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# Molecular structure and function of P2X receptors

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## A B S T R A C T

ATP-gated P2X receptors are trimeric ion channels selective to cations. Recent progress in the molecular biophysics of these channels enables a better understanding of their function. In particular, data obtained from biochemical, electrophysiological and molecular engineering in the light of recent X-ray structures now allow delineation of the principles of ligand binding, channel opening and allosteric modulation. However, although a picture emerges as to how ATP triggers channel opening, there are a number of intriguing questions that remain to be answered, in particular how the pore itself opens in response to ATP and how the intracellular domain, for which structural information is limited, moves during activation. In this review, we provide a summary of functional studies in the context of the post-structure era, aiming to clarify our understanding of the way in which P2X receptors function in response to ATP binding, as well as the mechanism by which allosteric modulators are able to regulate receptor function.

### List of chemical compounds:

ATP (PubChem CID: 5957)  
Suramin (PubChem CID: 5361)  
TNP-ATP (PubChem CID: 3035228)  
GW791343 (PubChem CID: 71576670)  
Ivermectin (PubChem CID:24278497)

### Keywords:

ATP  
Gating  
Ion channel  
Purinergic receptors  
Allosteric modulation

## Contents

1. Introduction .....	19
2. Global architecture .....	19
2.1. General topology of the ion channel .....	19
2.2. Location of the ATP sites .....	21
3. Structural point of view of the gating mechanism .....	21
3.1. Movement of the binding jaw .....	21
3.1.1. Reorganization of the binding loops following ATP binding .....	21
3.1.2. Intermediate activation states .....	22
3.2. ECD flexibility .....	23
3.3. Transmembrane motions and pore opening .....	23
4. Allosteric modulation of P2X receptors .....	24
4.1. Allosteric regulation within the ectodomain: the case of zinc .....	24
4.2. Allosteric regulation within the transmembrane domains: the case of ivermectin .....	25
4.3. Allosteric regulation within the intracellular C-terminus domain: the regulatory effects of phospholipids .....	26
5. Optogating of P2X receptors .....	26
6. Conclusion .....	28
References .....	28

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## 1. Introduction

Among the different transmembrane proteins involved in ionic transfer, the ligand-gated ion channels (LGICs) form an essential superfamily owing to the fact that they possess a number of key physiological roles, most notably the mediation of neuronal signalling (Albuquerque et al., 2009; Bardoni et al., 1997; Lalo et al., 2012; Paoletti et al., 2013; Taly et al., 2009). The binding of a ligand in the extracellular domain triggers conformational changes that open the pore and allow the passage of ions along their electrochemical gradients. LGICs comprise three families of receptors, differing primarily in their oligomeric assemblies (Khakh and North, 2006; Lemoine et al., 2012). Among these, trimeric P2X receptors, which are activated by extracellular ATP (Burnstock, 2006; North, 2002), are implicated in a diverse range of physiological and pathological processes, including pain sensation (Wirkner et al., 2007; Zhuo et al., 2011), inflammation (Idzko et al., 2014), taste (Kinnamon and Finger, 2013) and modulation of neurotransmitter release (Khakh and North, 2012; Lalo et al., 2012). These receptors are therefore promising therapeutic targets (Lemoine et al., 2012; North and Jarvis, 2013).

Thus far, seven different P2X subunits (P2X1-7) have been found in mammals, which assemble together in a homomeric and/or heteromeric fashion to form trimeric ion channel structures. Each subunit has several unifying features: C- and N-termini are located intracellularly, linked by two membrane-spanning segments (TM1 and TM2) and a large extracellular domain (ectodomain), where the ATP-binding sites are nestled (Fig. 1A). In response to the binding of extracellular ATP, these receptors switch between different conformational states (Browne et al., 2010; Giniatullin and Nistri, 2013; Jiang et al., 2013; Moffatt and Hume, 2007). Initial agonist binding causes the receptor to progress from a closed, resting state to an open, conducting state (named I1), allowing the flow of Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> across the membrane (Egan and Khakh, 2004). Prolonged exposure to ATP leads to the formation of a desensitized state, a temporary inactivation that terminates ion flux despite the fact that ATP remains bound to the receptor. The kinetics of desensitization vary between P2X subtypes – fast desensitization is observed only with P2X1 and P2X3 receptors, whereas slow desensitization is observed for P2X2, P2X4 and P2X5. In contrast, P2X7 receptors exhibit no apparent desensitization (North, 2002). Dissociation of ATP from the desensitized state, in turn, reverts the channel to the initial closed, resting state, in which it is able to be activated once again. This process is known as resensitization. A second conducting state (I2) is also found for selected P2X subtypes, whereby a dilation of the pore occurs, rendering the channel progressively permeable to larger cations and dyes (Khakh et al., 1999a; Virginio et al., 1999) (For a recent review see (Rokic and Stojilkovic, 2013)).

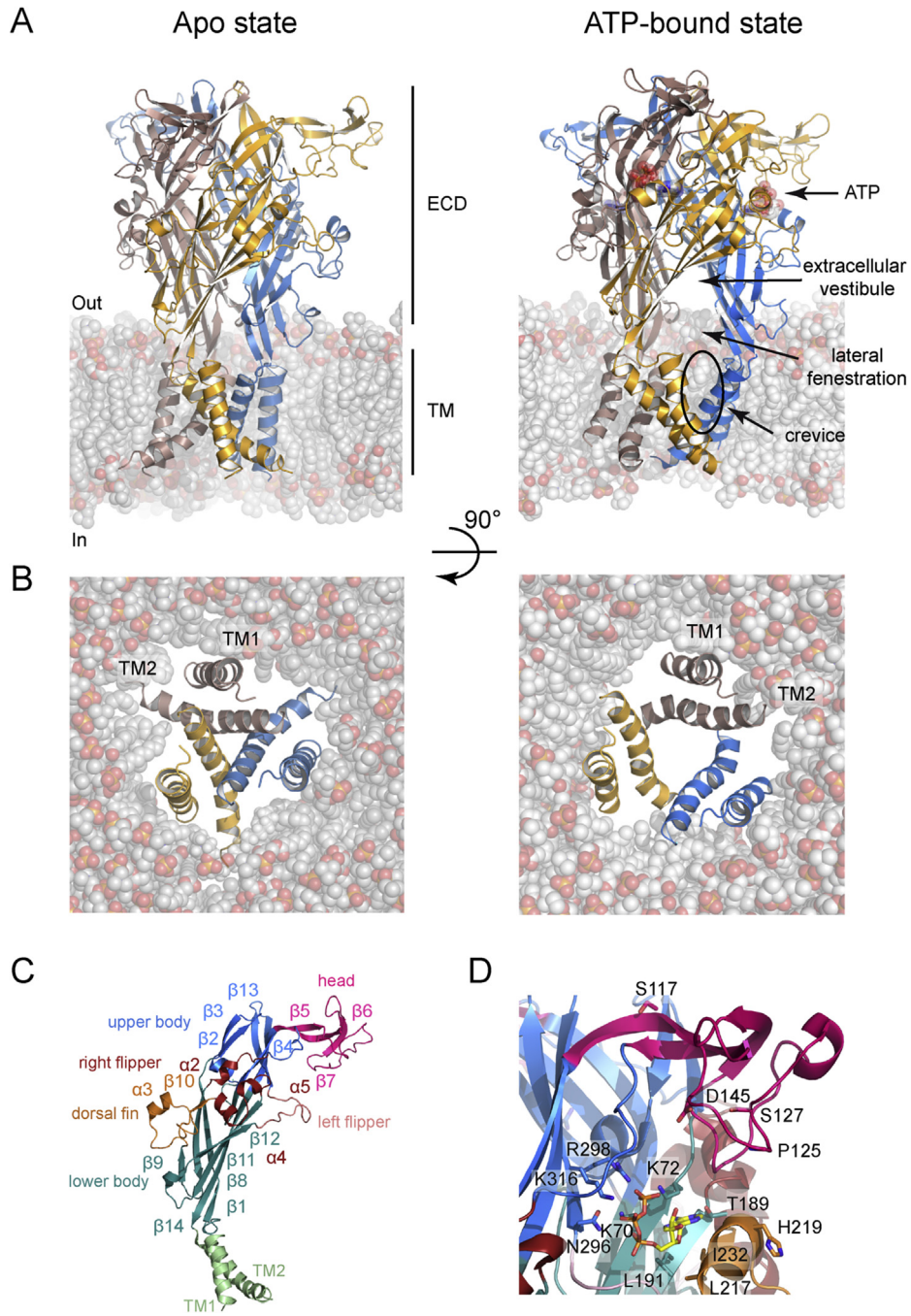
The recent determination of the crystal structures of the zebrafish P2X4 receptor (zfp2X4) in both an apo, closed-channel state (Kawate et al., 2009) and in an ATP-bound, presumably open-channel state (Hattori and Gouaux, 2012) represented a landmark achievement in the understanding of the molecular architecture of these receptors. These structures allowed the confirmation of a wealth of biochemical and electrophysiological research previously carried out to probe the structure and function of P2X receptors. In addition to this, comparison of structures in the apo and ATP-bound states has enabled identification of the extracellular motions which follow ATP binding, thus providing us with a plausible mechanism for channel activation. However, in addition to these advances, the X-ray structures have also raised a number of intriguing questions. In particular, the molecular mechanism involved in the opening of the transmembrane pore itself still

remains unclear and under debate. This may be due, in part, to the fact that the intracellular domains, known to be of critical importance for P2X function (Allsopp and Evans, 2011; Allsopp et al., 2013; Boue-Grabot et al., 2000; Robinson and Murrell-Lagnado, 2013; Schwarz et al., 2012; Xu et al., 2012), were removed for crystallization purposes. Additional factors, such as crystal packing and the presence of detergents for solubilisation, may also contribute to these uncertainties. This review will provide a summary of functional studies in the context of the post-structure era, aiming to clarify our understanding of the way in which P2X receptors function in response to ATP binding, as well as the mechanism by which allosteric modulators are able to regulate receptor function.

## 2. Global architecture

### 2.1. General topology of the ion channel

The concept of purinergic receptors was first proposed by Burnstock in the early seventies (Burnstock, 1972) but sustained studies in this field only began in 1994 following the first molecular identification of P2X receptors (Brake et al., 1994; Valera et al., 1994). As P2X receptors had no sequence homology with other known ion channels, molecular and biochemical studies were first used to extensively explore the molecular structure. The general pattern of hydrophobicity suggested the presence of two domains of sufficient length to span the membrane (Brake et al., 1994; Valera et al., 1994) and complementary approaches such as N-glycosylation site tagging (Newbolt et al., 1998) provided early evidence of the intracellular location of both the N- and C-termini. Substitutions of residues of the putative transmembrane domains to tryptophan (Silberberg et al., 2005), cysteine (Jiang et al., 2001; Rassendren et al., 1997) or alanine (Li et al., 2004) highlighted a relative periodicity compatible with an alpha-helical organization. The peripheral location of TM1 was suggested by calcium permeability studies (Samways and Egan, 2007; Samways et al., 2008), whilst methanethiosulfonate (MTS) accessibility of residues mutated to cysteine (Jiang et al., 2001; Li et al., 2008) showed that TM1 has a limited contribution to the ion conduction pathway. In terms of global receptor structure, the stoichiometry of these receptors was for a long time debated (for a detailed review see (Hausmann et al., 2015)). A trimeric assembly was first proposed following immunohistochemical studies using aldehyde-containing reagent-crosslinked receptors for P2X1 and P2X3 (Nicke et al., 1998), engineered concatemeric receptors of P2X2 (Stoop et al., 1999) and disulphide bond experiments on cysteine-substituted heteromeric P2X2/3 receptors (Jiang et al., 2003). Moreover, functional studies based on Hill slopes obtained from ATP dose–response curves and single-channel recordings (Ding and Sachs, 1999), along with low resolution structures of the receptor by atomic force microscopy and electron microscopy (Barrera et al., 2005; Mio et al., 2005, 2009; Nakazawa et al., 2005; Shinozaki et al., 2009), further supported the originally proposed trimeric assembly of P2X receptors. The determination of crystal structures of a truncated zebrafish P2X4 receptor ( $\Delta$ zfp2X4), firstly in 2009 resolved at 3.1 Å in the absence of ATP (Kawate et al., 2009) and subsequently in 2012 bound to ATP at a resolution of 2.8 Å (Hattori and Gouaux, 2012), definitively confirmed this stoichiometry, as well as the global architecture of P2X receptors. These structures revealed the precise three-dimensional organization of the receptor and the folding of each subunit (Fig. 1A–C). The overall shape of each individual subunit is likened to that of a dolphin, consisting of a body, head, tail, dorsal fin and left and right flippers (Fig. 1C). This comparison allows a clear distinction of structurally



**Fig. 1.** Molecular organization of P2X receptors. (A) Lateral view of the crystal structures of  $\Delta$ zfp2X4 receptor in a closed state (left, PDB code 4DW0) and in an ATP-bound state (right, PDB code 4DW1) embedded in a lipid bilayer. The three subunits are depicted in cartoon representation and shown in different colours. ATP is in stick representation, the membrane is represented by red, orange and white spheres. ECD and TM stand for extracellular and transmembrane domains, respectively. (B) The same structures as in (A), viewed from the extracellular side, along the axis of the central channel. (C) Ribbon representation of the folding of one subunit. Each domain is named according to a homology comparison to a dolphin. The extracellular domain is composed of 14  $\beta$ -sheets and 4  $\alpha$ -helices, whereas 2  $\alpha$ -helices form the transmembrane domain. (D) Close up view of the ATP-binding pocket coloured according to domains as in panel (C), the residues (zfp2X4 numbering) indicated are discussed in the text.

differing regions. The large glycosylated ectodomain represents the body of the dolphin, and is composed of highly conserved residues, organized into a rigid  $\beta$ -sheet structure and stabilized by five disulphide bonds. Branching out from this core body region are four more structurally flexible domains: the head, dorsal fin, left and right flippers (Fig. 1C). The transmembrane domains, likened to the dolphin tail, were confirmed to be organized into alpha-helices. TM1 is positioned peripherally to TM2 and the ion conducting pore is lined primarily by the association of three TM2 helices,

angled at  $45^\circ$  from the normal of the membrane plane (Fig. 1A and B). The gate of the channel is defined by the hydrophobic region between L340 and A347, centred at the residue A344, approximately halfway down the length of the TM2 helix. The structure of the zfp2X4 receptor solved in the absence of ATP, presumably in the closed state, reveals inter- and intra-subunit interfaces with extensive interactions in the upper body, each monomer twisting around its neighbours. Two potential pathways through which ions can access the pore were identified: a central pathway, whereby



ions enter the channel vertically *via* three connected vestibules positioned along the central axis of the receptor, or alternatively, a lateral pathway, whereby ions enter the channel through three fenestrations located above the gate region. However, MTS accessibility studies and electrostatic free energy calculations (Kawate et al., 2011; Samways et al., 2011) strongly suggest that extracellular ions use the lateral pathway, entering the channel preferentially through the fenestrations before flowing through the ion pore itself.

## 2.2. Location of the ATP sites

Systematic site-directed mutagenesis coupled with electrophysiology provided much of the information crucial for locating the ATP binding sites. Several recent reviews focused on this aspect of P2X function (Browne et al., 2010; Chataigneau et al., 2013; Evans, 2009). Briefly, a combination of several studies revealed that there are eight highly conserved residues critical for ATP binding (K70, K72, N96, F188, T189, F297, R298 and K316, zP2X4) insofar as substitutions at these homologous positions inhibit ATP currents in different P2X subtypes (Ennion et al., 2000; Fischer et al., 2007; Jiang et al., 2000; Roberts et al., 2008; Wilkinson et al., 2006; Yan et al., 2006; Zemkova et al., 2007). Evidence that the formation of a ligand-binding site requires the involvement of two subunits was provided by co-expression of rP2X3 with mutated K69A/K308A rP2X2 (a non-functional, “dead” subunit) (Wilkinson et al., 2006), in addition to SDS-page gels of K69C/F289C rP2X2 receptor in both presence and absence of ATP (Marquez-Klaka et al., 2009). Many strategies were also employed to determine the specific residues which are involved in binding ATP. Among them, charge-dependent effects induced by MTS labelling of cysteine introduced at position I67 (Jiang et al., 2000) and activation of the receptor following ADP-ribosylation at position R125 (P2X7) (Adriouch et al., 2008) suggested that these particular residues are in close proximity to the bound agonist. A strategy of site-directed affinity labelling was developed by Jiang et al., using a thiol-reactive probe (8-thiocyanato-ATP, NCS-ATP) to demonstrate the involvement of two residues (N140 and L186 in rP2X2) in the coordination of the adenine ring of ATP (Jiang et al., 2011). Voltage-clamp fluorometry (VCF) experiments coupled with site-directed fluorescence labelling allowed Lorinczi et al. to successfully orientate the ribose moiety of the ATP molecule in the binding cavity of rP2X1 (Lorinczi et al., 2012).

The crystal structures of zP2X4 confirm the implication of residues previously identified to bind ATP (Hattori and Gouaux, 2012). These residues form a tight cluster, located within an intersubunit cavity and surrounded by the head and left flipper of one subunit and by the dorsal fin of another (Fig. 1D). Several unanticipated interactions were also revealed: briefly, the residue L217 is involved in the recognition of the solvent-accessible ribose ring, the residues K70, K72, K316, N296 and R298 stabilize the triphosphate tail in an unusual U-shaped structure, K70 and T189 form hydrogen bonds with the adenine base, whilst L191 and I232 coordinate this adenine by means of hydrophobic interactions.

## 3. Structural point of view of the gating mechanism

Following the resolution of the crystal structures, a key issue for current research is the investigation into how ATP-binding can open the channel. In 2009, Shinozaki et al. provided the first time-lapse imaging of P2X4 receptor's structural changes in response to ATP application using fast-scanning atomic force microscopy (AFM) (Shinozaki et al., 2009). In the presence of ATP, individual subunits were observed, which draw away from one another to form the central pore. Since this study, several functional studies using the

substituted-cysteine accessibility method (SCAM) (Samways et al., 2014), engineered disulphide bonds (Du et al., 2012; Jiang et al., 2001; Kawate et al., 2011; Kowalski et al., 2014; Roberts et al., 2012; Stelmashenko et al., 2014; Zhao et al., 2014) or VCF (Lorinczi et al., 2012) have provided scattered information, giving a glimpse into the gating mechanism. The resolution of an ATP-bound state thus represented a major advance, as it provided an unrivalled picture of an open channel. *In silico* modelling of the two structures provided a basis for further investigations, and a plausible model of gating gradually began to take shape, which can be dissected into 5 key steps: ATP binding, tightening of the so-called binding “jaw”, flexing of the lower body regions, expansion of the lateral fenestrations and subsequent pore opening. ATP binding provokes a global structural movement, whereby subunits bend outward and rotate, inducing an expansion in the lower body region. As the lower body is directly connected to the transmembrane domain, this movement in turn induces a displacement of the outer ends of the TM helices, opening the pore in an iris-like motion (Browne et al., 2010). Here, we will focus on molecular changes in key domains during gating.

### 3.1. Movement of the binding jaw

#### 3.1.1. Reorganization of the binding loops following ATP binding

The ATP binding site, located in an inter-subunit cavity between the head domain of one subunit, and the dorsal fin of another, is often referred to as the “binding jaw”. Evidence that the closure of this binding jaw plays an important initial role in the gating process has been provided by multiple recent studies. First, the presence of a zinc-binding site, naturally formed by residues H120 and H213 in the rP2X2 receptor, was exploited to display this closing motion of the binding jaw's two lips. ATP-currents on the double cysteine mutant H120C/H213C (rP2X2) are dramatically decreased and DTT treatment rescues a high sensitivity state, indicating that these residues are found in close proximity (Nagaya et al., 2005). However, the distance between these positions as predicted by the closed structure is too great (15 Å) to form a zinc-binding site in the WT receptor or disulphide bridge in the double cysteine mutant, which implies that the head and the dorsal fin domains must come closer together during activation. Whilst this hypothesis readily supports zinc potentiation, it appears that a different mechanism must take place to explain data obtained from the double cysteine mutant. In the latter case, the disulphide bridges may block ATP responsiveness, most likely by locking the receptor in a state for which ATP binding is considerably reduced. VCF experiments coupled with fluorescence labelling of G115C and G124C (hP2X1) also revealed that agonist binding induces a movement of the head domain of P2X1 receptor (Lorinczi et al., 2012), a finding which was further supported by electron microscopy experiments (Roberts et al., 2012). Engineered histidine bridges formed between residues of the head domain and the right-flipper connecting loop (Jiang et al., 2012a), or disulphide bridges formed between the top of the lower body and the right flipper (hP2X1 190C-284C), or between residues of the head region from two different subunits (hP2X1 138C-181C) (Roberts et al., 2012) highlighted the critical role of these segments in ATP function. Finally, using the closed state structure with ATP docked in its binding site according to the results of NCS-ATP labelling (Jiang et al., 2011), two different sets of data were produced by normal mode analysis (NMA) (Du et al., 2012; Jiang et al., 2012a), both suggesting that a decrease in the distance between residues of the binding jaw located in two adjacent subunits occurs following ATP binding. These data combined thus demonstrate that ATP-induced tightening of the binding jaw correlates precisely with an opening of the channel.

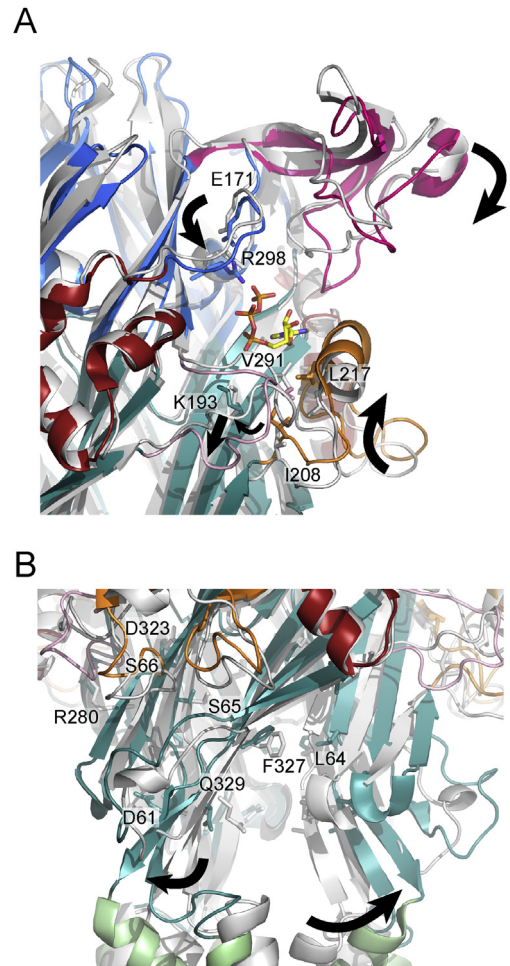
Although no structure with a competitive antagonist bound to

the receptor has been resolved to date, several studies suggest that antagonists act by binding to the upper jaw and thus impairing jaw closure. The position K138 of hP2X1 was first identified to be crucial in the binding of suramin (Sim et al., 2008). The use of hP2X1/DdP2X and hP2X1/hP2X2 chimeras subsequently allowed identification of a cluster of positively charged residues (KAKRK), including K138, in the head domain which contribute to suramin sensitivity (El-Ajouz et al., 2012). Moreover, the N140C mutant labelled with the NCS-ATP affinity probe displays decreased ATP-induced currents (Jiang et al., 2011) and binding of TNP-ATP, a presumably competitive P2X antagonist, blocks movement of the binding jaw (Jiang et al., 2012a). A recent study which uses NMA, principal component analysis (PCA) and short molecular dynamics (MD) simulations suggests the presence of at least three sites able to recognize the adenine group of ATP and thus three distinct recognition modes (Huang et al., 2014): the first mode being similar to that found in the ATP-bound crystal structure, the second featuring the adenine group buried in the interface of the rigid lower body domains and finally a third mode whereby adenine is positioned near the base of the head domain, which could explain the previous results obtained by engineered site-directed affinity labelling. Interestingly, only the first recognition mode can promote an effective downward motion of the head domain, whereas the other two modes are only able to induce outward or upward motions of the head. Jaw tightening therefore seems to be an essential step of the gating mechanism.

Other movements following ATP binding which occur in the loop regions surrounding the binding site were also identified as being critical for the initiation of pore opening. In particular, it has been shown that the left flipper must be pushed outward from the ATP binding pocket simultaneously to the cleft closure between the head and dorsal fin domains (Fig. 2A). To this end, Zhao et al. introduced cysteine or histidine residues on the left flipper (e.g. V288C rP2X4) and dorsal fin (e.g. T211C rP2X4) and observed an inter-domain disulphide bond or zinc bridge respectively, which impaired channel gating by preventing the relative motion of the two domains (Zhao et al., 2014). They predict, by MD simulations, that ATP binding may destroy the hydrophobic interactions between the left flipper and dorsal fin domains, induces repelling of V291 (zfpP2X4) from the ribose of ATP and provokes closing motions of K193, L217 and I208 around the molecule of ATP, thus causing the downward motion of the left flipper and upward movement of the dorsal fin (Zhao et al., 2014). Other studies have probed the reorganization of the binding pocket by using disulphide bond engineering to restrict movement between the upper body and right flipper domains (N284C/K190C hP2X1) or between the head domain and the adjacent subunit (K138C/E181C hP2X1), revealing that a counter-clockwise rotation of the subunits may occur after ATP binding (Roberts et al., 2012). These results are consistent with MD simulations, which show a leftward motion of the loop F139-G146 (zfpP2X4), situated within the head domain, and a rightward movement of the loop P169-A183 (zfpP2X4) (Fig. 2A), which is covalently linked to the head (Huang et al., 2014). In agreement with these movements, recent work has suggested that during ATP activation of the P2X2 receptor, a significant rearrangement of residues E167 and R290, which interact electrostatically in the closed state ATP binding site, enables R290 to form a new, strong ionic interaction with the  $\gamma$ -phosphate oxygen of ATP, thus breaking the original salt bridge (Hausmann et al., 2013). This electrostatic switch appears to be a critical step that leads to channel opening.

### 3.1.2. Intermediate activation states

Some recent works have suggested the existence of intermediate states in P2X receptors which precede pore opening. The idea of



**Fig. 2.** Structural rearrangements during gating mechanism. Superimposed structures in the resting state (grey) and the ATP-bound state (coloured) at the binding site (A) and the lower body (B). Black arrows show the relevant motions likely to occur during allosteric transition, leading to the opening of the channel. In panel B the indicated pairwise residues (L64-F327; D61-Q329; S65-R280; S66-D323), when mutated into cysteines on different P2X subtypes, are implicated in disulphide locking of the closed state (zfpP2X4 numbering).

such primed states in P2X receptors was first introduced by Moffatt and Hume who incorporated a so-called “flipped” intermediate state into the simple allosteric model to explain the delay between ATP-binding and the observation of ionic currents (Moffatt and Hume, 2007). This assumption was later confirmed by two crucial studies. The first of these showed that the agonist NCS-ATP tethered to L186C in rP2X2 receptor in which an allosteric reporter mutation, T339S, was introduced to observe spontaneous channel openings, does not lock the channel in the open state, but instead allows the channel to open in response to zinc (Jiang et al., 2012b), indicating that NCS-ATP actually may trap an intermediate state that precedes channel opening. A second study provides evidence that BzATP tethered in one of the three intersubunit ATP binding sites of P2X7 receptor induced conformational changes in the remaining sites, causing these sites to lose their high selectivity for ATP and to bind other pyrimidine and diphosphate nucleotide analogues (CTP, UTP and ADP) with greater efficiency (Browne and North, 2013).

The molecular movements underlying such primed states remain unclear, but recent works suggest that movements of the head domain may be able to occur not only as a result of ATP-binding, but equally in the absence of agonist. Normal mode

analysis of zfp2X4 and molecular dynamics experiments (Huang et al., 2014; Zhao et al., 2014) showed a possible spontaneous downward motion of the head domain, resulting in the closure of the jaw in the absence of ATP binding. This observation was confirmed by the spontaneous formation of a disulphide bond between residues of the dorsal fin and head domain (K113C and K201C, hp2X3), which impaired binding of a fluorescent ATP derivative, BODIPY-TR ATP (Kowalski et al., 2014). Such spontaneous movements of the binding jaw may therefore be related to the progress of the receptor towards these intermediate states.

It is known that strong cooperativity operates between the three binding sites of P2X receptors (Bhargava et al., 2012; Jiang et al., 2003; Yan et al., 2010), and that ATP association rates increase with the sequential binding of ATP. Experiments from heterotrimeric receptors and concatenated constructs (Stelmashenko et al., 2012) demonstrated that two molecules of agonist seem sufficient to open the channel. If this conclusion also stands true for NCS-ATP, one may speculate that the lack of irreversible trapping of the pore in the open state is due to the irreversible binding of only one NCS-ATP molecule. In this case, the binding of NCS-ATP to one of the three ATP-binding sites would allow the receptor to convert from the closed state to an intermediate state, and subsequent binding of a second molecule would eventually result in the full opening of the channel. Other experiments are needed to further clarify this issue.

### 3.2. ECD flexibility

The domains which form the ATP binding pocket are structurally coupled to the rigid body domain (Browne et al., 2010). As such, their movements are transmitted across the protein as a whole. But which residues in particular are essential in the gating mechanism? Among studies which explored the orthosteric sites, single-channel recordings revealed a difference in the role of K69 and K308 (rp2X2). Whilst the spontaneous opening of the K69A/T339S receptor was no different than that seen in T339S, this spontaneous activity disappeared in the mutant K308A/T339S (Cao et al., 2007) suggesting a critical role for K308 in channel gating. Interestingly, K308 (rp2X2) is located on the upper ends of  $\beta$ 14, a  $\beta$  strand located in the body domain, which is directly connected to TM2 (Fig. 1C). Du et al. also suggested a crucial role of this  $\beta$  strand and, following NMA simulations, proposed that the upper ends of  $\beta$  strands  $\beta$ 14 and  $\beta$ 1 of adjacent subunits undergo rotation in opposite senses, demonstrated by a decrease in the distance between K70 and K316 residues (zfp2X4). This rotation also causes the lower ends of these  $\beta$  strands to be pulled further apart from one another, a motion which in turn encourages the outward movements required for channel opening (Du et al., 2012).

Other studies have also demonstrated the necessity of this rotational motion of  $\beta$ 1 and  $\beta$ 14 strands of neighbouring subunits: the formation of disulphide bridges between I62C/D320C (hp2X1) (Roberts et al., 2012), E59C/Q321C (rp2X2) (Kawate et al., 2011) or S65C/D315C (rp2X2) (Stelmashenko et al., 2014) results in the inhibition of ATP-currents, which can be restored upon application of DTT. Moreover, in the lower part of the body domain, bridging of  $\beta$ 1 and  $\beta$ 8, which are connected to TM1, in the same subunit causes a decrease in gating (Stelmashenko et al., 2014), which suggests that the lower body region is involved in expansion movements not only between subunits but also within the subunits themselves (Fig. 2B). The flexibility of this region seems to be essential in the gating mechanism, and has been further illustrated by several studies. First, Jiang et al. showed the role of an extracellular salt-bridge between  $\beta$ 1 and  $\beta$ 12 strands (E63/R274 rp2X2) in stabilizing the closed state (Jiang et al., 2010). Second, it has been demonstrated that the segment spanning

G316–I333 (rp2X4) is essential in transmitting the signal for channel opening from the ATP binding site to the TM domains (Rokic et al., 2013). The residue G316 (rp2X4) is crucial for the flexibility of the lower body: an alanine substitution at this position, which rigidifies the structure, results in a decrease of ATP sensitivity as well as slower activation and desensitization kinetics, whereas a proline mutation causes only a variation in kinetics (Yan et al., 2006).

From the crystal structures, it can be seen that no large conformational changes occur in the upper body domain. However, all of the vestibules located in the central part of the trimeric assembly can be seen to expand, whilst the interface of the upper and the central vestibules, near P95 (zfp2X4), is kept relatively narrow. Previous SCAM studies had also suggested the expansion of the central and extracellular vestibules (Allsopp et al., 2011). Interestingly, mutation of F95 to leucine in hp2X7, a residue equivalent to zfp2X4 I94, results in an altered modulation of the ATP response by the allosteric modulator GW791343, and also favours the opening of the channel (Michel et al., 2008).

### 3.3. Transmembrane motions and pore opening

As previously discussed, combined movements of the head domain, dorsal fin and left flipper cause the rotation and outward flexing of the lower body domains, located near the membrane and connected to the TM helices, which, in turn, induce substantial expansion of the extracellular vestibule and pore opening (Hattori and Gouaux, 2012).

Prior to the publication of the apo and ATP-bound structures, multiple mutagenesis studies focused on the characterization of TM1 and TM2. Extensive SCAM studies provided information about the location of the gate in the TM domain, despite some discrepancies on the exact position of the narrowest point in the channel (for a complete review see (Samways et al., 2014)). From the crystal structures, the gate region can be determined to measure 8 Å in length, spanning from just below L340–N341 on the extracellular side to L346–A347 (zfp2X4) on the intracellular side. SCAM studies were also able to suggest a reorganization of the transmembrane domain, which likely involves extensive repacking and motions of both TM helices. Application of ivermectin (IVM), an allosteric modulator of selected P2X receptors, to mutants obtained by tryptophan scanning mutagenesis provided preliminary information about changes occurring in the interactions of TM1 and TM2 residues during gating, and the results led to the hypothesis that TM1 may rotate relative to TM2 (Silberberg et al., 2007). Following single-channel recordings of TM2 mutants, Cao et al. suggested a counter-clockwise rotation of several tens of degrees and separation of the TM2 helices (Cao et al., 2009). Structural constraints obtained from functional studies predicted conformational rearrangements. The expansion of TM1 and TM2 in the extracellular side was suggested by the inhibition of channel opening resulting from disulphide bond formation between V48C and I328C (rp2X2) (Jiang et al., 2003, 2001). In addition, by engineering metal bridges Li et al. proposed that the TM2 helix does not undergo large rotations, but instead, a relative straightening of TM2 occurs during gating, suggesting that the inner part of the TM2 domain must move toward the central axis of the pore during gating (Li et al., 2010). Moreover, using concatemeric channels carrying one, two or three T339K mutations in rp2X2 receptor, Browne et al. demonstrated that each subunit contributes symmetrically to the opening of the pore (Browne et al., 2011). From the crystal structures, it can be observed that TM1 and TM2 rotate by  $\sim 10^\circ$  and  $\sim 55^\circ$ , respectively, counterclockwise about the central pore axis, perpendicular to the membrane plane, and increase their tilt angle by  $\sim 8^\circ$  and  $\sim 2^\circ$ , respectively, about an axis parallel to the membrane



plane. This implies an iris-like movement, which leads to the opening of a 7 Å-wide pore. However, the large distance separating residues V343 or D349 is not compatible with data from metal bridge experiments in the inner section of the channel, which indicated that these residues should be found in close proximity to one another.

Furthermore, the structure revealed unexpectedly large intersubunit crevices within the transmembrane domain (Fig. 1A). The physiological relevance of these gaps remains unclear, but recent molecular dynamic simulations performed with the ATP-bound state structure indicated that lipids may diffuse into these gaps, thereby blocking the pore, suggesting that these crevices are inconsistent with ion conduction (Heymann et al., 2013).

The lack of cytosolic domain, which was truncated for crystallogenic purposes, may cause the local destabilization of the structure, as well as the absence of intersubunit interactions. A recent study proposes an improved model of the channel in the open state, generated by small rotations and a translation of TM2 toward the central axis, which decreases the size of the crevices and brings the intracellular ends of the TM helices closer together, narrowing the gaps between them. This new packing model restores intersubunit interactions between TM2 helices including the metal bridge formed at the threefold axis by V343C (rP2X2), and intrasubunit interactions between TM2 and TM1 helices (Heymann et al., 2013). These data suggest that the crystal structure most likely does not represent a native, functional open state.

#### 4. Allosteric modulation of P2X receptors

There are many allosteric modulators that regulate P2X function. These modulators can be as small as protons, metals or larger molecules such as IVM, neurosteroids, ethanol, phospholipids and kinases (Coddou et al., 2011a, 2011b; Evans, 2009). Here, we will consider only three examples of allosteric modulators that act on three different loci: the ectodomain, transmembrane domain and intracellular domain. The reader is referred to other extensive reviews for further details (Coddou et al., 2011a, 2011b; Evans, 2009; Huidobro-Toro et al., 2008).

##### 4.1. Allosteric regulation within the ectodomain: the case of zinc

Zinc is the second most abundant trace metal in the body after iron and consistently plays a major role in enzymatic reactions (Huidobro-Toro et al., 2008). In the blood plasma, the concentrations of trace metals including zinc reach 15 µM, and in the brain, zinc concentrations are as high as 100–150 µM. Zinc is co-released with neurotransmitters at some synapses and can act on several post-synaptic targets such as LGICs to modulate neuronal activity (reviewed in Huidobro-Toro et al. (2008), Sensi et al. (2009)).

Extracellular zinc modulates the function of some P2X receptors. It has a biphasic allosteric modulatory effect: at low concentrations (under 100 µM), pre-incubation with zinc has a potentiating effect on ionic currents flowing through rP2X2, rP2X3, rP2X4 and rP2X5 receptors, whereas at higher concentrations, in the millimolar range, zinc mainly has an inhibitory effect (Coddou et al., 2011b; Wildman et al., 2002; Wildman et al., 1998, 1999a, b). In rP2X1 and rP2X7 receptors, zinc exerts a purely concentration-dependent inhibitory effect (Acuna-Castillo et al., 2007; Liu et al., 2008; Virginio et al., 1997; Wildman et al., 2002, 1999b). The inhibitory allosteric effect is dependent upon a pre-incubation period, especially for rP2X2, rP2X3 and rP2X1.

The biphasic effect has been interpreted as a result of the existence of at least two distinct allosteric sites for zinc, one with a high affinity responsible for the potentiating effect and one with a low

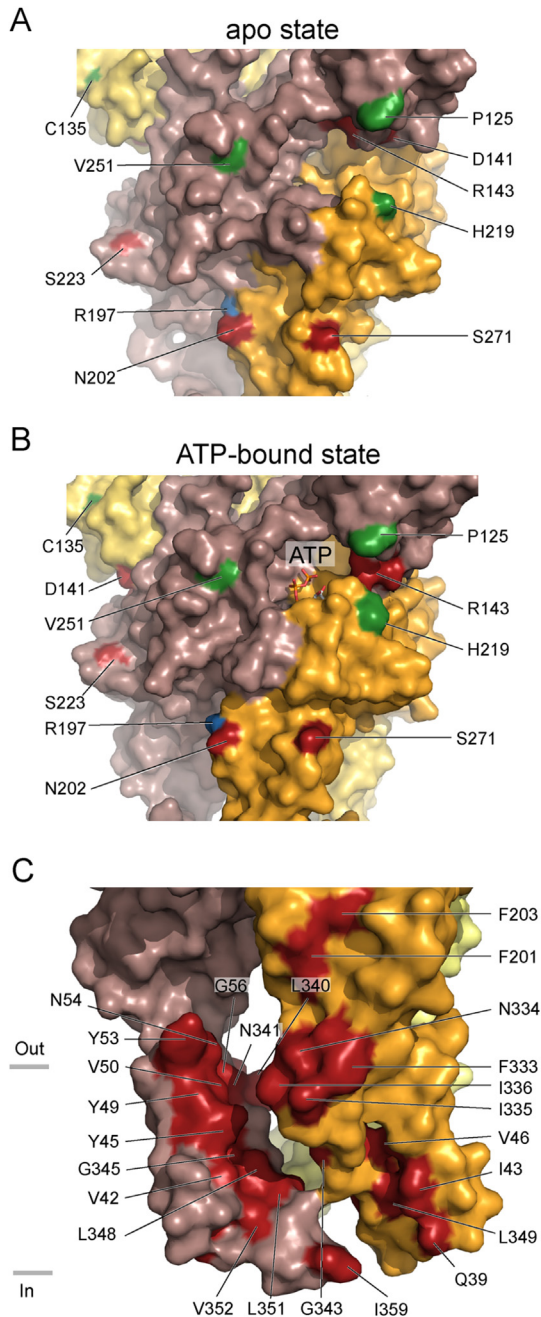
affinity involved in the inhibitory control of P2X receptors (Coddou et al., 2011b). Site-directed mutagenesis has been used to solve the role of specific amino acid residues involved in these modulatory effects. It has been observed that the residues H120 and H213 participate primarily to the allosteric metal binding site of rP2X2 receptor responsible for the potentiating effect of zinc (Clyne et al., 2002; Lorca et al., 2005; Nagaya et al., 2005; Tittle et al., 2007). In contrast to the potentiating effect observed on the rat P2X2, zinc inhibits the human P2X2 receptor activity with an IC<sub>50</sub> in the micromolar range (Tittle and Hume, 2008), highlighting striking opposite effects between rodents and humans. Analysis of the sequences indicated that only one of the two histidines essential for zinc potentiation in rP2X2 is present at the homologous position to rat H120 in hP2X2 (H132), with the position homologous to rat H213 instead having an arginine (R225). As zinc is likely coordinated directly by the two histidines in rodents, its ability to make such an interaction is abolished in humans because of the presence of the positively charged arginine residue. Notably, mutating R225 to cysteine was sufficient to confer zinc potentiation onto the human orthologue (Tittle and Hume, 2008), suggesting that zinc potentiation uses the same mechanism as that in the wild-type rP2X2 receptor. In the crystal structures, residues homologous to H120 (P125 in zfp2X4) and H213 (H219 in zfp2X4) belong to two adjacent subunits and are located, respectively, in the head and dorsal fin domains (Fig. 3A and B), positions which correspond to the two 'lips' of the ATP-binding jaw. In agreement with previous works (Jiang et al., 2012a) and with the recent crystal structure solved in the presence of ATP (Hattori and Gouaux, 2012), ATP binding promotes tightening of the two lips of the binding jaw and zinc stabilizes the conformation of closed jaws through coordination of the two histidine residues (Fig. 3B).

In addition, other histidines (H192 and H245) from rP2X2 (corresponding to R197 and V251 in zfp2X4, respectively) were also identified as potentially participating to the allosteric potentiating effect of zinc, although to a lesser extent than H120 and H213 (Lorca et al., 2005). However, mapping of the equivalent residues in the crystal structure shows that these residues are not close to each other and are far away from the established potentiating site involving H120 and H213 (Fig. 3A and B). The mechanisms of potentiation involving H192 and H245 thus remain to be clarified.

The location of the zinc inhibition sites also remains unclear. In rP2X2, neither the extracellular histidines nor glutamates or aspartates are required for low affinity zinc inhibition (Clyne et al., 2002; Friday and Hume, 2008). By contrast, mutating H204 and H209 to alanine in the hP2X2 receptor (corresponding to R197 and N202 in zfp2X4, respectively) has been shown to significantly reduce zinc inhibition, and mutating to cysteine restored wild-type inhibition (Tittle and Hume, 2008). However, the fact that the double alanine mutant was no more deficient than either single mutant, along with the fact that MTS reagents carrying opposite charges were nearly equipotent in affecting zinc effects in cysteine mutants, suggest that H204 and H209 are not directly involved in zinc inhibition. These data reveal the complexity of zinc modulation, since an equivalent position (rP2X2 H192; hP2X2 H204) is involved in either potentiation or inhibition depending on the species.

Additional residues from other P2X subtypes have been identified to be critically involved in zinc allosteric effects. For instance, in rP2X4 receptor, the residue C132 is involved in the potentiating allosteric effect of zinc, whereas D138 and H140 have been determined to participate in the inhibitory allosteric effect of zinc (Coddou et al., 2007, 2003). When mapped on the zfp2X4 crystal structure, D138 and H140 are close to each other and one may infer that zinc binding to these residues may restrict head domain mobility, and thus may block the ATP-induced response in an





**Fig. 3.** Mapping of residues implicated in allosteric modulation of P2X activity. Residues contributing to allosteric regulation induced by zinc application on a closed state (A) and an ATP-bound state of  $\Delta zfpP2X4$  (B). Residues implicated in the production of an inhibitory effect or a potentiation are coloured in red and green, respectively, and positions implicated in both effects are shown in blue. Amino acid residues mapped on the structure of  $\Delta zfpP2X4$  are indicated in brackets immediately after the equivalent residues of the indicated P2X species that are listed in the text: rP2X2 H120 (P125); rP2X4 C132 (C135); rP2X4 D138 (D141); rP2X4 H140 (R143); rP2X2 H192, hP2X2 H204 (R197); hP2X2 H209 (N202); rP2X2 H213, hP2X2 R225 (H219); rP2X7 H219 (S223); rP2X2 H245 (V251) and rP2X7 H267 (S271). (C) Location of residues associated with IVM potentiation (in red) in the ATP-bound state of  $\Delta zfpP2X4$ . Amino acid residues mapped on the structure of  $\Delta zfpP2X4$  are indicated in brackets immediately after the equivalent residues of the indicated P2X4 species that are listed in the text: rP2X4 Q36 (Q39); rP2X4 I39 (V42); rP2X4 L40 (I43); rP2X4 Y42, hP2X4 Y42 (Y45); rP2X4 V43, hP2X4 V43 (V46); rP2X4 W46 (Y49); rP2X4 V47 (V50); rP2X4 W50 (Y53); hP2X4 E51 (N54); rP2X4 G53 (G56); rP2X4 F198 (F201); rP2X4 F200 (F203); rP2X4 W330 (F333); rP2X4 D331 (N334); rP2X4 I332 (I335); rP2X4 I333 (I336); rP2X4 I337 (L340); rP2X4 N338 (N341); rP2X4 G340 (G343); rP2X4 G342 (G345); rP2X4 L345 (L348); rP2X4 L346 (L349); rP2X4 V348 (L351); rP2X4 A349 (V352) and rP2X4 I356 (I359). Note that

allosteric manner. Supporting this hypothesis, a recent NMR study of the structure of the rat P2X4 receptor head domain demonstrated that D138 and H140 are directly involved in the binding of zinc and copper, another modulator of the P2X4 receptor (Igawa et al., 2015). However, clear structural interpretation of the zinc potentiation involving the C132 residue is not easy, because this residue (Igawa et al., 2015), and its equivalent in zfpP2X4 (Hattori and Gouaux, 2012; Kawate et al., 2009), is already engaged in a naturally occurring disulphide bond. Additional investigations should be carried out in order to understand the specific role of C132 in zinc potentiation in rP2X4 receptor. In rP2X7 receptor, the inhibitory effect of zinc was abolished in H219A and H267A mutants, suggesting a major role of these two amino acids (Acuna-Castillo et al., 2007). As these residues are not conserved among P2X subtypes, these data suggest that P2X7 has undergone a separate adaptation to trace metal coordination (Huidobro-Toro et al., 2008).

#### 4.2. Allosteric regulation within the transmembrane domains: the case of ivermectin

Ivermectin (IVM) is a semi-synthetic macrocyclic lactone used as an antiparasitic in humans and animals (Silberberg et al., 2007). It is a positive allosteric modulator of P2X4 receptors from several different species (Agboh et al., 2004; Khakh et al., 1999b; Priel and Silberberg, 2004) as well as the human P2X7 receptor, but not murine P2X7 receptor (Norenberg et al., 2012). It acts only when applied from the extracellular side of the cell membrane but not from the inside, suggesting that it does not bind to intracellular domains (Norenberg et al., 2012; Priel and Silberberg, 2004). Consistent with its lipophilic character, the effects of IVM are slow to develop, suggesting that it might need first to partition into the membrane before reaching its binding sites, which are likely close to the extracellular face of the membrane. A detailed kinetic analysis suggested that IVM binds to separate sites in hP2X4 receptors. Binding to the higher affinity site increases current amplitude by reducing channel desensitization, whilst binding to the lower affinity site slows the deactivation of the current mainly by stabilizing the open channel state (Priel and Silberberg, 2004). Chimeras made between IVM-sensitive P2X4 receptor and IVM-insensitive P2X2 receptors first confirmed the critical role of the transmembrane domain. It has been initially found that chimeras of the ectodomain of P2X4 with helices and intracellular domains of rP2X2 are not sensitive to IVM (Jelinkova et al., 2006). In contrast, chimeras of the two transmembrane segments of rP2X4 receptor into rP2X2 receptor are sufficient to confer IVM sensitivity (Silberberg et al., 2007), indicating a major role of both transmembrane segments of P2X4 receptor in the positive allosteric effect of the modulator. Consistent with a transmembrane location, site-directed mutagenesis analysis of residues located in the putative TM domains (Jelinkova et al., 2008; Popova et al., 2013; Silberberg et al., 2007) indicated that V28, Q36, I39, L40, Y42, V43, V47, W50 (TM1) and N338, G340, G342, L345, L346, V348, A349, I356 (TM2) in rP2X4 are involved in IVM sensitivity of the channel (Jelinkova et al., 2008; Silberberg et al., 2007). A recent study confirmed the importance of some of these residues (L40, V43, V47 and W50) in the effect of IVM on deactivation in rP2X4, and further identified new ones (W46, G53, F330, D331, I332 and I337) (Zemkova et al., 2015). The mutation to tryptophan of the homologous residues of Y42 and V43 in human P2X4 receptor also reduced the allosteric effect of IVM (Samways et al., 2012). Although these results do not firmly

residue rP2X4 V28 is located out of the truncated crystal structure and therefore cannot be mapped. Each subunit is coloured differently.

demonstrate that these residues contribute directly to IVM binding sites, they support the conclusion that these residues are critically involved in the functional effect of IVM. Interestingly, mapping of the corresponding residues in the zfp2X4 crystal structure bound to ATP reveals that most of them are located on the putative transmembrane helices at the subunit interfaces, with their side chains facing one another (Fig. 3C). This suggests that IVM may indeed bind to these interfaces.

In support of this hypothesis, mutations of the amino acid W46 to other aromatic residues (tyrosine or phenylalanine) in heterologously expressed rP2X4 receptor did not modify IVM-induced potentiation of ATP-dependent currents, whereas substitution with non-aromatic residues (leucine, valine, alanine, asparagine, lysine or aspartate) switched the potentiation to inhibition (Popova et al., 2013). Therefore, it has been suggested that the presence of an aromatic group at position 46 is a critical determinant of IVM-induced allosteric effects, most probably by means of a cation- $\pi$  interaction (Popova et al., 2013). It was previously suggested that the interaction between W46 and M336 participates in a binding pocket for IVM (Asatryan et al., 2010). However, whilst both individual W46A and M336A mutations as well as the double mutation significantly reduced the inhibitory effect of ethanol, only the mutation W46A (or the combined mutation) modified the positive allosteric effect of IVM (Popova et al., 2013). Finally, the analysis of a new molecular model based on the ATP-bound structure of zfp2X4 suggested a prominent role of the amino acid Y42 of rP2X4 receptor, confirmed by the mutation Y42F which also switched the potentiating allosteric effect of IVM to inhibition (Popova et al., 2013). This is probably due to the lack of a hydrogen bond between IVM and the phenolic OH group of Y42 in the mutation. Interestingly, in another study, the mutation Y42A of rP2X4 receptor mimicked the allosteric action of IVM by decreasing the deactivation rate of the current (Zemkova et al., 2015). It has also been shown that removal of the fixed negative charge of the acidic amino acid E51 of hP2X4 receptor, by the mutation E51Q, markedly reduces the allosteric effect of IVM on current intensity and relative permeability to  $\text{Ca}^{2+}$  (Samways et al., 2012). As this amino acid is located at the lateral entrance to the transmembrane pore, it has been hypothesized that IVM may change the topology of these lateral portals (Samways et al., 2012).

In agreement with this hypothesis, the allosteric effect of IVM on current deactivation was significantly attenuated by the mutation of F198 and F200, two residues located in the fenestrations (Fig. 3C), whereas the effect was increased by the mutation F330A/S, another amino acid of the region (Gao et al., 2015).

Finally, a recent work investigated the role of IVM on P2X pore dilation (Zemkova et al., 2015). In this study, the influence of IVM on pore dilation was studied in both wild-type rP2X4 receptor and transmembrane domain receptor mutants. IVM-induced receptor dilation in the presence of ATP was reduced or lost in L40A, V43A, W46A, V47A, W50A, G53A, F330A, D331A, I332A, I333A and I337A mutants (Zemkova et al., 2015). The results established that the IVM-induced rescue of rP2X4 receptor from desensitization temporally coincides with pore dilation.

Altogether, these data highlight the notion that there are multiple sites of interaction for IVM in P2X4 receptors (Popova et al., 2013). IVM binding sites are believed to be close to the lateral fenestrations found at the membrane interface (Gao et al., 2015; Jiang et al., 2013). It is thus tempting to speculate that IVM acts by insertion between adjacent transmembrane helices, which are located just below these fenestrations, perhaps by slightly moving apart adjacent helices (Fig. 3C). This mechanism would be consistent with the slight increase of single-channel conductance observed for the lower affinity site and with the slow onset and washout of the effects of IVM (Priel and Silberberg, 2004). Crystal

structures of IVM bound to the receptor in the presence of ATP would definitively resolve this issue.

#### 4.3. Allosteric regulation within the intracellular C-terminus domain: the regulatory effects of phospholipids

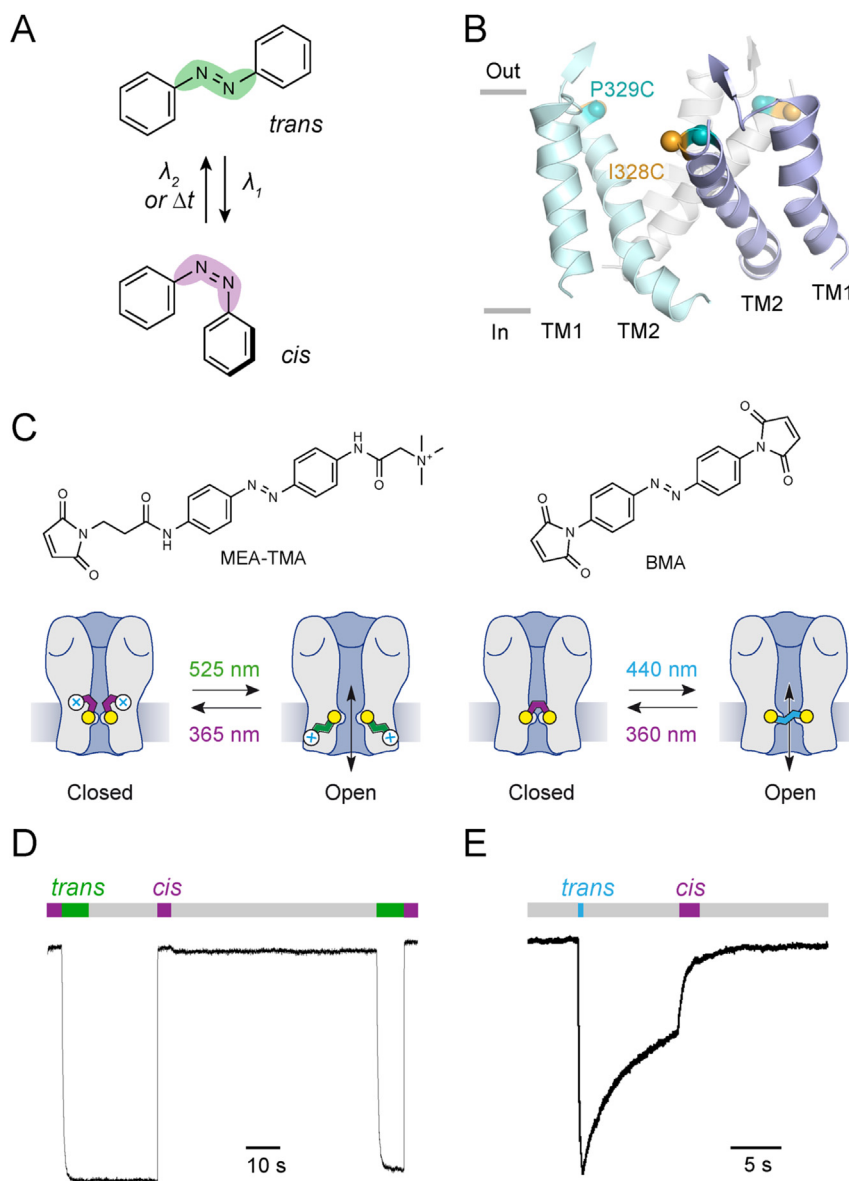
The particular allosteric regulation exerted by phospholipids at the carboxy terminal is considered as a post-translational mechanism of regulation of P2X receptor activity. The effects of phosphoinositides (PIPn) on P2X receptors have been reviewed recently (Bernier et al., 2013). PIPn increase P2X currents, most likely through a conformational change of the receptor, leading to an alteration of the gating (Bernier et al., 2013).

There are several important features in the regulatory effect exerted by PIPn on P2X receptors which have been mostly revealed by functional assays using the patch-clamp technique and/or by the binding of GST-fusion proteins containing the C-terminal domains of P2X receptors to PIPn coated on nitrocellulose membranes (PIP strip assays) (Bernier et al., 2013). From these studies, it has been shown that PIPn exert a direct effect on P2X1, P2X2 and P2X4 receptors as well as on the heteromeric P2X1/5 and P2X2/3 receptors (Bernier et al., 2008a, 2008b, 2012; Fujiwara and Kubo, 2006). These studies further identified a dual polybasic cluster motif in the C-terminal region of certain P2X subtypes, in which positively charged residues such as K359 and K364 in rP2X1 (Bernier et al., 2008b), K365 and K369 in rP2X2 (Fujiwara and Kubo, 2006) and K362, K363, K370 and K371 in P2X4 (Bernier et al., 2008a, 2012) play a major role in the binding of PIPn. These adjacent polybasic amino acid clusters, with a net positive charge, are assumed to electrostatically interact with PIPn, which are anionic phospholipids (Bernier et al., 2012). These clusters are located close to the plasma membrane (3 residues from TM2 in the cytosolic region of the C terminal) in close vicinity to the negative phosphate head groups of PIPn in the membrane (Bernier et al., 2012).

The two polybasic clusters are not completely conserved among P2X receptors; in particular, this microdomain is lacking in P2X3, P2X5 and P2X7 subunits (Bernier et al., 2012). However, it has been shown that P2X3 and P2X7 are functionally modulated by PIPn, suggesting that either an indirect regulation mechanism must take place for these receptors (Bernier et al., 2012; Zhao et al., 2007) or these receptors interact with PIPn through other yet unidentified residues. Furthermore, mammalian P2X5 receptors are not sensitive to PIPn regulation (Ase et al., 2010), but negative-to-positive mutations (through mutating negatively-charged residues into positively-charged ones) in the twin clusters were sufficient to confer PIPn-binding and positive regulatory effect on the engineered receptor (Bernier et al., 2012). The identification of the three-dimensional location of these residues is hindered by the lack of intracellular domain structure. Determination of the structure of a full-length receptor, including the intracellular domain, will, therefore, certainly advance in atomic detail the precise molecular mechanism underlying PIPn allosteric regulation.

## 5. Optogating of P2X receptors

It is clear that a combination of the previously discussed studies, using a range of techniques and methods, has been able to advance our understanding of the molecular structure of P2X receptors and its relationship to their function. And yet, despite the numerous important discoveries made over the years, there are many aspects of P2X structure and function which remain unclear, in particular, the precise mechanism of the gating process and the implications of gating at a molecular level. Progress in these areas is hindered by a lack of pharmacological tools which can be implemented for more in-depth study.



**Fig. 4.** Optical control of P2X function. (A) Isomerisation of the azobenzene from *trans* to *cis* occurs upon illumination of a particular wavelength  $\lambda_1$  (usually near UV light) and illumination at a different wavelength  $\lambda_2$  (in the visible light) or alternatively thermal relaxation ( $\Delta t$ ), leads to the isomerisation back to the thermodynamically favoured *trans* isomer. (B) Lateral view of the transmembrane region of a homology model of rP2X2 based on the  $\Delta zP2X4$  structure bound to ATP (4DW1) showing the location of I328C and P329C mutations that confer light sensitivity to the receptor following labelling with the appropriate photoswitchable probes. (C) Chemical structures of azobenzene compounds used to regulate P2X function by light. The scheme illustrates the light-induced gating mechanism of each photoswitchable probe. (D) Whole-cell currents evoked by light (*trans* induced by 525 nm and *cis* induced by 365 nm) in HEK cells expressing the I328C mutant treated by MEA-TMA (taken from Lemoine et al. (2013)). (E) Whole-cell currents evoked by light (*trans* induced by 440 nm and *cis* induced by 360 nm) in HEK cells expressing the P329C mutant treated by BMA (taken from Browne et al. (2014)).

However, new approaches based on optogenetic pharmacology principles may offer innovative, alternative ways of probing the structure and function of these ion channels. Optogenetic pharmacology, which involves the use of a synthetic photoisomerisable ligand covalently attached to a genetically modified ion channel, has given rise to a number of strategies which allow light-controlled gating of LGICs, such as nAChRs (Tochitsky et al., 2012), GABA<sub>A</sub> receptor (Lin et al., 2014) and iGluR6 (Volgraf et al., 2006). Azobenzene-containing reagents are typically employed, as the azobenzene group is well characterised in its photoisomerisation behaviour – isomerisation from *trans* to *cis* occurs upon irradiation of a particular wavelength,  $\lambda_1$ , and irradiation at a different wavelength,  $\lambda_2$ , or alternatively thermal relaxation, leads to isomerisation back to the thermodynamically favoured *trans* isomer

(Fig. 4A). The azobenzene group also offers a number of advantages for biological applications (Mourot et al., 2013): *cis* – *trans* isomerisation takes place on the picosecond scale, causes a significant change in geometry of the molecule, and the absorption spectra of the two isomers are sufficiently different to ensure that only one isomer is mostly (but rarely completely) obtained upon irradiation at the required wavelength. Using light as a stimulus for ion channel activation is particularly attractive, as it can be applied with high spatial and temporal precision, and is considered as an orthogonal stimulus unable to trigger other biological processes. Recently, several strategies have also been developed to allow light-controlled gating of P2X receptors.

The first of these is the optogating method, developed by Lemoine et al. in 2013. This strategy uses P2X2 receptors carrying a



genetically encoded cysteine residue in the TM domain, to which is covalently tethered the photoswitchable reagent. In this case, the photoswitch employed is MEA-TMA, comprising a maleimide moiety for tethering to the cysteine, the central azobenzene segment and a positively charged trimethyl ammonium extremity, vital for efficient labelling (Fig. 4C). Irradiation in the visible or near UV region of light, causes the tethered MEA-TMA to undergo isomerisation to the *trans* or *cis* isomer respectively, which in turn induces an opening or closing of the channel (Fig. 4D) (Lemoine et al., 2013). The exact mechanism of optogating remains unknown, but it can be reasonably assumed that the movement involved in this isomerisation process causes a change in the interactions between TM helices, resulting in the gating of the channel (Fig. 4C).

Depending on the exact position at which the tethering cysteine is introduced, the channel may be opened by the molecule in the *cis* or *trans* isomer, giving rise to “*cis*-opener” and “*trans*-opener” positions. The position found to offer the most robust and stable light-induced currents is I328C, a *trans*-opener (Fig. 4D).

An important characteristic of this system is the bistability of the bound MEA-TMA, which ensures a stable photoactivation, sustained in the absence of irradiation. As shown in Fig. 4D, optogated currents induced by isomerisation to the *trans* isomer following irradiation at 525 nm remain stable even when irradiation is ceased and the system left in the dark over the course of 20 s. Only upon irradiation at 365 nm and isomerisation back to the *cis* isomer is a closing of the channel observed. This is particularly pertinent property when considering the possible extension of optogating to *in vivo* systems, as it means that only a transient irradiation would be required, therefore limiting any photodamage to the surrounding tissue.

A second method for achieving light-controlled P2X gating was developed by Browne et al. This strategy, whilst similar to optogating in the choice of an azobenzene compound, BMA, as the photoswitch, differs in that the photoswitch contains two maleimide moieties at the extremities and is thus able to cross-link to two anchoring points of the receptor complex, on different subunits (Fig. 4C). A mechanism of light-induced activation different to that of optogating is therefore at play, whereby light controlled toggling between *cis* and *trans* BMA isomers acts to bring the subunits closer or further apart, thus closing or opening the channel (Fig. 4E) (Browne et al., 2014). Of note, is that this method has been developed using the tethering position P329C, just one residue down from I328C, the position for which the most robust light-gated currents were observed with the optogating strategy (Fig. 4B). This area, located toward the upper end of the TM2 helix, may therefore represent a point in the channel region particularly effective for manipulating the molecular motions involved in channel opening.

As the photoswitches employed here are intrinsically devoid of pharmacological activity, there is the possibility to extend these strategies not only to other P2X variants, but also to other LGICs, offering a flexibility over previously developed optogenetic pharmacology based methods, where photoswitches must feature a pharmacologically active ligand (agonist, antagonist or allosteric modulator) specific for each receptor in question. This has been demonstrated with the BMA system, shown to control gating in P2X3, P2X2/3 heteromers as well as the structurally related ASIC1a channel. Both strategies have also been successfully implemented in P2X2 channels carrying the K69A mutation (Browne et al., 2014; Lemoine et al., 2013), which are known to be rendered unresponsive to their endogenous ligand ATP (Jiang et al., 2000). These systems therefore present the opportunity to study P2X receptors with an exquisite spatiotemporal control, where the only channel activity observed originates from the application of light.

It would be desirable to extend these strategies further, in particular, to the P2X4 and P2X7 subtypes, which have been found to play an important role in a number of disorders such as neuropathic pain (Beggs et al., 2012) and chronic inflammation (Di Virgilio and Vuerich, 2015). An eventual goal which can be envisaged for this work would be the development of systems suitable for use *in vivo*, allowing elucidation of the precise pathological contribution of P2X activation in models of such diseases.

## 6. Conclusion

In this review, we provided a summary of functional and structural studies on P2X receptors. Although the determination of X-ray structures provided an unprecedented insight into the understanding of the molecular operation of P2X receptors, there are still many questions which are left unanswered. In particular, what is the structure of the intracellular domain and how does it contribute to the function of the receptor? How do the various allosteric modulators regulate the response to ATP? What are the mechanisms of allosteric transition? And how may the pore dynamically change its permeability to cations? Elucidation of the answers to these intriguing questions will better define the functional role of P2X receptors in normal and pathological states.

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