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Molecular Structure of Purinergic P2X Receptors and their Expression in the Hypothalamus and Pituitary

Short title: Structure and function of purinergic P2X receptors

Hana Zemková*, Ales Balík, Marie Jindrichová, Vojtech Vávra

Department of Cellular and Molecular Neuroendocrinology,

Institute of Physiology of the Academy of Sciences of the Czech Republic, v.v.i.

*To whom *correspondence and reprint requests should be addressed:* Institute of Physiology, ASCR v.v.i., Vídenská 1083, 142 20 Prague 4, Czech Republic.

Tel: 420 2 4106 2574, Fax: 420 2 4106 2488, E-mail: zemkova@biomed.cas.cz

Summary

Purinergic P2X receptors represent a novel structural type of ligand-gated ion channels activated by extracellular ATP. So far, seven P2X receptor subunits have been found in excitable as well as non-excitable tissues. Little is known about their structure, mechanism of channel opening, localization, and role in the central nervous system. The aim of this work is to summarize recent investigations and describe our contribution to elucidating the structure of the ATP binding site and transmembrane domains of the P2X receptor; we also discuss the expression and physiological roles played by the ATP and P2X receptors in the anterior pituitary and hypothalamus.

Key words: Purinergic P2X Receptors, ATP, Ivermectin, Hypothalamus, Anterior Pituitary

Introduction

ATP (adenosine-5′-triphosphate) is multifunctional intracellular molecule that serves as an energy source in all biological systems. Much less is known about its role as an extracellular messenger or neurotransmitter (Ralevic and Burnstock, 1998). Following the discovery of its release from sensory nerves in the rabbit ear, ATP became the first purine to be formally described as a neurotransmitter (Holton and Holton, 1953). Extracellular ATP acts on its plasma membrane receptors termed "purinergic receptors," which are activated not only by ATP, but also by uracil triphosphate (UTP) and products of their hydrolysis, adenosine diphosphate (ADP), uracil diphosphate (UDP) and adenosine. A comparison between the biological effects of ATP, adenosine, and their intermediaries showed that the degree of phosphorylation influenced both the intensity and type of response, implying the existence of various types of purinergic receptors. Formal recognition of purinergic receptors yielded nomenclature and categorization based upon the major natural agonist to the receptor (Burnstock, 1977): P1 receptors are activated primarily by adenosine, while P2 agonists are activated by ADP and ATP and in some cases pyrimidines, UTP or UDP. There are ionotropic and metabotropic ATP-sensitive purinergic receptors, a fact used to further classify the P2 receptors as P2X and P2Y, respectively (Khakh *et al.*, 2001). In vivo, ectonucleoside triphosphate diphosphohydrolases (NTPDases) control the concentration of endogenously released extracellular nucleotides (Grondal and Zimmermann, 1986).

The G-protein coupled P2Y receptors have been further subdivided into eight subclasses, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Abbracchio *et al.*, 2003), based upon the pharmacology and G-protein subunit coupling of the receptors.

The different subclasses of P2Y receptors are activated with varying potencies to ATP and ADP, however, some P2Y receptors, for instance $P2Y_4$ and $P2Y_6$ receptors, activate most potently to UTP, leading some to term these pyrimidinergic receptors (O'Connor *et al*., 1991). The role of ATP- and UTP-sensitive P2Y receptors has been studied extensively in erythrocytes, liver cells, and endothelial cells. P2Y receptors have been found to play a role in the relaxation of smooth muscle (Pinna *et al*., 2005), the repair of epithelial cell injury that involves Ca^{2+} signaling, cell communication, and migration. (Klepeis *et al*., 2004), and the increase of leukocyte adherence to endothelial cells (Dawicki *et al*., 1995).

The P2X receptors are ATP-gated cation channels, which allow the entry of Ca^{2+} in addition to monovalent cations such as Na^+ and K^+, and small organic cations (Valera *et al*., 1994). Their calcium permeability is relatively high, in the range from 2.7 % ($P2X_3$) to 12.4 % ($P2X_1$) of total ATP-induced current (Egan and Khakh, 2004). Cloned in the mid-1990s, seven distinct genes encode the P2X receptor subunits ($P2X_{1\text{-}7}$) (North, 1996), which form functional channels as homo- and heterotrimers (Nicke *et al*., 1998; Stoop *et al*., 1999). Subunit $P2X_6$ does not form functional homomeric channels as it is retained in the endoplasmic reticulum in the monomeric form (Barrera *et al*., 2005; Ormond *et al*., 2006), but its heteromeric assemblies are functional (Le *et al*., 1998; King *et al*., 2000). Using the baculovirus-Sf9 cell expression system, the $P2X_2$ receptor was expressed and purified, and its structure was observed using electron microscopy. These images showed that the $P2X_2$ receptor protein resembles an inverted three-sided pyramid 215 Å in height and 200 Å in side length, (Mio *et al*., 2005), providing visual evidence of the trimeric composition of the P2X receptor family.

Many cells express different types of P2X subunits simultaneously, indicating that native P2X receptors are usually heteromers. So far, seven combinations of heteromeric channels have been characterized in functional and biochemical studies: $P2X_1/P2X_2$ (Brown *et al.*, 2002), $P2X_1/P2X_4$ (Nicke *et al.*, 2005), $P2X_1/P2X_5$ (Torres *et al.*, 1998c; Le *et al.*, 1999; Surprenant *et al.*, 2000), $P2X_2/P2X_3$ (Lewis *et al.*, 1995; Radford *et al.*, 1997; Jiang *et al.*, 2003; Wilkinson *et al.*, 2006), $P2X_2/P2X_6$ (King *et al.*, 2000), $P2X_4/P2X_6$ (Le *et al.*, 1998) and $P2X_4/P2X_7$ (Guo *et al.*, 2007). All of these studies except one, however, do not answer the question of which ratio of assembly is favored by individual subunits; one study showed that heteromer $P2X_2/P2X_3$ is present in composition $P2X_2(P2X_3)_2$ (Jiang *et al.*, 2003). Whether or not a functional P2X channel can be formed from three different subunits is not yet known. Using co-immunoprecipitation techniques, formation of the following heteromers has been suggested: $P2X_1/P2X_3$, $P2X_1/P2X_6$, $P2X_2/P2X_5$, $P2X_3/P2X_5$, $P2X_4/P2X_5$ and $P2X_5/P2X_6$ (Torres *et al.*, 1999a); these heteromeric receptors have not yet been pharmacologically characterized. Functional studies with recombinant receptors have shown that heteromers exhibit different pharmacological properties compared to relevant homomeric receptors, indicating that heteromerization might be a common mechanism used by cells to fine tune receptor function and properties.

All P2X subunits share a similar structure consisting of two transmembrane domains (TM1 and TM2): a large extracellular loop, and intracellular N- and C- termini (Brake *et al.*, 1994; Valera *et al.*, 1994; Newbolt *et al.*, 1998; Torres *et al.*, 1998a). The subunits have between 379 and 595 amino acids and the pairwise homology of the seven

P2X receptor subunits that have been cloned in mammals is between 25 – 48 % (North, 2002).

The P2X receptors have been identified as the third class of ligand-gated ion channels (North, 1996), which is distinct from the first class, represented by the nicotinic acetylcholine receptor (nAChR), γ-aminobutyric acid receptor ($GABA_AR$), glycine receptor (GlyR) and 5-hydroxytryptamine receptor ($5\text{-}HT_3R$), and the second class, represented by glutamate receptors (GluR), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR). ATP-gated channels conduct more Ca^{2+} on average than nAChR or glutamate-gated AMPA channels and equal to or greater than the Ca^{2+} flux through NMDA channels (Burnashev *et al.*, 1995), which makes them physiologically important. The single channel conductance of P2X receptors is relatively low: the $P2X_4$ subunit conductance is in the range of 9-12 pS (Negulyaev and Markwardt, 2000; Silberberg *et al.*, 2005) and the $P2X_2$ subunit is approximately 30 pS (Ding and Sachs, 1999). For most channels, ion selectivity is constant during repetitive stimulation. One of the unique properties of P2X receptors is that their selectivity filter is highly dynamic, and the continuous presence of ATP causes the pore diameter to increase, allowing the channel to pass larger and larger molecules (Khakh *et al*., 1999a; Virginio *et al.*, 1999b). Consequently, permeability to larger organic cations, such as the N-methyl-D-glucamine ion ($NMDG^+$), increases and varies based upon the subunit composition and time of the sustained activation of the receptor (Virginio *et al*., 1999a). Cloning and expression studies eventually identified the receptor $P2X_7$ (Surprenant *et al*., 1996; Rassendren *et al*., 1997b; Virginio *et al*., 1999a) and two other receptors ($P2X_2$ and $P2X_4$) (Virginio *et al*., 1999b) as having this property.

In the case of the $P2X_7$ channel, the pore diameter is supposed to increase from about 8 Å up to 40 Å, and the permeability ratio of $NMDG^+$ and Na^+ (p_{NMDG}/p_{Na}) increases from 0.03 to 0.48 (Evans *et al*., 1996; Virginio *et al.*, 1999b; Eickhorst *et al*., 2002). Pore dilatation of the P2X receptor may be physiologically important, for example, in the body's response to skin sensitizers and allergens as well as for inflammation (Khakh and North, 2006).

The distinct P2X receptor subtypes are functionally differentiated by comparisons in calcium conductivity, sensitivity to agonists, antagonists, and allosteric modulators, and the rate of desensitization (North, 2002). For example, the $P2X_1$ receptor is highly sensitive to ATP and is also activated by αβ-methylene adenosine triphosphate and 2-methylthio-adenosine triphosphate (Ralevic and Burnstock, 1998). The $P2X_1$ receptor can be differentiated from the $P2X_3$ receptor by sensitivity to βγ-methylene adenosine triphosphate, where a 30-fold smaller concentration is required to activate $P2X_1$ than $P2X_3$ (Ralevic and Burnstock, 1998). Another effective agonist is 2',3'-O-(benzoyl-4-benzoyl)-adenosine triphosphate, which acts as a $P2X_7$ receptor-selective agonist (Bianchi *et al*., 1999; Roberts and Evans, 2004). Sensitivity to conventional P2X receptor antagonists, suramin, pyrodoxil phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (Nakazawa *et al.*, 1995), trivalent cations (Nakazawa *et al*., 1997), extracellular acidification to pH 6.3, Zn^{2+} (Wildman *et al*., 1999) and Cibacron blue (Khakh *et al*., 2001) and other drugs (Gever *et al.*, 2006) is also variable for different.P2X receptor subtypes. The most obvious difference among mammalian P2X receptors is their sensitivity to the positive modulatory effect of ivermectin (IVM), a member of a class of lipophilic compounds known as avermectins that are used as antiparasitic agents in

human and veterinary medicine (Burkhart, 2000). The $P2X_4$ receptor shows robust sensitivity to IVM, whereas other subtypes of P2X receptor family are IVM-insensitive (Khakh *et al*., 1999b).

P2X receptors are expressed across a wide range of organisms from amoeba to humans (Fountain *et al*., 2007). By mediating depolarization and Ca^{2+} influx, extracellular ATP has numerous functions. These involve functioning of nervous, cardiovascular, respiratory, immune, urogenital, neuromuscular, and gastrointestinal systems, and the production of cytokines; the P2X receptors are also important during development and aging (Mehta *et al*., 2001; Gourine *et al*., 2002; Labasi *et al*., 2002; Buckwalter *et al*., 2003; Gourine *et al*., 2003; Sueta *et al.*, 2003; Gourine *et al.*, 2004; Khakh and North, 2006). P2X receptors have also begun to provide novel therapeutic targets for a number of diseases such as muscular dystrophy (Ryten *et al*., 2004), irritable bowel syndrome (Galligan, 2004), cystic fibrosis (Zsembery *et a*l., 2004), chronic pain sensation (North, 2003), and cancer (White and Burnstock, 2006).

Ectodomain region and the ATP binding site.

The ectodomain of the P2X receptor probably contains three ATP binding sites (Bean *et al*., 1990), but their localization and conformational rearrangements during the opening and closing of channels are still not well understood. There are many ATP-binding proteins that bind ATP via a so-called Walker motif, but this canonical ATP-binding amino acid sequence is absent in P2X receptors. No crystal structure or homology model exists for these channels. In the absence of such data, the progress in characterization of

the ATP binding site has been slow, and molecular recombinant techniques and site directed mutagenesis must be used for identification of functional receptor domains.

The extracellular domain of all P2X receptors contains 10 conserved cysteines that form intramolecular disulfide bonds (Clyne *et al*., 2002; Ennion and Evans, 2002). Experimentally defined partners are the following pairs (for human receptor $P2X_1$): Cys^{117}-Cys^{165}, Cys^{126}-Cys^{149}, Cys^{132}-Cys^{159}, Cys^{217}-Cys^{227} a Cys^{261}-Cys^{270}. These disulfide bridges represent a unique characteristic of P2X receptors. Similar to other receptors, the extracellular domain of all P2X receptors is glycosylated. N-glycosylation is necessary for the assembly and surface expression of the receptors (Newbolt *et al*., 1998; Torres *et al*., 1998b; Rettinger *et al*., 2000). The number of conserved motifs for N-glycosylation varies from two to six for individual P2X receptors ($P2X_1$ – 4, $P2X_2$ – 3, $P2X_3$ – 4, $P2X_4$ – 6, $P2X_5$ – 2, $P2X_6$ - 3, $P2X_7$ -3 (North, 2002)). It has been shown that glycosylation of one conserved motif is sufficient for receptor functioning, while glycosylation of two arbitrary motifs is necessary for receptor expression in the plasma membrane (Newbolt *et al*., 1998; Rettinger *et al.*, 2000). Both disulfide bridges and glycosylation sites contribute to the secondary and tertiary structure of the P2X receptor ectodomain, but none of these sites has been shown to be critical for ATP binding.

Experiments with systematic substitution of conserved amino acids within the extracellular domain of $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_4$ receptors showed that several aromatic and charged amino acids (Lys^{67}, Lys^{69}, Phe^{185}, Lys^{190}, Phe^{230}, Asp^{280}, His^{286}, Asn^{293}, Arg^{295}, Arg^{308} and Lys^{313} ($P2X_4$ numbering) are important for ATP binding and/or channel gating (Ennion *et al*., 2000; Jiang *et al*., 2000; Nakazawa *et al*., 2004; Roberts and Evans, 2004; Yan *et al*., 2005; Roberts and Evans, 2006; Wilkinson *et al*.,

2006; Yan *et al*., 2006; Fischer *et al*., 2007). A model for the ATP binding domain based on targeted mutagenesis data of the $P2X_1$ receptor (Ennion *et al*., 2000; Jiang *et a*l., 2000; Roberts and Evans, 2004) and the crystal structure of rat synapsin II (Roberts *et al*., 2006) suggests that positively charged residues Lys^{68}, Arg^{292}, and Lys^{309} are in close proximity within the ectodomain structure and interact with the negatively charged phosphates in the tail of ATP, and that aromatic residues Phe^{185} and Phe^{291} could be associated with the binding of the adenine ring. Another model is a secondary structure prediction of the second half of the extracellular loop of the P2X receptor based on comparison with aminoacyl t-RNA synthetases (Freist *et al.*, 1998). Using this model as a template for the rational mutagenesis of $P2X_4$ receptor, the Asp^{280} residue has been suggested to coordinate ATP binding via the magnesium ion, the Phe^{230} residue coordinates the binding of the adenine ring of ATP, and the Lys^{190}, His^{286}, and Arg^{278} residues coordinate the actions of phosphate groups of ATP (Yan *et al.*, 2005). In this model, the Lys^{313}-Ile^{333} ectodomain sequence plays a role in transduction of signals to the channel gate (Yan *et al*., 2006). Both models are consistent with an intrasubunit localization of the ATP binding site. It is obvious that individual models are identical in some points but differ in many other points. This can be explained by the fact that they were created for different subunits of the P2X receptor, which differ in ATP sensitivity and pharmacological properties. Another possibility is that ATP binds between subunits and examination of one subunit is not sufficient to map the entire ATP binding region. Moreover, Yan's model was designed for only the second half of the $P2X_4$ receptor ectodomain (region Lys^{180}-Lys^{326}) and does not explain the role of the first half that is integrated into Evans' model. An intersubunit organization of ATP binding has been recently proposed, with

Lys^{69} and Lys^{308} ($P2X_2R$ numbering) playing a critical role in the formation of the binding pocket (Wilkinson *et al.*, 2006). In accordance with this hypothesis, histidines from different neighboring subunits have been found to contribute to the zinc binding site in the ectodomain of the $P2X_2$ receptor (Nagaya *et al*., 2005). In receptor $P2X_1$, the K67C and F294C mutants spontaneously form specific dimers and almost completely cross-link into trimers with the K67C/F294C double mutant (Marquez-Klaka *et al*., 2007), also supporting an intersubunit ATP binding.

Using ivermectin (IVM) as a pharmacological tool that increases maximum current amplitude and sensitivity to ATP by interacting with the transmembrane domains (Khakh *et al*., 1999b; Priel and Silberberg, 2004; Jelinkova *et al*., 2006; Silberberg *et al*., 2007), we studied the importance of aromatic and charged ectodomain residues for ATP binding properties in the $P2X_4$ receptor (Zemkova *et al.*, 2007). Several new pieces of knowledge were obtained in this study. Firstly, IVM partially or completely restored the responsiveness of all ectodomain mutants, including those that have been previously shown to be silent. This effect enabled us to compare individual ectodomain residues and to determine ATP EC_{50} values for low or non-responsive mutants. The EC_{50} values were about 1, 2, 4, 20, 60, 125, 270, 420, 1000 and 2300 μM ATP at D280A, R278A, F185A, K190A, R295K, K313R, R295A, K313A, K67A and K67R mutants, respectively. Differential potency of ATP for these mutants depended on their location within the extracellular domain. Three residues, Lys^{67}, Lys^{313} and Arg^{295}, which are on opposite ends of the extracellular loop, exhibited the most profound effects on ATP potency indicating that they play a critical role in forming the proper three-dimensional structure of the P2X4 receptor for agonist binding and/or channel gating. This could suggest the

intersubunit organization of the ATP binding domain, a hypothesis originally introduced by North's group (Wilkinson *et al.*, 2006). Alternatively, the loops from neighboring P2X subunits are in close proximity to the membrane, and such a topology is necessary for transduction of signals from the intrasubunit ATP binding site to the channel gate (Zemkova *et al.*, 2007). Further studies should clarify the role of these residues in agonist binding and/or channel gating.

Transmembrane domains and channel pore

The secondary structure of the transmembrane domains of P2X receptors has been examined using alanine, cysteine, or tryptophan scanning mutagenesis (Rassendren *et al*., 1997a; Egan *et al*., 1998; Haines *et al*., 2001a; Haines *et al*., 2001b; Jiang *et al*., 2001; Migita *et al*., 2001; Li *et al*., 2004; Khakh and Egan, 2005; Silberberg *et al*., 2005). These experiments showed that both TM1 and TM2 adopt an α-helical organization and that both helices move during gating (Li *et al*., 2004; Silberberg *et al.*, 2005). The P2X receptor trimer apparently forms a parallel six-helix bundle, in the center of which is an aqueous cavity (Duckwitz *et al*., 2006). However, the TM1 and TM2 helices may play distinct roles in the structure and function of channel pores. The contribution of TM1 to the pore formation is postulated to be less than that of TM2 (Samways *et al*., 2007). The TM2 region is dominant for channel function and is also critical for trimer assembly, as it serves as a hydrophobic anchor by which the receptor is fixed in the membrane (Torres *et al.*, 1999b). In particular, conserved TM2 residue Asp^{355} ($hP2X_5$ numbering) has been shown to be important for this function as it initiates oligomerization of subunits in the membrane (Duckwitz *et al.*, 2006).

Conductivity and permeability of ion channels depend not only on the size of the pore, but also on the selectivity filter and gating properties. The selectivity filter is a region that transiently binds ions during passage of the channel pore. Usually it is a narrow region in the channel pore lined with charged or polar amino acids that undergo electrostatic interactions with the selected ion (Hille, 1975). Once open, the P2X channel pore allows rapid permeation of small cations and Ca^{2+} but selects against anions. The mechanism of cation selectivity is not known. One factor that likely contributes is TM2 residues Thr^{339} and Ser^{340}. Their substitutions cause changes in ion permeability so that the order of ions is similar to the relative mobility of ions in water. Moreover, substitution with residues of different hydrophobicity or volume properties caused specific changes in permeability for calcium, indicating that residues Thr^{336}, Thr^{339} and Ser^{340} could contribute to formation of the selective filter in P2X receptors (Migita *et al.*, 2001; Egan and Khakh, 2004). These residues, however, are not conserved among all subunits, indicating that other P2X channels might have different residues in the selectivity filter. In addition, conserved TM1 residue Tyr^{43} ($P2X_2$ receptor) has been suggested to control Ca^{2+} permeability and to act as an inter-pore binding site for Ca^{2+} (Samways and Egan, 2007). Other factors that likely contribute to the high Ca^{2+} permeability of $P2X_1$ and $P2X_4$ channels are negatively charged ectodomain residues glutamate and aspartate, localized near the membrane at the end of TM1 and at the beginning of TM2 (Samways and Egan, 2007). However, negatively charged residues are also present at the same positions in $P2X_3$ and $P2X_7$ receptors, which exhibit relatively low Ca^{2+} permeability (Samways and Egan, 2007), arguing against this hypothesis.

$P2X_2$, $P2X_4$ and $P2X_7$ receptors display a time- and activation-dependent increase in large cation permeability (Virginio *et al.*, 1999b). The fact that permeability changes depend on receptor subtype evokes the question of what is the mechanism of pore dilatation and which residues are responsible for this function. Dilation could proceed by different mechanisms: i) dilatation of an existing pore, ii) subunit clustering followed by oligomerization of preexisting monomers generating a new pore and iii) activation of a new permeability pathway (Eickhorst *et al.,* 2002; Fisher *et al*., 2004; Jiang *et al.,* 2005; Egan *et al.*, 2006). So far, the third possibility is only theoretical and has no experimental proof. The second possibility seems to be unlikely because stoichiometric changes do not occur during channel activation. Results of alanine scanning mutagenesis performed in the $P2X_2$ receptor support the first possibility and indicate that three TM1 residues (Phe^{31}, Arg^{33} and Gln^{37}) and six TM2 residues (Ile^{328}, Ile^{332}, Ser^{340}, Gly^{342}, Trp^{350} and Leu^{352}) could be involved in pore dilation (Khakh and Egan, 2005). These data also indicate that pore dilation might be due to channel rearrangements that occur at the interface between TM1 and TM2 of neighboring subunits (Jiang *et al.*, 2003; Khakh and Egan, 2005).

The gating properties of an ion channel are defined as a conformational change between open-channel and closed-channel states evoked by specific stimuli (Colquhoun, 1998). Numerous mutations in transmembrane domains of P2X receptors affect channel gating by ATP. For example, an alanine mutation of conserved TM2 residue Gly^{342} exhibited a rightward shift in the EC_{50} in the $P2X_2$ receptor (Li *et al*., 2004). This residue has been suggested to play a special role in helix motion as a point of local flexibility - a hinge - between the lower and upper part of TM2 (Khakh and Egan, 2005). The region between residues Gly^{347} and Asp^{354} most probably contributes to formation of the channel

pore gate (Egan *et al*., 1998). Mutations in transmembrane domains also affect ATP sensitivity. For example, an alanine substitution of conserved TM1 residue Tyr^{43} ($P2X_2$ receptor) generated a constitutively active channel that exhibited enhanced ATP sensitivity (Haines *et al.*, 2001b; Li *et al*., 2004). The mechanism by which this TM1 mutant controls ligand binding is unknown.

Using IVM, we attempted to examine the orientation of the transmembrane helices of the $P2X_4$ receptor in the membrane bilayer (Jelinkova *et al*., 2008). IVM is a large lipophilic molecule that affects $P2X_4$ receptor function when applied extracellularly (Khakh *et al*., 1999b). The transfer of the $P2X_4$ ectodomain sequences to the backbone of the $P2X_2$ receptor did not transfer the sensitivity for IVM (Fig.1), indicating that IVM interacts with transmembrane domains (Jelinkova *et al*., 2006; Silberberg *et al*., 2007). The regions of helices involved in IVM binding have to be relatively large and lipid-oriented, which raised the possibility of experimentally determining which transmembrane residues are responsible for recognition of IVM. Using cysteine scanning mutagenesis, we found that the following $P2X_4$ receptor residues could participate in recognition of IVM molecule: Arg^{33}, Gln^{36}, Leu^{40}, Val^{43}, Val^{47} and Trp^{50} of TM1, and Asn^{338}, Gly^{342}, Leu^{346}, Ala^{349}, Cys^{353}, and Ile^{356} residues of TM2 (Jelinkova *et al*., 2008). These residues could be lipid-oriented, and the majority of them were substitution resistant in the absence of IVM (Fig. 2). Mutations of residues Gly^{29}, Met^{31}, Tyr^{42}, Gly^{45}, and Val^{49} of TM1 and Gly^{340}, Ser^{341}, Leu^{343}, Ala^{344}, Gly^{347}, Thr^{350}, Asp^{354} and Val^{357} of TM2 led to changes in current amplitude and ATP EC_{50}, indicating the relevance of these residues for other receptor functions such as channel gating. These residues are suggested to face the hydrophilic pore (Fig.1) or protein in the channel open state (Jelinkova *et al*.,

2008). It is clear that different residues are involved in gating the other P2X subtypes because their helices are only 39-55% identical with the $P2X_4$ subunit (Rassendren *et al.*, 1997; Egan *et al.,* 1998; Jiang *et al*., 2001; North, 2002). However, the predominantly non-polar residues identified as IVM-sensitive are also present in the IVM-sensitive *Schistosoma mansoni* P2X subunit (Agboh *et al.*, 2004). These results suggest that IVM provides an additional tool for studies on the organization of transmembrane domains around the pore of the P2X receptor.

Functional role of P2X receptors in the brain

Originally, P2X receptors were believed to be located mostly in the periphery; it is now known, however, that of all the tissues investigated, the mammalian brain has the highest levels of purines and the greatest variety of purinergic receptors (Buell *et al*., 1996; Collo *et al.*, 1996; Seguela *et al*., 1996). Both neurons and glial cells express P2X receptors (Fields and Stevens, 2000; Raivich, 2005; Inoue *et al*., 2007). Neurons release ATP together with other neurotransmitters such as GABA, glycine, and glutamate (Robertson *et al.*, 2001; Sokolova *et al*., 2001). Glia release ATP in response to mechanical and electrical stimulation (Newman, 2003). The most frequent receptor forms in the brain are $P2X_2$, $P2X_4$, and $P2X_6$ as well as heteromers composed of $P2X_2/X_6$, $P2X_4/X_6$, and perhaps $P2X_1/X_4$ receptors (Buell *et al*., 1996; Collo *et a*l., 1996).

In excitable cells, P2X receptor activation causes an increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) via two distinct mechanisms: by membrane depolarization resulting in voltage-dependent Ca^{2+} entry and by Ca^{2+} entry through the P2X receptor itself. Stimulation of receptors by extracellular ATP in the brain might thus have

numerous physiological consequences. ATP acts as a neurotransmitter and P2X receptor activation has been shown to increase neuronal excitability (Khakh and Henderson, 1998; Khakh *et al*., 2003), mediating fast synaptic transmission in both the peripheral (Evans *et al.*, 1992) and central nervous systems (Edwards *et al.*, 1992; Pankratov *et al*., 1998; Pankratov *et al*., 2003), and affecting long-term potentiation (Sim *et al*., 2006). Extracellular ATP might also act as a trophic factor on growing axons during development (Heine *et al*., 2006). ATP is the dominant messenger for neuron-glia communication (Guthrie *et al*., 1999; Newman, 2003; Fields and Burnstock, 2006).

P2X receptors in the hypothalamus

Purinergic P2X receptors are also expressed throughout the hypothalamus where ATP appears to be involved in the regulation of hormone secretion (Kapoor and Sladek, 2000) and control of specific autonomic functions including the central mechanism of body temperature regulation (Gourine *et al*., 2002). $P2X_2$, $P2X_4$ and $P2X_6$ receptor, but not $P2X_5$ receptor mRNAs have been identified in the supraoptic nucleus, ventromedial nucleus, paraventricular nucleus, arcuate nucleus, suprachiasmatic nucleus, and several other hypothalamic areas using in situ hybridization; the $P2X_1$, $P2X_3$ and $P2X_7$ receptors were not tested in this study (Collo *et al*., 1996). Another PCR analysis revealed that $P2X_2$, $P2X_3$, $P2X_4$, $P2X_6$, and $P2X_7$ receptor mRNAs are expressed in the rat supraoptic neurons; $P2X_1$ and $P2X_5$ mRNAs were not found (Shibuya *et al*., 1999). In the preoptic area of the rhesus monkey, the $P2X_2$, $P2X_4$ and $P2X_7$ receptor mRNAs, but not $P2X_1$, $P2X_3$ and $P2X_5$ receptor mRNAs, were identified; subunit $P2X_6$ was not examined (Terasawa *et al.*, 2005). Interestingly, immunohistochemistry shows local differences in

P2X receptor protein distribution in the rat hypothalamus. For example, the $P2X_2$ receptor immunoreactive neurons and nerve fibers are localized in the paraventricular nucleus, arcuate nucleus, retrochiasmatic area, periventricular nucleus, the ventral part of tuber cinereum area, supraoptic, circular, and ventral tuberomammillary nuclei, organum vasculosum, and median eminence (Xiang *et al*., 1998; Yao *et al*., 2003), but are absent in the ventromedial nucleus (Vulchanova *et al*., 1996; Xiang *et al*., 1998). We also found $P2X_2$ receptor immunoreactive neurons in the supraoptic but not in suprachiasmatic nuclei of the rat hypothalamus (Fig. 2).

Functional studies showed that ATP evokes an increase in $[Ca^{2+}]_i$ in cultured hypothalamic neurons (Chen *et al*. 1994), and local ATP application increases excitability of SON neurons in the hypothalamus (Day *et al*., 1993). ATP stimulation increases the release of vasopressin and oxytocin in isolated rat neurohypophysial nerve terminals, which is attenuated by the P2X receptor antagonist PPADS (Kapoor and Sladek, 2000). It is supposed that ATP, co-released with neuropeptides, could act as a paracrine-autocrine messenger, stimulating Ca^{2+} entry through $P2X_2$ receptors (Troadec *et al*., 1998). These lines of evidence suggest that ATP plays a crucial role in the regulation of SON neurons at both the soma and the terminals. However, the functional role of other receptor subtypes mediating the ATP effect in the SON remains unclear. ATP synchronizes spontaneous intracellular Ca^{2+} oscillations in primary cultures of luteinizing hormone-releasing hormone (LhRH) neurons, derived from the olfactory placode region of monkey embryos, through $P2X_2$ and $P2X_4$ receptor activation (Terasawa *et al*., 2005). GT1 neurons, a cell line of mouse hypothalamic LhRH neurons, release ATP under resting conditions, and ATP is hydrolyzed by ectonucleotidase-2,

suggesting that the extracellular ATP effect might be regulated in the hypothalamus (He *et al.*, 2005b).

The specific role of P2X receptors and the importance of extracellular ATP in the hypothalamus is still being elucidated. It is not yet known how ATP functionally interacts within the hypothalamus, what is the reason for local differences in distribution of P2X receptor subtypes, how they are regulated by a multisynaptic pathway connecting the brain and the hypothalamus, and how ATP release from hypothalamic cells is regulated.

P2X receptors in the pituitary

The release of hormones from the anterior pituitary gland is controlled by hypothalamic peptides and neurotransmitters that reach the pituitary via hypophysial portal blood, as well as by extracellular ATP (Chen *et al*., 1995) that is released by the anterior pituitary itself in a regulated manner (Tomic *et al*., 1996; Lazarowski *et al*., 2000; Stojilkovic and Koshimizu, 2001; He *et al*., 2005b). The mRNA transcripts for several P2X subunits ($P2X_2$, $P2X_3$, $P2X_4$, and $P2X_7$) were identified in anterior pituitary cells from neonatal (Zemkova *et al.*, 2006) and adult rats (Koshimizu *et al.*, 2000a; Koshimizu *et al.*, 2000b; Stojilkovic and Koshimizu, 2001), including two spliced forms of the $P2X_2$ subunit. Experiments with the plasma membrane-targeted luciferase expressed in HEK cells or ACN neuroblastoma cells indicated that endogenous extracellular ATP concentrations are in the range of 100-200 μM, which is more than sufficient to activate all types of P2X receptors (Pellegatti *et al.*, 2005). *In vivo*, the ATP action on gonadotroph functions could be controlled by ectonucleotidases 1-3, which are expressed in pituitary cells (He *et al.*, 2005a) and provide an effective pathway for the control of ATP's extracellular actions.

Most anterior pituitary cells express functional P2X receptors (Carew *et al*., 1994; Villalobos *et al*., 1997; Koshimizu *et al*., 2000a). The $P2X_2$ receptors were identified in pituitary gonadotrophs (Tomic *et al.*, 1996; Koshimizu *et al*., 2000a; Zemkova *et al*., 2006) and somatotrophs (Koshimizu *et al.,* 2000a). Lactotrophs most probably express functional $P2X_4$ (Carew *et al*., 1994; He *et al*., 2003) as well as $P2X_7$ receptor subtypes (He *et al.*, 2003). Corticotrophs seem not to respond to extracellular ATP directly by stimulation of any P2X receptor (Zhao *et al.*, 2006), and identification of P2X receptors in remaining anterior pituitary cell type, thyreotrophs, has not yet been done. In gonadotrophs, ATP induces a non-oscillatory, depolarizing, slowly desensitizing, and rapidly deactivating current that has been identified as the $P2X_2$ current (Fig. 3A&B). ATP also induces an increase in the frequency of action potentials (Fig. 3C) in gonadotrophs, indicating that $P2X_2$ receptors could operate as pacemaking channels in the pituitary (Zemkova *et al.*, 2006).

ATP could play the role of a local paracrine/autocrine modulator in the anterior pituitary (Stojilkovic and Koshimizu, 2001), which may serve as a synchronizer of spontaneous electrical activity (Zemkova *et al*., 2006). ATP may also initiate intercellular Ca^{2+} waves, as observed in other cell types (Guthrie *et al.*, 1999). Finally, Ca^{2+} influx through the pore of $P2X_2$ receptors and the associated voltage-gated Ca^{2+} influx could play a role as a modulator of G-protein coupled receptor-stimulated Ca^{2+} signaling by refilling of intracellular calcium stores (Zemkova *et al.*, 2006). Such an action of extracellular ATP could provide a mechanism for the amplification of the effects of hypothalamic peptides on calcium signaling and secretion. Further experiments should clarify to what extent these capacities of P2X receptors are utilized *in vivo*.

Conclusion

In the past ten years, the number of studies on P2X receptors has increased as investigators have begun to determine the physiological roles played by extracellular ATP and specific P2X receptor subtypes. It is already known that purinergic signaling is a key mechanism in pain sensation, brain injury, and inflammation. P2X receptors and investigations of the physiological relevance of ATP in the control of hypothalamic and pituitary functions have started. Termination of ATP action by ectonucleotidases is well established and has been examined experimentally, but the precise physiological roles played by these enzymes in the modulation of P2 receptor signaling remain unclear. In addition, the mechanisms of endogenous ATP release and its regulation are not yet known. Detailed knowledge about these events and the structure of purinergic receptors evoke hope that a door will be opened for development of new drugs that could prevent chronic pain and would be effective in protection against many diseases.

Text to figures

Fig.1

Models of TM1 and TM2 helix of $P2X_4R$. A, The plot of the TM1 sequence from Gly^{29} to Trp^{50} and TM2 sequence from Asn^{338} to Leu^{358} of the purinergic $P2X_4$ receptor onto helical wheel projections. *Left,* The amino acid residues Leu^{40}, Val^{43}, Val^{47} and Trp^{50}, which were tolerant to substitution by cysteine but were important for the effect of IVM, map specifically to one side of the predicted α-helix that could be lipid-oriented (black

asterisks). The substitution-sensitive Gln^{36} and Arg^{33} residues that were important for the effect of IVM also map on the same side, whereas four other substitution-sensitive residues are located on the opposite side of the TM1 α-helix that might line the wall of the pore (red asterisks). *Right,* In parallel*,* the substitution-resistant Asn^{338}, $Gly^{342,}$ Leu^{346}, Ala^{349}, and Ile^{356} TM2 residues, as well as the Cys^{353} residue that was important for the effect of IVM, are located on the same side of TM2 α-helix, whereas the substitution-sensitive residues map on the opposite side of α-helix. Colors of circles indicate hydrophobic nonpolar residues (yellow), glycine or polar uncharged residues (green), and charged basic (blue) and acidic (red) residues. B, Schematic comparison of the sizes of IVM molecule and the transmembrane segments of $P2X_4R$ modeled as regular α-helices. IVM-sensitive residues which were tolerant to alanine substitution and might be lipid-oriented are green, substitution-sensitive residues are red. For details see: (Jelinkova *et al*., 2008).

Fig.2

Representative photomicrographs demonstrating the presence of the $P2X_2$ receptor in supraoptic nucelus (SON) and its absence fin the suprachiasmatic nucleus (SCN). Experiments were performed on rat brain slices using an immunoreactive antibody for neuronal nuclei (NeuN, Chemicon, red), glial cells (GFAP, Chemicon, blue) and the $P2X_2$ receptor (Alomone Labs, green). Abbreviations are as follows: 3V, third ventricle; opt, optic tract. (Vávra , unpublished).

Fig.3

Identification of the $P2X_2$ receptor in pituitary gonadotrophs. A, Gonadotrophs can be identified in a mixed population of pituitary cells because of their specific GnRH-induced calcium oscillations monitored as outward calcium-activated potassium current ($I_{K\text{-}Ca}$). B, The sensitivity of ATP-induced current to reactive blue 2 (RB2), pyridoxal 5-phosphate 6-azophenyl-2',4'-disulphonic acid (PPADS) and suramine, but not ivermectin (IVM), shows that these cells express the $P2X_2$ receptor. Gray areas indicate the duration of drug application, and horizontal bars indicate the duration of ATP application. C, Stimulation of electrical activity by application of ATP to identified gonadotrophs. For details see: (Zemkova *et al.*, 2006).

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Fig. 1

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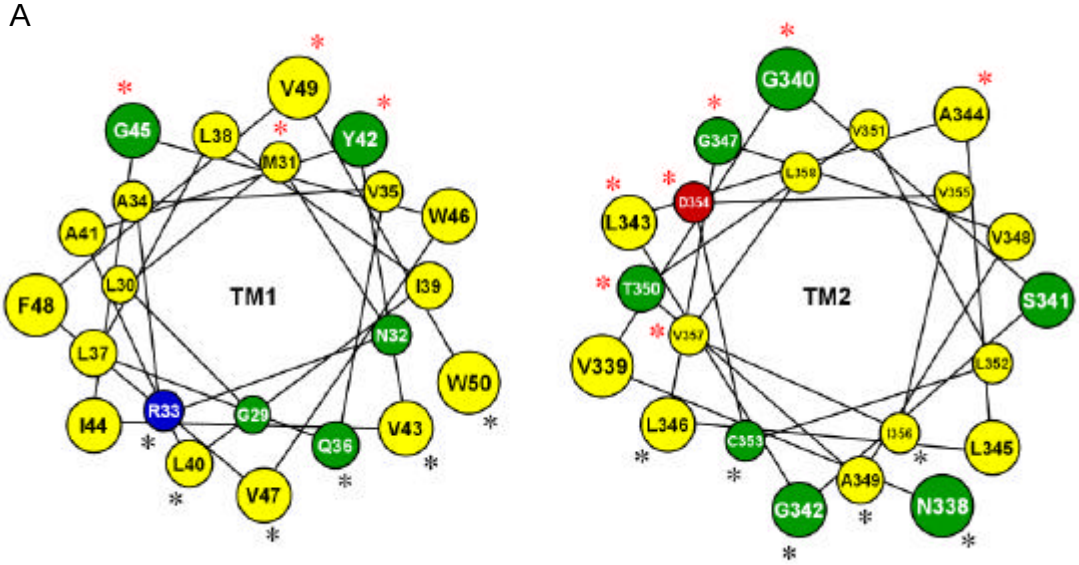
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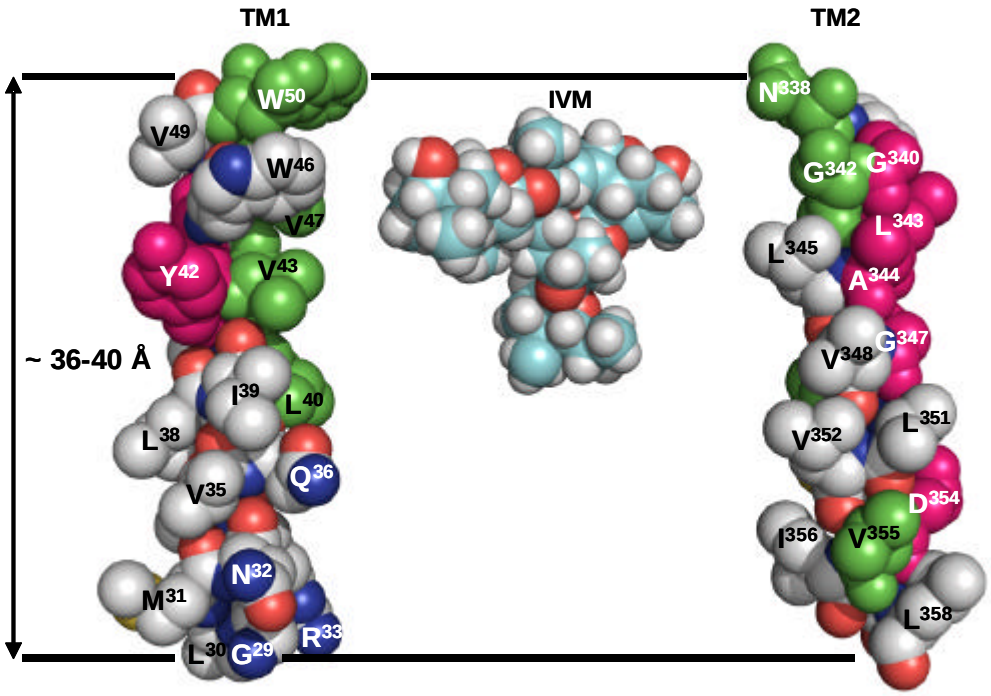
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Fig. 2

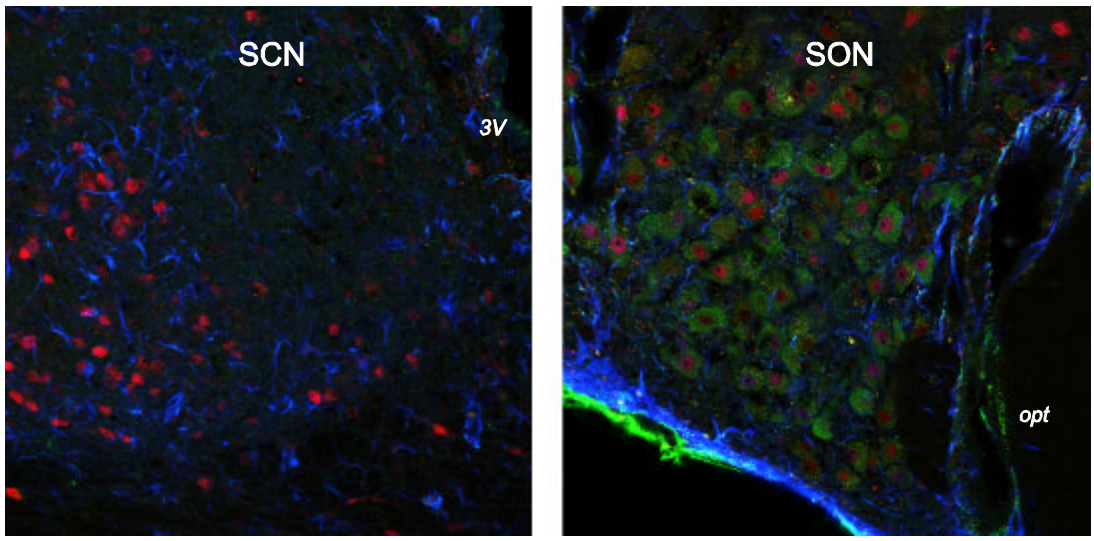
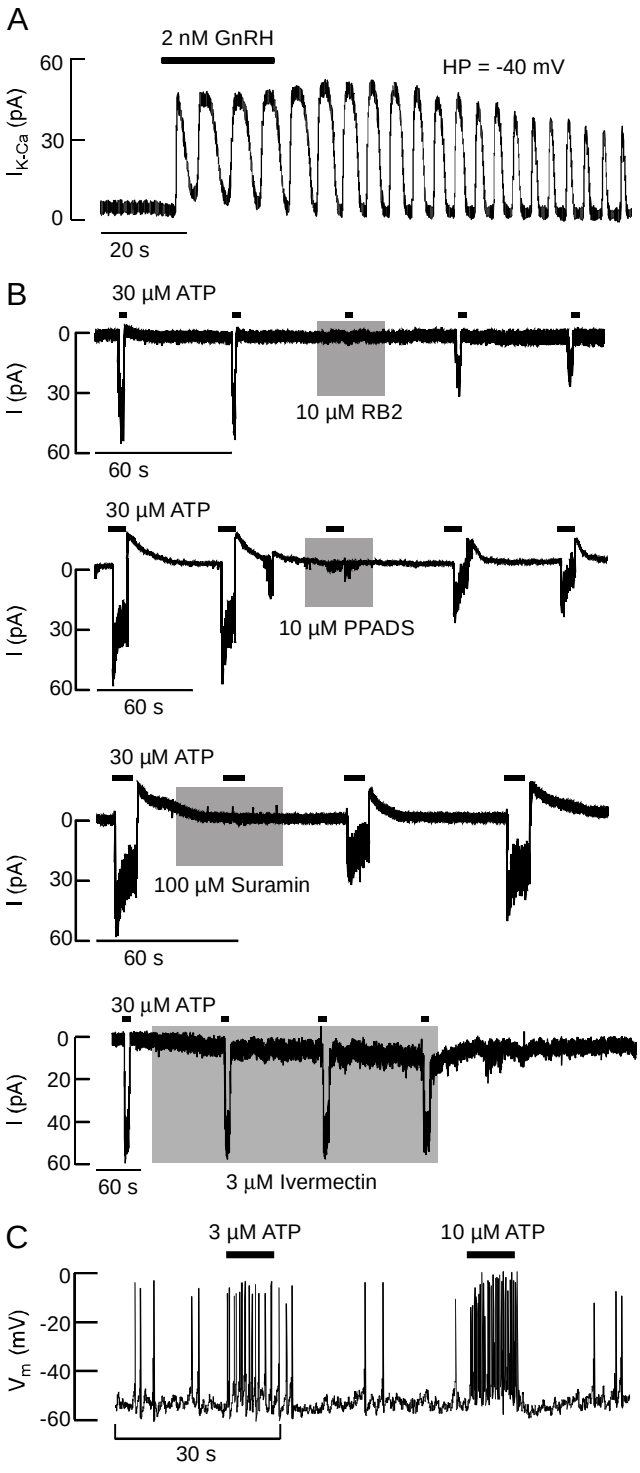


Fig. 3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60 s
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