibited by protons, and His286 mediates this effect (Clarke *et al.*, 2000). Interestingly, although different histidine residues are involved in proton inhibition at P2X3 and P2X4 receptors, the residues are predicted to be on the surface of the receptor (Figure 3) in the ring below the predicted ATP binding site, where mutants may have an effect on ATP potency. This raises the possibility that protonation of the histidine reduces gating and thus potency (see above). At P2X7 receptors, His130 was involved in proton sensitivity (Acuna-castillo *et al.*, 2007), and this residue forms part of the cysteine rich region (Figure 3) that also contributes to divalent cation sensitivity.

Antagonist binding sites

P2X receptor antagonists have been suggested to have therapeutic potential in several diseases (Burnstock, 2006b) and a range of subtype selective compounds have been described, for example P2X1 receptor selective NF449 (Braun *et al.*, 2001) and P2X3 receptor selective A317491 (Jarvis *et al.*, 2002). However, our understanding of the molecular basis of antagonist action is limited and focused on the relatively non-selective antagonists pyridoxal 5-phosphate 6-azophenyl-2',4'-disulphonic acid (PPADS) and suramin (North and Surprenant, 2000).

PPADS acts non-competitively, and at high concentrations depresses maximal responses to ATP. The rat P2X4 (rP2X4) receptor is insensitive to commonly used antagonists (Buell *et al.*, 1996). PPADS can form a Schiff base with lysine residues, and a lysine present in PPADS sensitive P2X1 and P2X2 receptors is replaced by a glutamic acid residue in the rP2X4 receptor. Mutation of this glutamic acid residue at position 249 in the rP2X4 receptor to lysine recovered sensitivity to PPADS (Buell *et al.*, 1996). However, the reverse mutation in the P2X2 receptor (Lys246Glu) did not remove PPADS inhibition. Furthermore, this lysine residue is not present in PPADS-sensitive human P2X3 and P2X7 receptors, and therefore other residues are also likely to contribute to the antagonist binding site (Buell *et al.*, 1996). Subsequent cloning of the human and mouse P2X4 receptors showed that they were more sensitive to PPADS than the rat form (Garcia-Guzman *et al.*, 1997b; Jones *et al.*, 2000); however, they showed essentially identical amino acid sequence around residue 249, suggesting that another region of the receptor was also involved in determining PPADS sensitivity. A chimeric approach was used to determine what was responsible for the PPADS sensitivity differences between human and rat P2X4 receptors, which showed residues in the region 81–183 also contribute to PPADS sensitivity (Garcia-Guzman *et al.*, 1997a). Species differences in PPADS sensitivity at P2X7 receptors were also utilized to generate chimeras and point mutations that identified a contribution of Arg126 to PPADS action (Michel *et al.*, 2008). The mutated residues shown to have an effect on PPADS sensitivity are positively charged, which fits with a role in coordination of the negative charge of the antagonists PPADS. Mapping these residues (equivalent to Arg 249 from the P2X4 and 126 from the P2X7 receptor) onto a homology model structure of the P2X1 receptor shows that they are located either side of the proposed agonist binding site

(Figure 1B–D). Binding of PPADS at either of these sites could therefore restrict access of ATP to its binding site and account for its antagonistic effect.

Suramin appears to be a competitive antagonist, and insight into its binding site has come from differences in properties between species variants of P2X receptor subunits. For the P2X4 receptor, point mutation showed residue 78 was associated with differences between rat and human receptors; for the mutant rat P2X4 Gln78Lys, the sensitivity was increased ~10-fold (Garcia-Guzman *et al.*, 1997a). However, this was still >100-fold lower than suramin affinity at the hP2X1 receptor (Roberts and Evans, 2004), suggesting that additional residues also contribute substantially to sensitivity. The marked differences in sensitivity of human and mouse P2X1 receptors to suramin have also been used to identify Lys138 as contributing to the antagonist action of suramin and its derivative NF449 that is effective at low nanomolar concentrations (Braun *et al.*, 2001; Sim *et al.*, 2008). However, this residue is not conserved among suramin sensitive receptors, and it has been suggested that it is the localized positive charge that is important in modulating the actions of the negatively charged suramin (Braun *et al.*, 2001; Sim *et al.*, 2008).

Suramin can act as a partial agonist at constitutively active P2X2 receptors (Cao *et al.*, 2007), and radioligand binding studies have shown that suramin can competitively displace radioligands from P2X receptors (Michel *et al.*, 1996). This raises the possibility that the same residues may be involved in suramin and ATP binding. However, alanine mutants of the conserved positively charged Lys, Arg and Phe residues in the P2X1 receptor, which reduced ATP potency by >100-fold, had little (Arg292Ala) or no effect (Lys68Ala, Lys70Ala and Lys309Ala) (Ennion *et al.*, 2000) or actually increased (Phe291Ala) (Roberts and Evans, 2004) suramin sensitivity. This shows that key residues involved in ATP potency do not have an effect on suramin sensitivity, and indicate there is not significant overlap in the particular residues involved in suramin and ATP binding. The homology model of the hP2X1 receptor also suggests that the mutants that can change suramin sensitivity do not appear to interact directly with residues that form the proposed ATP binding site. The Lys138 side chain, shown to regulate suramin sensitivity at the P2X1 receptor (Sim *et al.*, 2008), does not line the ATP binding pocket, but faces away from it and is on the other side of the cysteine rich region (Figure 1). The proximity to, but not direct interaction with the proposed ATP binding site, suggests that suramin may act by stabilizing particular conformational states of the receptor or its binding occludes subsequent ATP binding and either of these mechanisms could account for the competitive antagonism.

Suramin and PPADS are effective antagonists at most, but not all P2X receptors. Therefore, it is likely that each antagonist may share key core conserved residues involved in binding across subunits in addition to those residues described above that can regulate sensitivity. However, whether this is the case, or there is a more variable/diffuse binding site of non-conserved residues in a general area remains to be determined. The zP2X4 receptor structure has given an insight into where antagonists may act and highlights adjacent regions of interest for future mutagenesis studies.

Transmembrane regions

Cysteine scanning mutagenesis studies showed that both TMs could contribute to the permeation properties of P2X receptors (Rassendren *et al.*, 1997; Egan *et al.*, 1998; Jiang *et al.*, 2001; Haines *et al.*, 2001b; Li *et al.*, 2008). Determination of the rates of modification of introduced cysteine residues suggested that TM2 lined the pore and contained the channel gate, and that TM1 was peripheral to the pore region (Li *et al.*, 2008). These predictions are supported by the zP2X4 receptor structure (Kawate *et al.*, 2009), and, interestingly, the arrangement of the TMs is essentially the same as trimeric two TM ASIC channels (Gonzales *et al.*, 2009). While it is clear that the TM2 region forms the ionic permeation pathway, TM1 also contributes to control of the gating of the channel as shown by a chimera of TM1 of P2X1 added to P2X2 increasing sensitivity to α,β -methylene ATP (Haines *et al.*, 2001a), and the converse chimera with TM1 of P2X2 changing P2X1 from a desensitizing to non-desensitizing phenotype (Rettinger and Schmalzing, 2004). Elegant single-channel analysis of TM2 mutants have demonstrated residues that play a role in the gating, single-channel conductance and rectification of the P2X2 receptor and have given an important insight into the gate and channel opening of P2X receptors (Cao *et al.*, 2007; Cao *et al.*, 2009). Studies on the P2X receptor TM domains have clearly shown that they both play a role in determining ATP responsiveness, and it is clear that TM derived differences in gating between the receptors could contribute to the variation in agonist sensitivity.

Future directions

The structure of the zP2X4 receptor has provided a major advance in our understanding of P2X receptors, insight into the interpretation of mutagenesis studies and a model of where ATP binds to the receptor. This review provides an overview of mutagenesis studies on several P2X receptor subunits that supports a conserved consensus core ATP binding pocket at P2X receptors, with the negative charge of the phosphate groups coordinated by conserved Lys residues, and an AsnPheArg motif that may regulate binding of the adenine ring. The differences in ligand sensitivity between subunits (for review, see North and Surprenant, 2000), however, demonstrate clearly that variant amino acids also contribute to, and regulate, pharmacological properties. The variation could arise from single amino acid changes to disrupt gating and/or interaction with the ligand, and/or involve more complex structural changes as suggested by the variation in the distance between introduced cysteine residues in different subunits (Marquez-klaka *et al.*, 2009). A range of studies, including the use of differences in properties between species orthologues, chimaeric receptors and mutations, have identified residues that contribute to the heterogeneity of receptor properties. However, to date, no clear picture has begun to emerge on what underlies the variations in pharmacology, for example the differences in ATP potency, why α,β -meATP is effective at P2X1 and P2X3 but not other P2X receptor subunits, or the mechanism of action of selective antagonists. Additional P2X receptor structures would provide another

leap forward in our understanding of this family of receptors and give an insight into subtype dependent differences. Of particular interest would be structures with agonist or antagonist bound to develop models of drug action, aid in the understanding of the subtype dependent basis of pharmacological properties, and gain insight into structural changes in the receptor on activation. However, in their absence, there are a number of areas that can be addressed. (i) Structural-based modelling of drug action: Now that a structure for the zP2X4 receptor is available, molecular-docking programmes can be used to develop models of how ligand binding to the receptor. These models could also be developed to understand the structural basis for differences in pharmacology between subunits and in rational drug design. (ii) *Conformational changes*: The structure of zP2X4 is in the closed conformation in the absence of ATP and provides a snapshot of one of the conformations that the receptor can adopt. A major question is how ATP binding leads to gating of the channel pore? Techniques to look at changes in accessibility of regions of the protein will be useful, and voltage clamp fluorometry could be used to give some temporal resolution to conformational changes (Pless and Lynch, 2009). (iii) *Intracellular domains*: Mutations in the intracellular domains can have profound effects on the properties of P2X receptors, and how these are transmitted to the rest of the channel remains to be determined. The association of P2X receptors with interacting proteins (e.g. Kim *et al.*, 2001) provides an added layer of complexity to understand the molecular basis of how the intracellular domains regulate channel function.

Acknowledgements

This work was supported by the Wellcome Trust. Thanks to Dr A. Gingras for help with PyMOL, Ms Olivia Presley and Dr R. Schmid for work on the hP2X1 receptor homology model, and Dr B.D. Grubb and Professors M.P. Mahaut Smith and I.D. Forsythe for helpful comments.

Conflict of interest

There is no conflict of interest for the paper.

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