

An antagonist-insensitive P_{2X} receptor expressed in epithelia and brain

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A cDNA was cloned which encodes a new ATP-gated ion channel (P_{2X₄} receptor). ATP induces a cationic current in HEK293 cells transfected with the P_{2X₄} receptor. However, the current is almost completely insensitive to antagonists effective at other P_{2X} receptors. Sensitivity to two of these antagonists (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid and pyridoxal 5-phosphate) is restored by replacement of Glu249 by lysine, which occurs at the equivalent position in P_{2X₁} and P_{2X₂} receptors. P_{2X₄} RNA is found by *in situ* hybridization in the brain, peripheral ganglia and epithelia including serosal cells of salivary glands. Recordings from rat submandibular gland cells showed ATP-induced currents that are also insensitive to antagonists. These results define a further member of P_{2X} receptor family, and they identify an amino acid residue involved in antagonist binding. They also introduce a new phenotype for ATP responses at P_{2X} receptors—insensitivity to currently known antagonists.

Keywords: ATP/epithelia/ligand-gated ion channel/P_{2X} receptor

Introduction

ATP is a fast excitatory neurotransmitter between neurons in the central and peripheral nervous system (Edwards *et al.*, 1992; Evans *et al.*, 1992; Galligan and Bertrand, 1994) and from sympathetic nerves to smooth muscle (Sneddon and Westfall, 1984; Evans and Surprenant, 1992; Jobling, 1994) (for reviews, see Burnstock, 1990; Bean, 1992; Surprenant *et al.*, 1995). This fast transmission is mediated through P_{2X} receptors, which are integral membrane ion channels permeable to cations including calcium. The identification of ATP as a synaptic transmitter has relied greatly on two approaches. First, α,β -methylene-ATP ($\alpha\beta$ meATP) is a desensitizing receptor blocker that also inhibits the response to nerve stimulation (Kasakov and Burnstock, 1983). Second, suramin (Dunn and Blakeley, 1988) and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; Ziganshin *et al.*, 1994) antagonize the responses to ATP and nerve stimulation in parallel. Such approaches have limitations. For example, it is already clear that one subgroup of the receptors is very insensitive to $\alpha\beta$ meATP; this includes receptors on pheochromocytoma (PC12) cells (Nakazawa *et al.*, 1990), rat superior cervical ganglion cells (Cloues *et al.*, 1993;

Nakazawa, 1994; Khakh *et al.*, 1995) and nucleus tractus solitarius neurons (Ueno *et al.*, 1992).

The first two P_{2X} receptor cDNAs were cloned from vas deferens smooth muscle (P_{2X₁}) and pheochromocytoma cells (P_{2X₂}). The encoded proteins (P_{2X} receptor subunits) are 38% identical and define a new family of membrane proteins (Brake *et al.*, 1994; Valera *et al.*, 1994) which is structurally unrelated to other ligand-gated ion channels. Each P_{2X} receptor subunit appears to have intracellular N- and C-termini, with the bulk of the protein (almost 300 amino acids) lying on the extracellular surface between two presumed membrane-spanning domains. In overall molecular architecture, although not in primary sequence, the P_{2X} receptor thus resembles a subunit of the amiloride-sensitive epithelial sodium channel (ENaC) (see Surprenant *et al.*, 1995); as for ENaC, the number of subunits that form a channel is unknown.

Heterologous expression of either P_{2X} receptor cDNA results in channels with properties which closely resemble those seen in the native cells. In the case of P_{2X₁} and vas deferens cells, $\alpha\beta$ meATP is an agonist and desensitization is marked; for P_{2X₂} and PC12 cells, $\alpha\beta$ meATP has little or no effect and the response to ATP undergoes much less desensitization (Brake *et al.*, 1994; Valera *et al.*, 1994; Evans *et al.*, 1995).

A third receptor (P_{2X₃}) cDNA has recently been isolated that is selectively expressed in sensory ganglia (Chen *et al.*, 1995; Lewis *et al.*, 1995). Expression of P_{2X₃} subunits alone in HEK293 cells does not reproduce the functional responses of sensory ganglion cells ($\alpha\beta$ meATP-sensitive, non-desensitizing); we recently reported studies which demonstrate that heteropolymerization of P_{2X₂} with P_{2X₃} most likely accounts for this neuronal phenotype (Lewis *et al.*, 1995).

There is considerable functional evidence for P_{2X} receptors in the central nervous system (reviewed by Edwards, 1994; Zimmermann, 1994; Surprenant *et al.*, 1995) but RNA levels of P_{2X₁}, P_{2X₂} and P_{2X₃} receptors are low or absent in adult brain (Brake *et al.*, 1994; Valera *et al.*, 1994; Lewis *et al.*, 1995). Thus, one purpose of the present work was to obtain further related clones that encoded neuronal receptors. The cDNA isolated (P_{2X₄}) was found to be expressed not only in neurons but also in some epithelial tissues; heterologous expression showed that it provides a previously unknown phenotype, being insensitive both to $\alpha\beta$ meATP and the antagonists.

Results

A new receptor cDNA

The P_{2X₄} receptor cDNA was isolated from a rat superior cervical ganglion cDNA library. The encoded protein has 388 amino acids and is 49, 44 and 45% identical with the vas deferens (P_{2X₁}), PC12 (P_{2X₂}) and sensory ganglion

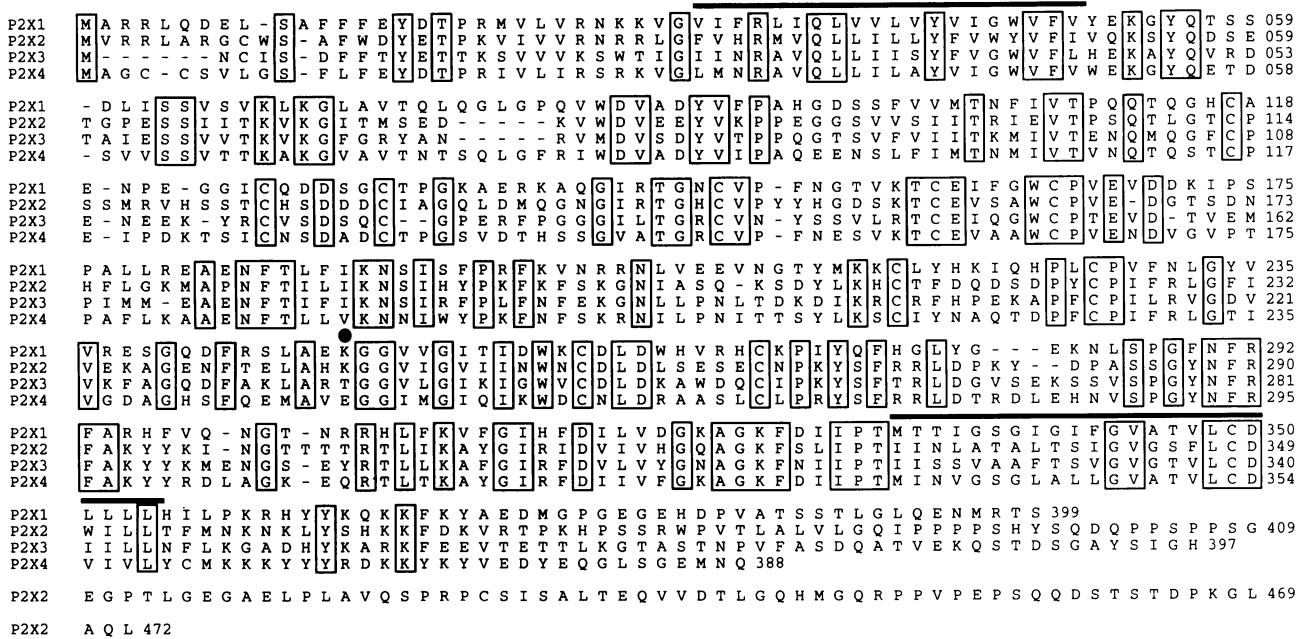


Fig. 1. Alignment of the predicted amino acid sequences for four P_{2X} receptors. Overlines indicate probable transmembrane domains, boxes indicate residues identical in all sequences, and the filled circle indicates the position of the lysine/glutamate substitutions.

(P2X₃) forms of the receptor (Figure 1). The overall pattern of hydrophobicity for the P2X₄ receptor agrees well with that for the other receptors, and is consistent with the model of relatively short intracellular N- and C-termini, two transmembrane regions, and a large extracellular domain. Within the putative extracellular loop there is conservation among all three receptors of the ten cysteine residues (Figure 1). It is probable that disulfide bonds contribute to the tertiary structure. In fourteen positions, glycine is conserved, and in seven positions there is a conserved lysine residue (Figure 1). These residues are commonly associated with nucleotide-binding sites (Traut, 1994).

RNA distribution in neurons and salivary glands

Northern blot analysis with P2X₄ riboprobe detected an RNA of 2 kb with a considerably more widespread tissue distribution than seen for the other receptor RNAs (data not shown) (Brake et al., 1994; Valera et al., 1994). In initial studies, this same P2X₄ riboprobe was used for *in situ* hybridization in the E19 rat embryo. Strong specific staining for P2X₄ RNA was found in the parotid and submandibular gland, as well as in the nasal and laryngeal epithelia (Figure 2A and B). A weak, diffuse hybridization signal was also detected in the embryonic brain; however, no staining was observed in any peripheral ganglia at E19.

In situ analysis in the adult rat confirmed this epithelial distribution. Submandibular glands from adult rats showed strong staining of the serosal epithelial cells, whereas adjacent mucosal epithelial cells from the same gland were only faintly positive (Figure 2C and D). Duct cells, identified by counter staining, were uniformly negative for P2X₄ RNA. Bronchial, though not tracheal, epithelium was also moderately stained. The abundance of the RNA in one subset of acinar cells strongly suggests a physiological role, and we therefore further characterized the

actions of ATP and analogs on rat submandibular gland cells by whole-cell recording (see below).

In the adult rat brain, hybridization was observed in several regions. Specific staining was found in the olfactory bulb, particularly in mitral cells, tufted cells and inner granular cells; external and internal plexiform layers were not stained (Figure 2E and F). In the hippocampus, strong hybridization was detected primarily in the pyramidal layer of CA1, CA2 and CA3 subfields, the mossy cells of the hippocampal hilus and the granule cells of the dentate gyrus (Figure 2G and H). Staining was also observed in the medial habenula, Purkinje cells of the cerebellum and some cranial nerve motor nuclei (facial and hypoglossal). No signal was detected in the basal ganglia or lateral thalamic nuclei. Sensory neurons of the trigeminal, nodose and dorsal root ganglia (Lewis et al., 1995), and sympathetic neurons of the superior cervical and coeliac ganglia were well-stained with the P2X₄ riboprobe, in contrast to the absence of signal in the embryo. Further detailed description of neuronal localization of the P2X₄ receptor (which is outside the scope of this study) is currently in preparation.

Functional expression of $\alpha\beta$ meATP-insensitive currents

The properties of the P2X₄ receptor were determined by whole-cell recordings from HEK293 cells after transient transfection. Responses to ATP and ATP analogs resembled closely those seen for the PC12 form of the receptor (P2X₂) (Figure 3) (Brake et al., 1994; Evans et al., 1995). ATP (30 μ M) induced an inward current with a latency to onset of 1–8 ms (3.3 ± 0.5 ms, $n = 20$) which showed little desensitization during agonist applications of up to 2 s (time constant of decay = 17.6 ± 1.5 s, $n = 14$). The ATP current was due to activation of a non-selective cationic conductance as determined by reversal potential

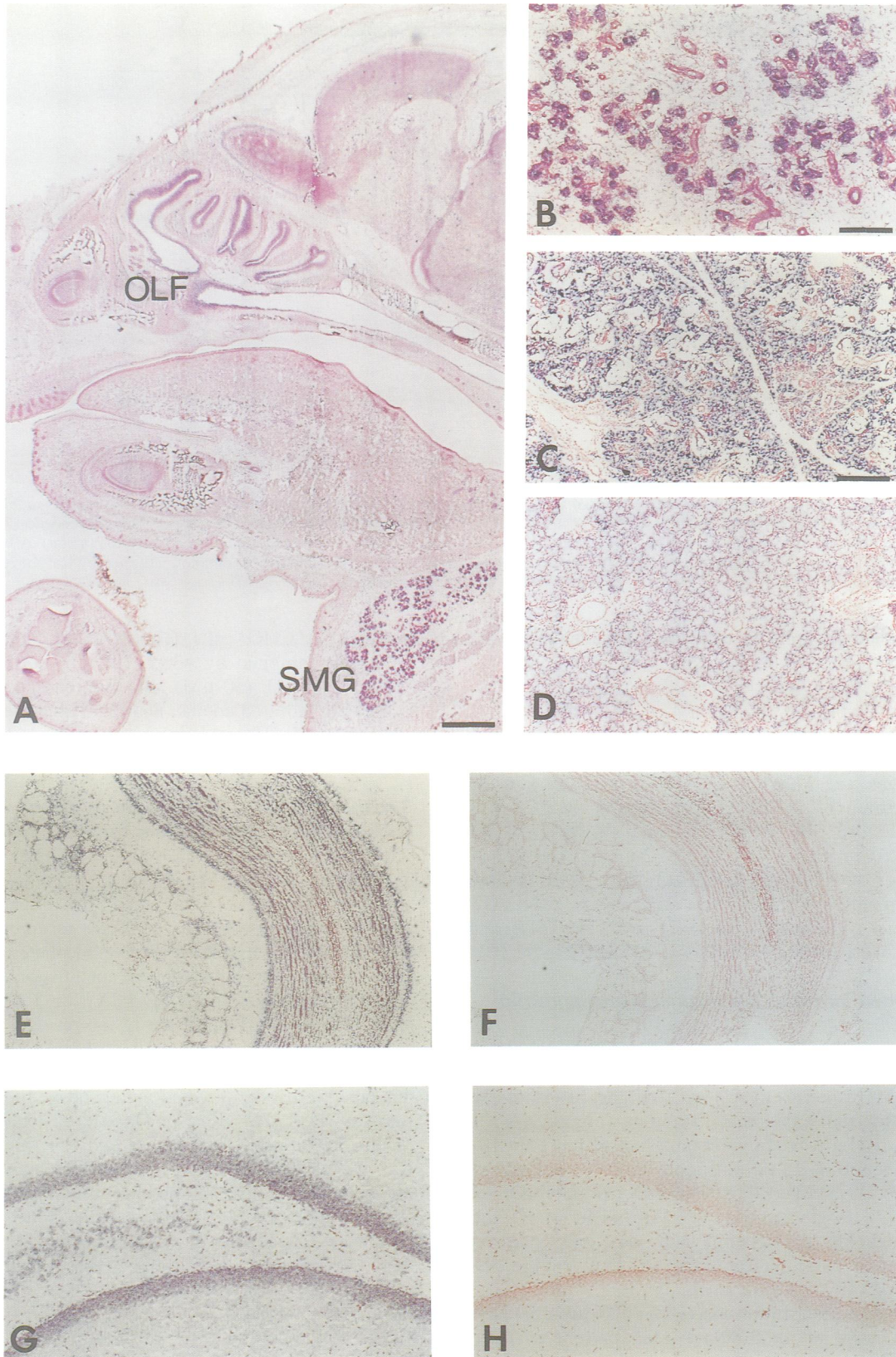


Fig. 2. Cellular localization of P_{2X₄} receptor mRNA by *in situ* hybridization. Panels show digoxigenin-riboprobe hybridization in (A) olfactory epithelium (OLF) and submandibular gland (SMG) of an E19 rat embryo, (B) acinar cells of embryonic rat submandibular gland, (C) serous and (D) mucous acinar cells of an adult rat submandibular gland; (E) olfactory bulb and (G) hippocampus of adult rat brain. (F) and (H) are representative sense strand hybridization for olfactory bulb and hippocampus. Scale bars are (A) 1 mm, (B) 50 μ m and (C–H) 100 μ m. In all sections mRNA staining is purple while pink is neutral red counterstain for cell body nuclei.

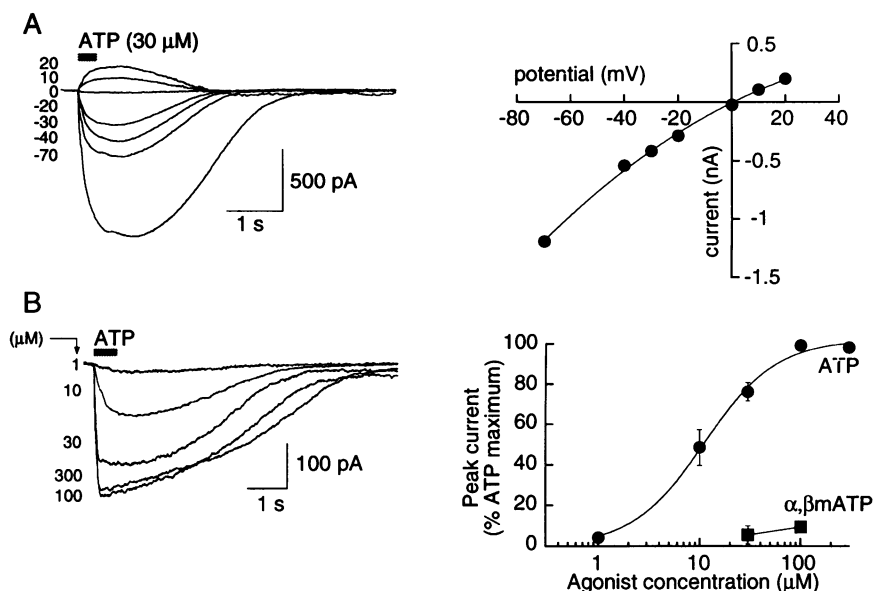


Fig. 3. Functional properties of heterologously expressed P2X₄ purinoceptors. (A) Voltage-dependence. Left; currents in response to ATP at membrane potentials indicated (mV). Right; current-voltage relation obtained from experiment shown. (B) Concentration-dependence. Left; currents in response to indicated concentrations of ATP. Right; concentration-response curve for all experiments ($n = 8-12$ for each point). All recordings made from HEK 293 cells transiently transfected with cDNA encoding P2X₄ receptor. ATP applied for duration indicated by bar above traces, in this and subsequent figures.

measurements (Figure 3A) with ion replacements; under bi-ionic conditions, reversal potentials in Na_i/Na_o, K_i/Na_o, Cs_i/Na_o, or Na_i/Ca_o were 0.9 ± 0.7 mV ($n = 8$), 0.2 ± 0.8 mV ($n = 8$), 6.3 ± 0.5 mV ($n = 7$) and 11.8 ± 1.2 mV ($n = 6$) respectively. Thus, permeability ratios for Na⁺:K⁺:Cs⁺:Ca⁺⁺ were 1: 1: 0.8: 4.2 (corrected for ion activities, see Valera *et al.*, 1995). ATP evoked the maximal current (400–3000 pA) at 100 μM; the EC₅₀ was 10 μM (Figure 3B). The rank order of potency for other effective agonists was ATP = 2-methylthio-ATP > adenosine 5'-O-(3-thiotriphosphate) >> ADP (Figure 3B). The methylene substituted ATP analogs, αβmeATP and L- and D-αβmeATP, as well as UTP, GTP and adenosine, were ineffective at 100 or 300 μM (Figure 3B).

ATP-induced currents are insensitive to suramin and PPADS

In one respect, the P2X₄ receptor differed strikingly in its properties from the P2X₁ and P2X₂ receptors (Evans *et al.*, 1995); responses to ATP were almost insensitive to blockade by the antagonists suramin (Figure 4A–C), PPADS (1–100 μM) and pyridoxal 5-phosphate (P5P; 10–300 μM; Trezise *et al.*, 1995) (Figure 4D). By comparison, 10 μM PPADS blocked P2X₁ and P2X₂ (Figure 4D; see Evans *et al.*, 1995) receptors by 90%. This block was slow to develop (5–10 min) and was only partially reversible with washing for up to 40 min (Figure 5D) (Evans *et al.*, 1995; Khakh *et al.*, 1995).

At very high concentrations (100–1000 μM), PPADS often did cause an inhibition of the current at P2X₄ receptors (20–60%). However, this occurred more rapidly; it developed within 30 s and washed out within 2 min, and was therefore clearly distinguishable from the slower inhibition observed with the P2X₁ and P2X₂ receptors. These concentrations of PPADS also inhibited currents through other ligand-gated currents with a similar rapid time course. Thus, at 5-HT₃ receptors expressed hetero-

logously in CHO cells (Hussy *et al.*, 1994) PPADS (100 μM) inhibited by $43 \pm 10\%$ ($n = 6$) the current evoked by 5-HT (30 μM), and at nicotinic receptors in guinea-pig cultured coeliac neurons (Khakh *et al.*, 1995), where PPADS (100 μM) inhibited by $36 \pm 11\%$ ($n = 6$) the current evoked by acetylcholine (100 μM).

Sensitivity to PPADS restored by lysine substitution into P2X₄ receptor

The slow time course of onset and reversal of blockade by PPADS (1–100 μM) led us to hypothesize that the aldehyde group on position 4 may form a Schiff base with a lysine residue in the P2X₁ and P2X₂ receptor. Consistent with this notion, pyridoxamine 5-phosphate, in which an amine replaces this aldehyde, did not antagonize the action of ATP in cells expressing P2X₁ or P2X₂ receptors (100 μM, $n = 4$). One of the lysine residues common to the P2X₁ and P2X₂ receptors is replaced by glutamate in the P2X₄ receptor (Glu²⁴⁹). We introduced a lysine into this position by site-directed mutagenesis. In this receptor [termed P2X₄(E249K)], ATP-induced currents were readily antagonized by PPADS and P5P, and this block was again only slowly reversible (Figure 5A and B). In this mutated P2X₄ receptor, concentrations of PPADS and P5P that inhibited by 50% the current induced by 30 μM ATP were 2.6 μM ($n = 5$) and 36 μM ($n = 3$) respectively; these concentrations are similar to those required to block wild-type P2X₂ receptors (Figure 4D) or P2X₁ receptors (Evans *et al.*, 1995).

The reciprocal mutation, substitution of glutamate into the P2X₂ receptor [P2X₂(K246E)], did not simply remove sensitivity to P5P and PPADS. It converted their action from the characteristic slow inhibition to a block that was rapidly reversible (Figure 5C and D). The results imply that the lysine residue is essential for the irreversible component of the block, but it is not the sole residue contributing to the differences between the P2X₂ and P2X₄

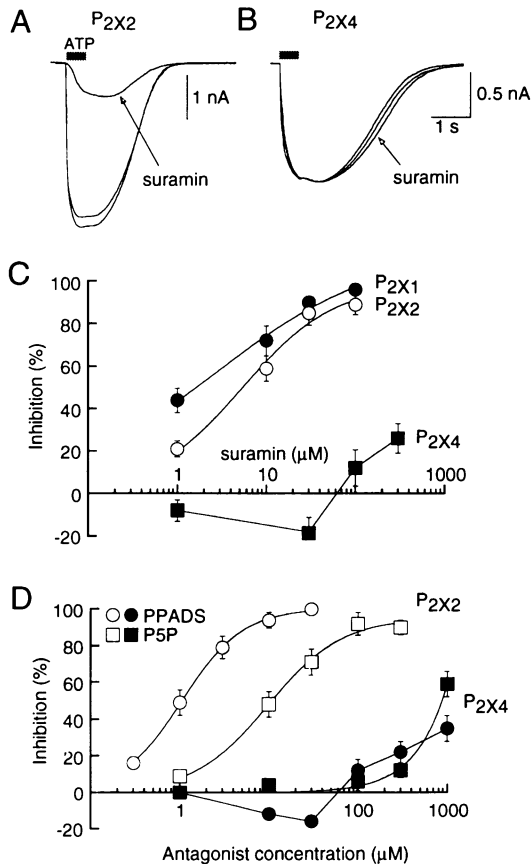


Fig. 4. P_{2X4} receptors are insensitive to blockade by P_{2X} receptor antagonists. (A and B) Each set of traces shows currents before, during and after application of suramin (30 μM), in HEK293 cells transfected with the P_{2X2} receptor (A) or the P_{2X4} receptor (B). (C) Inhibition of ATP-induced current by suramin in HEK293 cells expressing P_{2X1}, P_{2X2} or P_{2X4} receptors; each point is mean ± s.e.m. for 6–10 cells. (D) Inhibition of ATP-induced current by PPADS (circles) and P5P (squares) of P_{2X2} receptor (open symbols) and P_{2X4} receptor (closed symbols); each point is mean ± s.e.m. from 4–12 cells. ATP concentration was 30 μM throughout.

receptors in binding these antagonists. The P_{2X3} receptor has a Thr residue in the equivalent position (Figure 1); PPADS at low μM concentrations inhibits ATP action with quick onset and reversibility (Lewis *et al.*, 1995), which is also consistent with the absence of Lys. Presumably other residues are important for the initial binding of PPADS and P5P, but when a lysine is present at position 249 (in P_{2X4}) then a Schiff base can form with the aldehyde. In neither mutant was the sensitivity to ATP significantly altered (EC₅₀s 10–15 μM). This indicates that this lysine is probably not a critical contributor to agonist binding; lysines are commonly involved in binding to the oxygens of the γ-phosphate of ATP (Traut, 1994).

Suramin did not block the action of ATP in the wild-type P_{2X4} receptor (Figure 4B and C) nor in the P_{2X4}(E249K) receptor (8 ± 1% and 22 ± 6% inhibition at 30 and 100 μM, *n* = 9). Moreover, suramin blocked the response of the P_{2X2}(K246E) receptor (67 ± 7% and 81 ± 8% inhibition at 30 and 100 μM, *n* = 4) as was previously found for the wild-type P_{2X2} receptor (Figure 4C; and see Evans *et al.*, 1995). These results are not unexpected since suramin is a polysulfonic antagonist and lacks an aldehyde moiety. They indicate that other differences

between the P_{2X4} and the P_{2X2} receptors, as yet undetermined, must underlie the lack of sensitivity to suramin.

Submandibular glands also express antagonist-insensitive ATP responses

On the basis of the RNA distribution (see above), we hypothesized that epithelial cells should express ATP-gated cation channels that are not activated by αβmeATP and not blocked by the antagonists. Whole-cell recordings were made from dissociated acini of rat submandibular glands. ATP evoked fast inward currents in ~25% of cells examined (21 of 92 cells). The latency to onset (4.2 ± 0.8 ms, *n* = 9) and reversal potential (1.7 ± 0.6, *n* = 6) were the same as for the heterologously expressed P_{2X4} receptor (Figure 6A). The currents were concentration-dependent (EC₅₀ 10 μM; Figure 6B) and 2-methylthio-ATP was equal in effect to ATP (*n* = 3, data not shown); αβmeATP, L-βmeATP and UTP were all ineffective (100–300 μM). As for the cloned receptor in HEK293 cells, these ATP-induced inward currents were not blocked by suramin, P5P (30, 100 μM, *n* = 2) or PPADS (10–100 μM, *n* = 4) (Figure 6C).

Discussion

The P_{2X4} receptor is the first of this family to be found widely expressed in brain regions. Dense staining for P_{2X4} mRNA was found in olfactory bulb, hippocampal pyramidal cells, medial habenula neurones and cerebellar Purkinje cells. Only in the medial habenula has ATP been shown to activate P_{2X}-purinoceptors (Edwards *et al.*, 1992). In contrast to the αβmeATP-insensitive, antagonist-insensitive current activated by heterologously expressed P_{2X4} receptors, the P_{2X} receptor in medial habenula neurones is sensitive to both αβmeATP and suramin (Edwards *et al.*, 1994). It may be that the endogenous P_{2X} receptor in these neurones is formed by a distinct gene product or by co-assembly of the P_{2X4} subunit with another, as yet unidentified, subunit.

We also found a previously unsuspected distribution of P_{2X4} mRNA in epithelia, particularly serosal epithelia of submandibular glands. ATP is known to directly activate ligand-gated cationic channels in lacrimal gland cells (Sasaki and Gallacher, 1990; Vincent, 1990) but αβmeATP and antagonist actions at these sites are unknown. Presence of P_{2X} receptors in other epithelial cells has not been reported previously. Based on the epithelial localization of mRNA for the P_{2X4} receptor, we sought and found an ATP-gated cationic channel in rat submandibular gland cells. Here, the properties of the endogenous P_{2X} receptor matched those of the cloned P_{2X4} receptor, suggesting that assembly of homopolymeric channels could form the P_{2X} receptor in these cells. Our results also provide an explanation for the observation that, in rat submandibular gland cells, ATP causes a rise in intracellular calcium due to calcium entry from the extracellular solution (Hurley *et al.*, 1994); this could result from entry through P_{2X4} receptors. One important implication of the present findings is that transmission from postganglionic sympathetic fibers to epithelia might have a significant purinergic component. However, the present findings indicate that, unlike the transmission from sympathetic fibers to smooth muscle (Dunn and Blakeley, 1988; Evans and Surprenant,

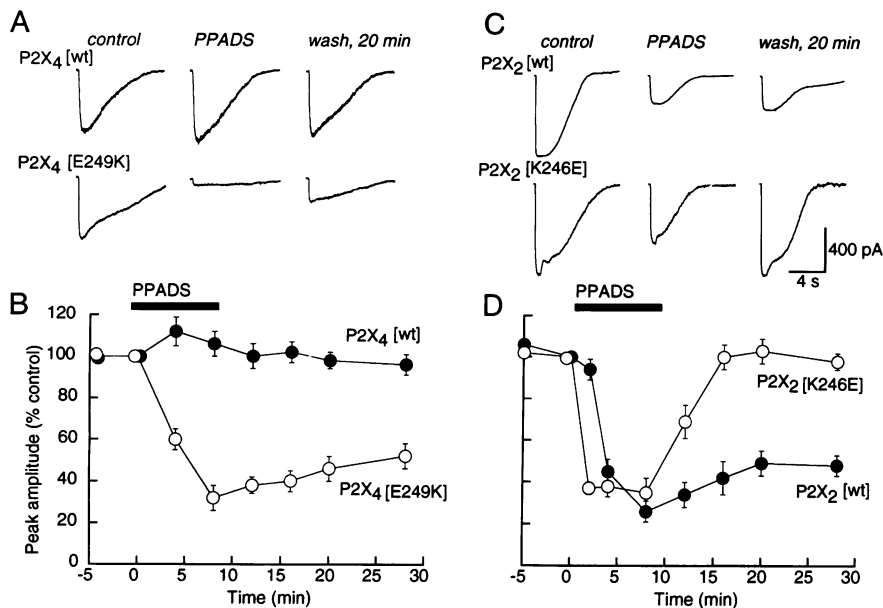


Fig. 5. Point mutation in P2X₄ receptor restores sensitivity to antagonist. (A) Recordings from HEK293 cells expressing wild-type P2X₄ or P2X₄(E249K) receptors. Currents are responses to ATP (30 μ M, 500 ms) in control solution, PPADS (10 μ M, after 8 min) and wash (20 min). (B) Time course of inhibition by PPADS (10 μ M) on wild-type P2X₄ receptor (filled circles) and P2X₄(E249K) (open circles); results are plotted as % of control amplitude in each cell. Each point is mean \pm s.e.m. of 4–7 experiments. (C and D) Similar experiments to those shown in (A) and (B), on wild-type P2X₂ receptor and on P2X₂(K246E). Note that the inhibition is now rapid in onset and recovery.

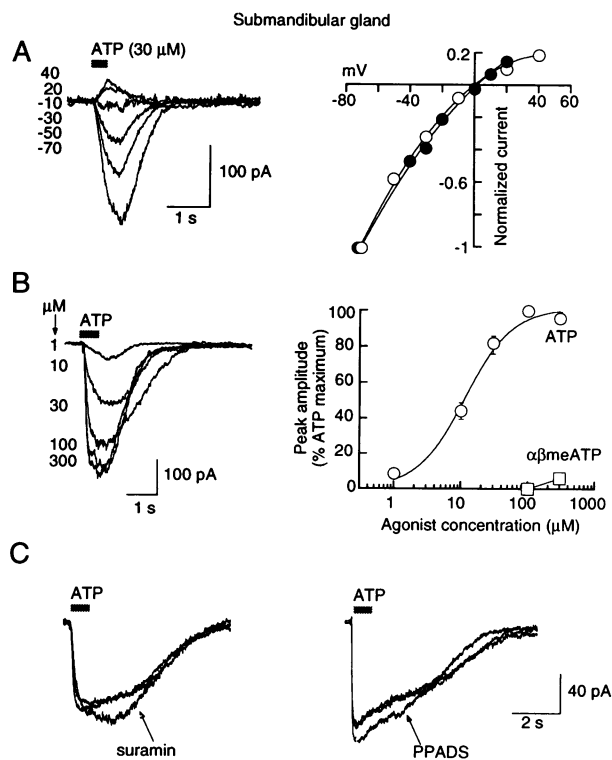


Fig. 6. P_{2X} receptors in submandibular gland cells have the same properties as cloned P2X₄ receptors. All recordings are from acutely dissociated rat submandibular gland cells. (A) Currents in response to ATP application at various membrane potentials; graph is current-voltage relation for gland (open circles) and for cloned P2X₄ receptor (closed circles) from data illustrated in Figure 3A; currents are normalized to response to -70 mV. (B) Superimposed currents in response to increasing concentrations of ATP and summary of agonist responses; $n = 3$ for each point. (C) Currents recorded before, during and after application of suramin (left traces) or PPADS (right traces); antagonist and ATP concentrations were 30 μ M.

1992), this could not be detected with currently available antagonists.

The discovery of a P_{2X} receptor that is insensitive to the commonly used antagonists (PPADS and suramin) has several implications. First, it brings to four the number of readily distinguishable phenotypes of ATP responses at P_{2X} receptors. These are: (i) strongly desensitizing, mimicked by $\alpha\beta$ meATP and blocked by the antagonists (e.g. vas deferens and expressed P2X₁ receptors); (ii) minimally desensitizing, not mimicked by $\alpha\beta$ meATP, and blocked by antagonists (e.g. PC12 cells and expressed P2X₂ receptors); (iii) minimally desensitizing, not mimicked by $\alpha\beta$ meATP, and not blocked by antagonists (e.g. submandibular gland cells and expressed P2X₄ receptors); (iv) minimally desensitizing, mimicked by $\alpha\beta$ meATP, and blocked by antagonists. The fourth type of response has been observed in dorsal root ganglion cells and nodose ganglion neurons (Khakh *et al.*, 1995) and appears to arise by subtype specific heteropolymerization of P2X₂ and P2X₃ (Lewis *et al.*, 1995). With respect to P2X₁, P2X₂ and P2X₄, the overall correspondence between the properties of the receptor in native tissue and the properties of the receptor when heterologously expressed remains consistent with the notion that native channels contain a single subunit, presumably as a homopolymer.

The further implication of the present work arises from the finding that one lysine residue plays a critical role in the slowly reversible block by PPADS of the P2X₁ and P2X₂ receptor. This provides the first direct evidence that this region of the molecule lies on the extracellular aspect of the membrane. In the case of other ligand-gated channels, identification of antagonist binding sites has been an important step towards developing a picture of molecular structure, and it may now be possible to refine our view of the pyridoxal binding site at the P_{2X} receptor by a combination of modelling and mutagenesis.

Finally, blockade by antagonists has been a primary criterion in the identification of chemical transmitters in general, and of ATP in particular (see Introduction). The finding of P_{2X} receptors that are insensitive to known antagonists will therefore complicate the assessment of their physiological and pathological roles. This will be particularly important in the central nervous system where, to judge from the widespread distribution of the P_{2X}₄ receptor RNA, more extensive functional roles await discovery.

Materials and methods

Inosine-containing degenerate oligonucleotides [TG(T/C)GA(G/A) (G/A)TIT(T/C)IG(G/C)ITGGT(G/T)CC and GC(A/G)AA(T/C) CT(G/A) AA(G/A)TT(G/A)(T/A)AICC, based on the conserved peptide sequences CEVAAWCP and GYNFRFA; Figure 1] were tested for their ability to amplify DNA with sequences related to P_{2X}₁ and P_{2X}₂ cDNAs. A 440 bp fragment of P_{2X}₄ cDNA was initially isolated from rat testis cDNA and was used as a hybridization probe to screen a λ gt10 cDNA library from mRNA of the rat superior cervical ganglia. A 1997 bp cDNA was subcloned from lambda into pcDNA3 (Invitrogen, San Diego, CA) and sequenced (accession No. X87763) by fluorescent DNA sequencing (Applied Biosystems).

For *in situ* hybridization, sagittal cryosections (14 μ m) were hybridized at high stringency (50% formamide + 5X SSC, at 72°C) with 50 ng/ml digoxigenin-UTP-labeled full-length cRNA probe for P_{2X}₄ receptor (Schaeren-Wiemers and Gerfin-Moser, 1993). Hybridization was detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim). Results are shown for 18 h phosphatase reaction. Sections were counter-stained with neutral red which indicates nuclei in cell bodies.

Primary cultures of submandibular gland acinar cells were made from four week old rats (Doyle *et al.*, 1994). The dissected tissue was disaggregated by digestion for 30 min at 37°C with calcium-free, modified Hank's balanced solution (Gibco BRL) containing collagenase (75 U/ml; Gibco BRL) and hyaluronidase (1 mg/ml; Sigma). Dispersed cells were collected by centrifugation, washed twice and resuspended in medium DMEM/F12 (Gibco BRL) supplemented with fetal bovine serum (1%), HEPES (25 mM), gentamycin (50 μ g/ml), epidermal growth factor (80 ng/ml) (all from Gibco BRL), insulin (5 μ g/ml), transferrin (5 μ g/ml), dexamethasone (100 nM), retinol (100 nM), thyroxine (200 pM), selenium dioxide (30 nM), carbachol (300 μ M), molybdc acid (1 nM), nickel chloride (5 pM), ammonium metavanadate (5 nM) and stannous chloride (5 pM) (all from Sigma). Cells were plated on coverslides pre-coated with collagen (200 μ g/ml) and laminin (50 μ g/ml) (both from Boehringer Mannheim) and incubated at 37°C for 4 h before recordings.

Whole-cell recordings were obtained at room temperature with 5–7 M Ω patch electrodes filled with (mM): K-aspartate or Cs-aspartate 140, NaCl 20, EGTA 10, and HEPES 5. The external solution was (mM): NaCl 147, CaCl₂ 2, MgCl₂ 1, KCl 2, HEPES 10 and glucose 12. Agonists were applied by fast-flow U-tube method (Fenwick *et al.*, 1982); antagonists were applied in both fast-flow and superfusion solutions. HEK293 cells were transfected with 1 μ g cDNA/8 \times 10⁴ cells using lipofectin, and recordings were made 12–72 h later (Evans *et al.*, 1995). The time constant of decay was estimated from an exponential fit to the current during 2 s or 5 s applications of ATP; longer applications were not examined.

Monovalent cation permeability ratios were determined under bi-ionic conditions, using the simplified Goldman–Hodgkin–Katz equation (Hille, 1992). For calcium permeability measurements, internal solution was (mM): NaCl 145, HEPES 10 and EGTA 5; external solution was (mM): CaCl₂ 110, Ca(OH)₂ 1.8, HEPES 6.5 and glucose 11.1.

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