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Structural Insights into the Function of P2X4: An ATP-Gated Cation Channel of Neuroendocrine Cells

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Abstract

The P2X4 receptor (P2X4R) is a member of a family of ATP-gated cation channels that are composed of three subunits. Each subunit has two transmembrane (TM) domains linked by a large extracellular loop and intracellularly located N- and C-termini. The receptors are expressed in excitable and non-excitable cells and have been implicated in the modulation of membrane excitability, calcium signaling, neurotransmitter and hormone release, and pain physiology. P2X4Rs activate rapidly and desensitize within the seconds of agonist application, both with the rates dependent on ATP concentrations, and deactivate rapidly and independently of ATP concentration. Disruption of conserved cysteine ectodomain residues affects ATP binding and gating. Several ectodomain residues of P2X4R were identified as critical for ATP binding, including K67, K313, and R295. Ectodomain residues also account for the allosteric regulation of P2X4R; H140 is responsible for copper binding and H286 regulates receptor functions with protons. Ivermectin sensitized receptors, amplified the current amplitude, and slowed receptor deactivation by binding in the TM region. Scanning mutagenesis of TMs revealed the helical topology of both domains, and suggested that receptor function is critically dependent on the conserved Y42 residue. In this brief article, we summarize this study and re-interpret it using a model based on crystallization of the zebrafish P2X4.1 receptor.

Keywords

P2X receptors; ATP; Gating; Orthosteric and allosteric regulation; Ivermectin; Trace metals; Scanning mutagenesis

Introduction

P2X receptors (P2XRs) are ATP-gated cation channels with only two TMs, the N- and C-termini facing the cytoplasm, and a large extracellular loop. Seven mammalian receptor subunits, denoted P2X1 through P2X7, and several spliced forms of these subunits have been identified. These subunits assemble together as homo- or heterotrimers to make functional receptors (North 2002). P2XRs likely have three intersubunit binding sites and their full occupancy appears to be required to activate the receptors (Browne et al. 2010). In addition to their classical agonist binding sites (orthosteric), P2XRs have also regulatory (allosteric) sites (Evans 2009). The P2XR subtypes differ in Ca^{2+} permeability, rates of desensitization, resensitization, and deactivation. The subtypes have also shown different sensitivity to agonists, antagonists, and allosteric modulators (Khakh and North 2006).

The P2X4R was identified as a distinct member of the P2XR family of channels when it was shown that a single gene product was sufficient to give rise to ion channels with distinct biophysical and pharmacological properties (Bo et al. 1995; Soto et al. 1996). These receptors are widely expressed in the brain (Rubio and Soto 2001). We have reported recently, that P2X4R are also expressed in the anterior pituitary gland, specifically in lactotrophs and their activation led to stimulation of electrical activity, promotion of voltage-gated and voltage-insensitive Ca^{2+} influx and prolactin release (Zemkova et al. 2010). Recent studies have also shown that P2X4R are expressed in microglia and alveolar macrophages (Bowler et al. 2003) and that up-regulation of these receptors in activated microglia located in the dorsal horn of the spinal cord contributes to neuropathic pain (Tsuda et al. 2003; Ulmann et al. 2008). P2X4R are also expressed in human lung mast cells (Wareham et al. 2009) and PC12 cells (Sun et al. 2007). Immortalized C8-B4 cells derived from cerebellar microglia also express P2X4R and were used to show that antidepressants do not affect the gating of these receptors (Toulme et al. 2010). The P2X4R knockout mice have high blood pressure, likely reflecting the role of these channels on endothelial cells (Yamamoto et al. 2006), and show a reduced amplitude in long-term potentiation in the hippocampus (Sim et al. 2006).

P2X4 subunits also form functional heteromers with P2X1 (Nicke et al. 2005) and P2X6 subunits (Le et al. 1998; Ormond et al. 2006), and the P2X4R also interacts with P2X7R (Nicke 2008; Alqallaf et al. 2009; Boumechache et al. 2009). Homomeric channels bathed in physiological solutions activated rapidly, desensitized with a moderate rate, and displayed inwardly rectifying current–voltage relations that reversed at 0 mV (Khakh et al. 1999a; Fountain and North 2006). Pharmacological experiments revealed two distinct features of P2X4R: relative resistance to P2XR antagonists, such as suramin and PPADS (Buell et al. 1996), and sensitivity to ivermectin (IVM), which acts as an allosteric regulator of this receptor in either a homomeric or a heteromeric configuration but does not regulate other P2XRs (Khakh et al. 1999b). Activated P2X4R undergoes relatively rapid endocytosis and traffic to lysosomes (Bobanovic et al. 2002; Royle et al. 2002). The recently solved structure of zebrafish P2X4.1R at 3.5 Å resolution by Gouaux and colleagues confirmed the trimeric organization of the receptors, the formation of disulfide bonds among conserved ectodomain cysteines as well as the helical organization of TMs and suggested potential residues for coordinating ATP binding (Kawate et al. 2009).

Here, we briefly review work on mammalian P2X4Rs. Based on the crystal structure of zebrafish P2X4.1R at a 3.5 Å resolution, we have also generated a model of rat P2X4R and re-analyzed published data by our own group and other groups that have been working on the structural and functional characterization of this receptor.

Gating and Internalization of P2X4R

The majority of studies on recombinant P2X4R were completed using rat receptor constructs expressed in mammalian HEK293 and GT1 cells or *Xenopus* oocytes. The wild type receptor expresses well in these cells and exhibits comparable biophysical and pharmacological properties. The noticeable difference in the two expression systems is time-dependent enhanced permeability to organic cations and dyes, which was observed in the *Xenopus* system but not in mammalian cells (Khakh et al. 1999a).

To follow the expression and trafficking pattern of wild type and mutant receptors, GFP-tagged P2X4Rs were developed. GFP translated from the same bicistronic mRNA as P2X4R was homogeneously distributed within the HEK293 cells, whereas P2X4R-fused GFP was localized in the plasma membrane and in intracellular structures (Fig. 1a). The tagged receptor was fully functional; Fig. 1b shows typical current traces elicited by 100 μ M ATP in cells expressing P2X4R (left) and cells expressing the GFP-tagged P2X4R (right). There was no obvious difference in the rise time and peak amplitude of current responses between the two receptors. Furthermore, in all cases, decaying currents were observed with highly comparable time constants of desensitization. The patterns of Ca^{2+} signaling (Fig. 1c) and the ATP potency for the two receptors were highly comparable with EC_{50} values ranging between 1 and 5 μ M. Together, these experiments confirmed that visualization of P2X4R with GFP tagging at the C-terminal end has no obvious effects on receptor function (Yan et al. 2005).

The P2X4R current response consists of three phases: a rapid rising phase of inward current induced by application of ATP (activation phase), a slowly developing decay phase in the presence of an agonist (desensitization phase), and a relatively rapid decay of current after ATP is washed (deactivation phase). Because activation and deactivation of P2X4R occurs rapidly, we used an ultrafast solution-switching system with a resolution time of about 1 ms (Yan et al. 2006). Deactivation efficiency was independent of ATP concentration and did not correlate with the activation and desensitization kinetics of the receptor (Fig. 2a). In contrast, the activation efficiency increased with increase in ATP concentration and the activation time was inversely correlated with ATP concentration (Fig. 2b). Similar to the activation phase, there was an inverse relationship between desensitization time and ATP concentration (Fig. 2c, d, main panels). Consequently, there was a strong linear correlation between activation and desensitization time values (Fig. 2c, d, insets).

GFP-tagged receptors were also used to show that P2X4R, but not P2X2R, undergo rapid constitutive internalization and subsequent reinsertion into the plasma membrane in a dynamin-dependent manner. Internalization of P2X4/6 heterodimers was also observed, suggesting that one or two P2X4 subunits are sufficient to govern the trafficking properties of the receptor (Bobanovic et al. 2002). The C-terminal YXXGL motif serves as a non-canonical tyrosine-based sorting signal that is necessary for efficient endocytosis of this receptor (Royle et al. 2002). Endogenous P2X4Rs in cultured rat microglia, vascular endothelial cells and freshly prepared peritoneal macrophages are localized predominantly to lysosomes where the receptors can retain their function and subsequently travel out of lysosomes to the plasma membrane (Qureshi et al. 2007). Unstimulated macrophages express very low levels of functional P2X4R, but expression of the receptors at the plasma membrane was enhanced by activation of phagocytosis (Stokes and Surprenant 2009).

Role of Conserved Cysteines in Receptor Structure and Function

The extracellular loop of all mammalian P2X subunits contains 10 conserved cysteine residues that have been predicted to form bonds in the following order (P2X4R numbering): 116–165 (SS1), 126–149 (SS2), 132–159 (SS3), 217–227 (SS4), and 261–270 (SS5) (Clyne

et al. 2002; Ennion and Evans 2002; Rokic et al. 2010). Direct evidence for the existence of these disulfide bonds was provided by solving the crystal structure of zebrafish P2X4.1R (Kawate et al. 2009). In P2X1R, the individual bonds are not essential for receptor function (Ennion and Evans 2002). For P2X2R, however, the SS1–4 bonds are individually needed for proper operation of the receptor (Clyne et al. 2002). Disruption of the SS1, SS2, and SS4-P2X4R bonds by substituting both cysteines with threonine generated less sensitive receptors. Of these three bonds, mutation of the SS4 cysteines residue generated the most profound changes in receptor function (Rokic et al. 2010).

The mammalian model of P2X4R based on the crystal structure of zebrafish P2X4.1R (Kawate et al. 2009) provides some rationale for the specific roles of the SS1–5 disulfide bonds in P2X4R function (Fig. 3). It shows that SS1–3 are located within the head domain above the predicted ATP binding pocket, whereas SS4 is below the ATP binding pocket. The model also shows that the SS5 bond is located relatively far from the putative ATP binding site but close to the extracellular vestibule above the TM domains. Together, these findings indicate that the SS1, SS2, and SS4 bonds contribute substantially to the structure of the ligand binding pocket, while the SS5 bond located towards the TM domains could contribute to receptor gating. The SS4 bond is absent in the simple eukaryote *Ostreococcus*, and SS2, SS3, SS4, and SS5 bonds are absent in *Dictyostelium* (Jarvis and Khakh 2009; Surprenant and North 2009). These receptors are functional, but a high concentration of agonist is required for their activation, suggesting that formation of the SS bonds was an important step in the evolution of P2XR proteins.

Identification of Residues Contributing to ATP Binding Sites

A search for the ATP binding sites was predominantly conducted by Evans' group using P2X1R (Evans 2009) and by North's group using P2X2R (Browne et al. 2010). The investigation in our laboratory was focused on P2X4R. We initially started experiments on this topic using variable chimeric receptors. The P2X2/X3R chimeras containing the Val⁶⁰–Phe³⁰¹ sequence of P2X3R instead of the Ile⁶⁶–Tyr³¹⁰ sequence of P2X2R not only preserved ATP binding but developed two intrinsic functions of P2X3R: sensitivity to $\alpha\beta$ -methylene ATP and ecto-ATPase-dependent recovery from endogenous desensitization. Furthermore, the corresponding P2X2/X4R chimera displayed gain of function, as indicated by increased sensitivity to ligands and faster desensitization compared with both parental receptors (He et al. 2003a). Finally, the P2X2/X7R chimera showed sensitivity to ATP between the sensitivities observed in cells expressing the parental receptors (He et al. 2002). These experiments indicated that the Lys⁶⁷–Lys³¹³ ectodomain sequence not only contains an ATP binding domain, but also accounts in part for the receptor agonist specificity of these sites.

Looking for the candidate residues responsible for ATP binding sites in this domain, we initially relied on the secondary structure similarities between P2X4R and class II aminoacyl-tRNA synthases. This study revealed that receptor function was practically lost in the K190A, R278A, and D280A mutants (Yan et al. 2005). The subsequent study indicated that receptor function was also dramatically affected in the K313-P2X4R mutants (Yan et al. 2006). To examine whether loss of function reflects a decreased sensitivity of the mutants for ATP, we treated cells with IVM, an antiparasitic agent that enhances the responsiveness of this receptor (see below). In addition to the K190, R278, D280, and K313 mutants, we also studied K67, F185, and R295 mutants because the importance of these residues in ATP binding was suggested for P2X1R (Ennion et al. 2000) and P2X2R (Jiang et al. 2000). In the presence of IVM, all low or non-responsive mutants responded to ATP in a dose-dependent manner with EC₅₀ values for ATP of about 1, 2, 4, 20, 60, 125, 270, 420, 1000, and 2300 μ M for the D280A, R278A, F185A, K190A, R295K, K313R, R295A, K313A, K67A, and

K67R mutants, respectively. These results indicate that lysines 67 and 313 as well as arginine 295 could play a critical role in forming the proper three-dimensional structure of P2X4R for agonist binding.

A breakthrough in understanding the structure of the ATP binding site came when two studies found that the site is localized between two subunits (Wilkinson et al. 2006; Marquez-Klaka et al. 2007). Crystallization of P2X4R was solved in the absence of ATP. Thus, verification of the residues responsible for the coordination of ATP has yet to be completed. The homology model of rat P2X4R (Fig. 3) suggested that conserved residues K67 and K69 of one subunit and N293, R295, and K313 of the neighboring subunit could make a pocket for ATP, whereas F185 and K190 are in proximity of ATP binding sites. For a detailed description of putative ATP binding sites see (Browne et al. 2010).

Allosteric Modulation of P2X4R

In addition to their orthosteric binding sites, all P2XRs have allosteric sites. These sites are independent and physically separated from the ligand-binding domain of receptors. Among P2XRs, allosteric regulation of P2X4R is the best characterized. The unique characteristic of P2X4R is its differential modulation by trace metals such as zinc and copper. Zinc potentiates the receptor's activity and copper inhibits it (Acuna-Castillo et al. 2000). This receptor contains only three extracellular histidines in its ectodomain. Among them, only mutation of H140 abolished the inhibitory action of copper and modified the pattern of zinc action from a bell-shaped curve, typical of biphasic modulation, to a sigmoid curve accompanied with an increase in the amplitude of the response (Coddou et al. 2003). It is also possible that D138 is a second residue contributing to the copper inhibitory site, whereas C132 could account for the zinc positive allosteric site (Coddou et al. 2007).

Cadmium potentiated P2X2R and P2X4R (Coddou et al. 2005; Lorca et al. 2005) and inhibited P2X1R, P2X3R, and P2X7R (Virginio et al. 1997). In P2X2R and P2X4R, this metal seemed to bind allosteric sites identified for zinc (Coddou et al. 2005; Lorca et al. 2005). Other metals (lead, barium, palladium, silver, platinum and gallium), had no effect on the ATP-evoked currents of the P2X2R or the P2X4R, indicating that the modulator allosteric sites have strict structural requirements (Coddou et al. 2005; Lorca et al. 2005). Acidification caused a decrease in the potency of ATP for P2X4R (Stoop et al. 1997; He et al. 2003b), while an increase to pH 8 enhanced the current amplitude (Clarke et al. 2000). Mutation of H286 removed the extracellular pH sensitivity of this receptor subtype (Clarke et al. 2000; Yan et al. 2005). P2X4R gating was also affected by ethanol and two residues, D331 and M336, play an important role in the ethanol sensitivity of P2X4R (Xiong et al. 2000; Popova et al. 2010).

IVM, a semisynthetic derivative of the natural fermentation products of *Streptomyces avermitilis*, is a member of a class of lipophilic compounds known as avermectins. These compounds are widely used in human and veterinary medicine as an antiparasitic drug. IVM was also found to modulate several ligand-gated channels including mammalian homomeric and heteromeric P2X4R (Khakh et al. 1999b). When applied extracellularly, IVM increases the sensitivity of P2X4R to ATP, enhances the maximum current amplitudes, and greatly prolongs the deactivation kinetics. Initial experiments that focused on identification of IVM binding sites were performed on chimeric P2X2/4Rs (Jelinkova et al. 2006). These experiments suggested that the TMs and the nearby ectodomain region contribute to the effects of IVM on channel gating, a hypothesis confirmed recently using TM domain chimeras between P2X4R and P2X2R (Silberberg et al. 2007). In our study, the IVM-sensitive hits were: Q36, L40, V43, V47, W50, N338, G342, L346, A349, and I356

(Jelinkova et al. 2006; 2008). The potential position of IVM-sensitive residues in the TMs of the rat P2X4R homology model is shown in Fig. 4.

Helical Organization of TMs

Perturbation of receptor function caused by cysteine and tryptophan scanning mutagenesis of P2X4-TMs suggested that TMs adopt α -helical structures (Silberberg et al. 2005; Jelinkova et al. 2008). The same conclusion was reached by cysteine and alanine scanning mutagenesis of P2X2-TMs (Rassendren et al. 1997; Egan et al. 1998; Haines et al. 2001a; b; Jiang et al. 2001; Li et al. 2004). Crystallization of zebrafish P2X4.1R confirmed these predictions (Kawate et al. 2009). Consistent with the prediction that the TM2 region plays a dominant role in receptor function, channel assembly, gating, ion selectivity, and permeability for divalent ions (Egan et al. 1998; Migita et al. 2001; Egan and Khakh 2004; Li et al. 2004; Khakh and Egan 2005; Samways and Egan 2007), the homology model of rat P2X4R shows that TM2 domains form a triangle, which is the pore of the channel in a closed state, whereas three TM1 domains are located more peripherally (Fig. 4). Our scanning mutagenesis of P2X4R resulted in eight hits in the TM2-P2X4R domain, G340, S341, L343, A344, G347, T350, D354, and V357, which suggested their potential relevance in receptor functions (Jelinkova et al. 2008). Experiments with P2X2R indicated that T(S)341 and T(A)344, together with S(L)345 (P2X4R numbering), are located in the narrowest part of the permeation pathway (Rassendren et al. 1997; Egan et al. 1998; Li et al. 2008) and that the channel gate is between T(S)341 and T(A)344 (Cao et al. 2009; Kracun et al. 2010). None of the residues that appear to contribute to the formation of the P2X2R pore gate were conserved, clearly indicating that further structural information is required to understand the orientation of the TM2 residues in closed and open states of P2X4R.

Alanine and cysteine scanning of the P2X4R-TM1 domain indicated the G29, M31, Y42, G45, V49 residues are mutation-sensitive (Jelinkova et al. 2008; Jindrichova et al. 2009). Among these residues, it appeared that the conserved Y42 residue played the most important role in receptor function. Originally, it was suggested that this residue affected the permeability of P2X2R for cations, but in an indirect manner (Samways et al. 2008). The P2X4R-Y42 mutant indicated increased sensitivity of the receptor to ATP and decreased maximum amplitude of the current response (Silberberg et al. 2005; Jindrichova et al. 2009). In P2X1R, replacement of this residue with alanine resulted in a non-functional channel, further supporting the importance of this residue in the receptor's function. The sensitivity of the P2X3R-Y37A mutant to ATP also increased. We also observed a transition of $\alpha\beta$ -meATP from partial to full agonist for these two receptors with increased sensitivity. In contrast, conserved TM1 tyrosine mutant of P2X7R did not show a gain in sensitivity to ATP with our experimental conditions (Jindrichova et al. 2009). The homology P2X4R model suggests that Y42 is located at the level of the membrane where the TM2 helices cross each other and the TM pore is the narrowest (Fig. 5). The model shows the position of the W50 and W46 residues that have been shown to influence the function of the Y42 residue. Furthermore, the model suggests that M336 of the second subunit may interact with Y42 in the channel closed state; a possibility that should be tested experimentally.

Concluding Remarks

The site-directed mutagenesis of mammalian P2X4R species combined with data on crystal structure of the zebrafish P2X4.1 receptor and a model of mammalian P2X4R provided solid structural insights into the function of this particular receptor subtype. It is reasonable to suggest that P2X4R will serve as a receptor model for further studies on the structural–functional characterization of P2XRs and for the development of receptor-specific antagonists and allosteric regulators to be used in functional studies and therapeutics.

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Fig. 1.

Attaching GFP to C-terminus did not affect the expression and gating of P2X4R. **a** Confocal images of GFP fluorescence in transfected HEK293 cells. Distribution of GFP without P2X4 fusion (*left*) and P2X4-fused GFP (*right*). **b** Typical patterns of ATP-induced currents in HEK293 cells expressing P2X4R (*left*) and GFP-tagged P2X4R (*right*). The traces shown are from different cells. The numbers below the traces show mean values $\pm$ SEM for the time constants of receptor desensitization (τ_{des}) and peak amplitude (I). **c** The averaged ATP-induced Ca^{2+} responses in GT1 neurons expressing P2X4R (*left*) and P2X4R-GFP (*right*). The numbers above the traces indicate the time constants of calcium signal desensitization

Fig. 2.

Characterization of P2X4R gating. **a** The 10–90% deactivation time values are independent of ATP concentration. *Horizontal line* illustrates the mean value for deactivation time. **b** The inverse relationship between 10 and 90% activation time and ATP concentration. **c, d** main panels, The inverse relationship between the values for 10% (**c**) and 90% (**d**) desensitization time and ATP concentration. *Insets*: the correlation between 10 and 90% activation time and 10% (**c**) or 90% (**d**) desensitization time. R , coefficient of correlation

Fig. 3.

Structural model of rat P2X4R with bound ATP. The predicted ATP binding site is located between two neighboring subunits (Kawate et al. 2009). Residues predicted to be involved in ATP binding are shown as sticks. In addition, five disulfide bridges (SS1–SS5) are also shown as sticks. The three-dimensional model of rat P2X4R (sequence Arg33–Val355) was generated using the Modeller 9v7 package (Sali and Blundell 1993) and the crystal structure of zebra fish P2X4R solved at 3.5 Å resolution (PDB access code 3I5D) (Kawate et al. 2009). Missing side-chains were built using the DeepView/Swiss-PdbViewer v4.0.1 program (Guex and Peitsch 1997). The final model was validated by PROCHECK and bad contacts were corrected manually. The resulting model was energy-minimized using the DeepView/Swiss-PdbViewer with the GROMOS96 43B1 parameters set. The ATP docking started with manual docking of the ATP molecule into the predicted binding site of P2X4R (Kawate et al. 2009). Subsequently, AutoDock v4.2 (Morris et al. 2009) was used to predict the structure of the rat P2X4-ATP complex. This figure as well as Figs. 4 and 5 were generated using Pymol v0.99 (<http://www.pymol.org>)

Fig. 4. View of the rat P2X4R model parallel to the molecular threefold axis from the intracellular side of the membrane. Residues that have been shown to alter the IVM effects on rat P2X4R are shown in *yellow/light gray* (Jelinkova et al. 2008). Only IVM-sensitive residues of one subunit are shown as *sticks* (Color figure online)

Fig. 5.
Cartoon representation of the transmembrane domains of rat P2X4R viewed parallel to the membrane plane. Residues in the vicinity of the conserved Y42 residue of one subunit are shown as *sticks*