Cloning of P2X₅ and P2X₆ Receptors and the Distribution and Properties of an Extended Family of ATP-Gated Ion Channels

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Two new P2X receptor cDNAs (P2X₅ and P2X₆) were isolated and expressed. All six proteins are 36–48% identical and seem to have two transmembrane segments with a large extracellular loop. Functionally, P2X₅ and P2X₆ receptors most resemble P2X₂ and P2X₄; they desensitize only slowly and do not respond to $\alpha\beta$ -methylene-ATP. P2X₆ receptors, like P2X₄ receptors, are not blocked by the antagonists suramin and pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonic acid. P2X₆ and P2X₅ receptors express at lower levels than P2X₁–P2X₄ receptors do, perhaps indicating that they do not normally form homomultimeric channels. P2X₆ and P2X₄ are the receptors expressed

most heavily in brain, where their RNAs have a widespread and extensively overlapping distribution. The spinal cord expresses all receptors except P2X₃. P2X₂, P2X₄, and P2X₆ are the most abundant in the dorsal horn. Sensory neurons of the trigeminal, dorsal root, and nodose ganglia express all six RNAs; P2X₃ is found only there. The functional properties and tissue distribution of these six P2X receptors indicate new roles for ATP-gated ion channels.

Key words: adenosine 5'-triphosphate; P2X receptors; *in situ* hybridization; ion channels; RNA distribution; functional expression

P2X receptors are ligand-gated ion channels that bind extracellular ATP. ATP activates these channels in many excitable and nonexcitable cells, ranging from neurons and muscle and gland cells to hair cells, astrocytes, microglia, and B lymphocytes (Nakagawa et al., 1990; Walz et al., 1994; Bretschneider et al., 1995; for reviews, see Burnstock, 1990; Bean, 1992; Zimmermann, 1994). Although the physiological role of ATP in many of these locations is not well understood, it is clear that one function of ATP is as a synaptic transmitter. Thus, ATP released from presynaptic nerves causes a typical fast excitatory postsynaptic potential both in neurons (Edwards et al., 1992; Evans et al., 1992; Galligan and Bertrand, 1994) and in smooth muscle (Sneddon and Westfall, 1984; Dunn and Blakeley, 1988; Evans and Surprenant, 1992). The observation that ATP opens cation-selective channels in several regions of the CNS and peripheral nervous system (Zimmermann, 1994; Surprenant et al., 1995) suggests that such a transmitter role may be more widespread. Therefore, an important approach is to map the distribution of P2X receptor RNAs in brain and other tissues, and this has recently become possible with the cloning of the corresponding cDNAs. This was one aim of the present work.

Four P2X receptor cDNAs have been cloned previously (P2X₁: Valera et al., 1994; P2X₂: Brake et al., 1994; P2X₃: Chen et al., 1995; Lewis et al., 1995; P2X₄: Bo et al., 1995; Buell et al., 1996; Seguela et al., 1996; Soto et al., *in press*). The receptors constitute a related group of proteins, each of which can form ATP-gated cation-selective channels when expressed in heterologous cells. There are phenotypic differences among the expressed receptors,

particularly with respect to the actions of $\alpha\beta$ -methylene-ATP ($\alpha\beta$ meATP), the rate of desensitization, and the effectiveness of antagonists (Evans et al., 1995; Surprenant et al., 1995; Buell et al., 1996). These phenotypes do not always correspond to those observed in native tissues, suggesting that the native channels might be heteropolymers of the cloned subunits or that they contain additional subunits not yet cloned. Heteropolymerization can occur; coexpression of P2X₂ and P2X₃ is required to reproduce the responses to ATP of sensory neurons (Lewis et al., 1995). The poor correlation between the phenotypes reported for cloned receptors P2X₁–P2X₄ and responses of some central neurons also implied that additional P2X genes may exist. Thus, a second aim of this work was to isolate and express cDNAs encoding additional members of the family.

MATERIALS AND METHODS

Cloning and mutagenesis. P2X₅ and P2X₆ cDNAs were found by PCR, using degenerate primers based on the amino acid sequences CEVAAWCP and GYNFRFA described previously. The sequences of these inosine-containing oligos have been reported previously (Buell et al., 1996). Approximately 440 bp PCR fragments were amplified for P2X₅ and P2X₆ cDNAs from celiac and superior cervical ganglia mRNAs, respectively. λ gt10 cDNA libraries were constructed from these same mRNAs, and the PCR fragments were used as hybridization probes to isolate the corresponding cDNAs. The cDNAs were subcloned into pcDNA3 (Invitrogen, San Diego, CA) and sequenced by fluorescent DNA sequencing (Applied Biosystems, Foster City, CA). Single point mutations were made as described (Buell et al., 1996). The accession numbers of the six cDNAs used in this study are P2X₁, X80477; P2X₂, U14414; P2X₃, X91167; P2X₄, X87763; P2X₅, X92069; and P2X₆, X92070.

Expression of P2X receptors. For transient transfections, cDNA (1 μ g/ml) and lipofectin dissolved in Optimum (Gibco, Gaithersburg, MD) were placed in Petri dishes containing coverslips onto which HEK293 cells were plated at a density of 8×10^4 /coverslip. Cells were washed after 5 hr and maintained in normal culture medium at 37°C. Recordings were made 16–48 hr later. Approximately 90–95% of all cells from which recordings were made showed inward currents to ATP when cDNAs encoding P2X₁–P2X₅ were used, but only 0–15% of cells transfected with

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	P2X1	P2X2	P2X3	P2X4	P2X5	P2X6
P2X1	-	36.8	40.5	48.4	40.5	41.7
P2X2	54.3	-	44.3	41.2	44.1	36.6
P2X3	55.1	55.6	-	42.0	42.8	37.4
P2X4	64.2	58.2	58.2	-	46.6	42.1
P2X5	56.4	59.5	57.2	63.4	-	45.4
P2X6	53.1	53.1	47.2	55.1	59.5	-

HEPES, and 12 glucose. The time course of agonist applications from the U-tube was estimated by measuring changes in junction potential occurring when distilled water was applied (no cell present). The latency from valve activation to response onset (measured as time to 5% of peak amplitude) was 4–20 msec, and the equilibration time (measured as time to advance from 10 to 90% of peak response) was relatively invariant at 10–12 msec. Agonist concentration–response curves were constructed from transfected HEK293 cells. EC₅₀ values were obtained by constructing complete concentration–response curves (five to six concentrations) in a single cell. In all experiments, a maximum concentration of ATP (usually 30 μ M) was applied at the beginning and the end of the experi-

Figure 2. Functional expression of P2X₆ and P2X₅ receptors. *A*, Inward currents to 1 sec applications of ATP and ATP analogs in cells transfected with P2X₆ cDNA. All concentrations are 30 μM except for ADP and αβmATP, which are 100 μM. *B*, Inward currents in response to 2 sec applications of increasing concentrations of ATP in cells transfected with P2X₅ cDNA. Note small amplitude currents compared with P2X₆. *C* and *D*, Concentration-response curves for activation of P2X₆ and P2X₅ receptors. At P2X₆ receptors, EC₅₀ values were (in μM): ATP (●) 12 ± 2 (*n* = 4), 2-MeSATP (■) 9 ± 1.3 (*n* = 4), 2-chloro-ATP (▲) 10 ± 2 (*n* = 4), ATP-γ-S (▼) 16 ± 2 (*n* = 4), and ADP (◆) 126 ± 21 (*n* = 4). At P2X₅ receptors, half-maximal concentrations were (in μM): ATP 15.4 ± 1.2 (*n* = 8), 2-MeSATP 20 ± 3 (*n* = 4), ATP-γ-S 9.3 ± 1 (*n* = 4), and ADP 270 ± 35 (*n* = 4). *E*, Peak amplitude of response to maximum concentration of ATP (30 or 100 μM) for P2X receptors expressed in HEK293 cells. Numbers above each bar indicate numbers of cells. *F*, Expression rate of P2X receptors heterologously expressed in HEK293 cells. Numbers above each bar indicate numbers of transient transfections. At least eight cells/transfection were examined, except for P2X₆ receptor, in which case >25 cells/transfection were examined.

Figure 3. Antagonist actions at P2X₆ and P2X₅ receptors. **A**, P2X₆ receptors. Superimposed records before, during, and after the application of suramin or PPADS. **B**, Recordings from cells expressing P2X₆[L251K] mutation. Suramin (30 μ M) remains ineffective, but PPADS (10 μ M) causes a largely irreversible antagonism. **C**, P2X₅ receptors. Currents are inhibited by suramin (30 μ M) and PPADS (10 μ M). **D**, Summary of results with suramin (●) and PPADS (■) at P2X₅ (right) and P2X₆ (left) receptors; open symbols are results for P2X₆[L251K] mutant. Results are plotted as percentage inhibition from control current evoked by 30 μ M ATP; $n = 4$ for each point except for L251K mutant, in which case $n = 3$. All results obtained from holding potential of -70 mV; agonist concentration was 30 μ M for all experiments in A–D.

Table 1. Distinguishing properties of P2X receptors when individual subunits are heterologously expressed

	P2X ₁	P2X ₂	P2X ₃	P2X ₄	P2X ₅	P2X ₆
ATP	0.9	8	0.7	10	15 ± 12(8)	12 ± 2(5)
αβmeATP	2.2	>100	0.9	>100	>100	>100
Suramin	1	5	3	>100	4	>100
PPADS	1	2	1	>100	2.6 ± 0.3(4)	>100
Desensitization	0% ^a	78%	9% ^b	64%	87 ± 5%(8)	60 ± 4%(8)

Values are EC₅₀ (μM) for agonists (ATP, αβmeATP), IC₅₀ (μM) for antagonists (suramin, PPADS). Values for P2X₅ and P2X₆ are mean ± SEM for number of experiments in parentheses; values for P2X₁–P2X₄ from Valera et al. (1994), Buell et al. (1995), Evans et al. (1995), and Lewis et al. (1995). Desensitization is measured from currents induced by 2 sec application of 30 μM ATP and is expressed as percentage current remaining at the end of the 2 sec application. Boxes highlight similar properties.

^aCurrent decay fitted by single exponential of time constant 300 msec.

^bCurrent decay fitted by two exponentials with time constants 100 msec and 1.3 sec.

ment; data were used only when the amplitude of the currents that were evoked differed by <5%. Hyperbolic functions were fit by least-squares to calculate EC₅₀. EC₅₀ values from individual cells so obtained were then averaged to provide values stated in text. For the P2X₁ and P2X₃ receptors, the current declined almost completely during application of ATP (30 μM) for 2 sec. Desensitization at these receptors has therefore been described by fitting exponentials (one for P2X₁; two were better for P2X₃). For P2X₂, P2X₄, P2X₅, and P2X₆ receptors, the currents evoked by ATP (30 μM) declined much less during the 2 sec period. In these

cases, desensitization was measured as the percentage of peak current remaining at the end of the 2 sec application period. All results in text and figures are mean ± SEM for the number of observations in parentheses.

In situ hybridization. Frozen sections of brain and other tissues from 3-week-old rats (14 μm thick) were mounted on polylysine-coated microscope slides and incubated with either sense or antisense digoxigenin-UTP-labeled cRNA probes at 50–150 ng/ml of probe (Schaeren-Wiemers and Gerfin-Moser, 1993). After 16 hr at 72°C, hybridization was detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase

Figure 4. P2X₆ and P2X₄ are the predominant brain P2X receptors. Low-magnification photomicrographs (2.5×) showing the distribution of hybridization signals for P2X₄ (A), P2X₅ (B), and P2X₆ (C) receptors. At this level (−3.4 mm from bregma), P2X₄ and P2X₆ RNAs were found in several areas, but no P2X₅ signal was detected. Scale bar (shown in A): 1 mm. D, Schematics for high-magnification photomicrographs shown in Figures 5–7. Half-brain maps ordered rostrocaudally; distance from bregma (B) in millimeters (Swanson, 1992). CG, Cingulate cortex; SON, supraoptic nucleus; MHb, medial habenula; CA1–CA3, CA1 and CA3 hippocampal fields; Purkinje cells, Purkinje layer of cerebellar cortex; Me5, mesencephalic trigeminal nucleus; NTS, nucleus tractus solitarius; X, dorsal motor nucleus vagus nerve; XII, hypoglossal nucleus.

Figure 5. P2X₅ RNA is only in Me5 neurons in brain; P2X₆ and P2X₄ RNAs are in Purkinje cells. Photomicrographs show *in situ* hybridization signals for P2X₄ (A–C), P2X₅ (D–F), and P2X₆ (G–I) in the mesencephalic trigeminal nucleus (Me5) and the Purkinje cell layer of cerebellum. Sections containing Me5 were incubated with digoxigenin antisense riboprobes (A, D, G) or sense riboprobe controls (B, E, H). Antisense probe hybridizations are shown for the cerebellar Purkinje neurons (C, F, I). See schematic of Figure 4D. Sections were counter-stained with neutral red. Scale bar (shown in A): 50 μ m.

(Boehringer Mannheim, Indianapolis, IN) using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Boehringer). Results are shown for an 18 hr phosphatase reaction. Sections were counter-stained with neutral red (Sigma, St. Louis, MO). For each probe the sense strand was generated as negative control. P2X_{1–4} riboprobes corresponded to full-length cDNAs (nucleotides 1–1837, 1–1831, 1–1762, and 1–1997, respectively). The P2X₅ and P2X₆ riboprobes corresponded to nucleotides 742–2436 and 1–2017 of the respective cDNAs. Before use, all probes were alkali-hydrolyzed to an average length of 300 bp to enhance their penetration and specificity. The criterion used to define the presence of an RNA was the observation of more than three positive cell-like profiles per microscopy field (20 $\times$). Some animals were injected with sodium kainate (10 mg/kg, i.p.) or saline 48 hr before they were killed.

RESULTS

Two new cDNAs were cloned and expressed. Their properties are described in detail and compared with those of P2X₁–P2X₄. The RNA distribution of all six clones was determined by *in situ* hybridization.

P2X₆ receptor

The P2X₆ receptor cDNA (2243 bp) was isolated from a superior cervical ganglion cDNA library. The predicted receptor protein has 379 amino acids (Fig. 1A), and the overall hydrophobicity conforms to that of the other receptors (Fig. 1B). The putative

extracellular loop has the 10 conserved cysteines and 13 conserved glycines of receptors P2X₁–P2X₄.

Currents evoked by ATP in cells expressing P2X₆ receptors resembled those seen for the P2X₂ receptor (Table 1) (Evans et al., 1995). ATP and other effective agonists induced quickly rising currents that showed little desensitization during agonist applications of 0.5–2.0 sec (Fig. 2A,B; Table 1). The time to peak (measured from 10–90%) in response to supramaximal concentration of ATP (100 μ M) was 14 ± 2 msec ($n = 7$), which is approximately the limit of resolution of the application system (see Materials and Methods). ATP, 2-methylthio-ATP, 2-chloroATP, and ADP were full agonists, ATP-5'-O-(3-thiotriphosphate)(ATP- γ -S) was a partial agonist, and α meATP had no effect (Fig. 2C). The current reversed at 0.6 ± 0.7 mV ($n = 5$). Although the currents in cells expressing the P2X₆ receptor were large, ranging from 380 to 1800 pA in response to 30 μ M ATP ($n = 11$) (Fig. 2E), they were observed in only a small fraction of cells examined (Fig. 2F). A similarly low fraction of cells expressed a construct of the P2X₆ receptor in which the 5' untranslated region was reengineered to remove a guanosine- and cytosine-rich segment and insert an optimal Kozak translation initiation sequence.

Figure 6. Overlapping distribution of P2X₄ and P2X₆ RNAs. Staining shown in cingulate cortex (*A, B*); medial habenula (*C, D*); supraoptic nucleus (*E, F*); nucleus tractus solitarius (*nts*), dorsal motor nucleus vagus nerve (*X*), and hypoglossal nucleus (*XII*) (*G, H*). Signals were obtained with riboprobes for P2X₄ (*A, C, E, G*) and P2X₆ (*B, D, F, H*). Hybridization for both probes was localized in neuron-like cells of II, III, and V cortical layers (*A, B*), in small neuron-like profiles of medial habenula (*C, D*), in the large peptidergic neurons of supraoptic nucleus (*E, F*), in motoneurons of visceral (*X*) and somatic (*XII*) cranial nerves, and in neurons of *nts* and area postrema (*G, H*). Arrowhead indicates ependyma. See schematic of Figure 4*D*. Scale bars: *A–F*, 50 μ m; *G, H*, 100 μ m.

Currents evoked by ATP were unaffected by suramin (1–30 μ M) or pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (1–30 μ M), when the antagonists were present in the bath for up to 15 min (Fig. 3*A*). These concentrations and durations of application inhibit currents through P2X₁, P2X₂,

and P2X₃ receptors by 80–100% (Evans et al., 1995; Lewis et al., 1995; Buell et al., 1996). Longer applications (30–60 min) resulted in partial inhibition of currents (10 μ M PPADS, $59 \pm 4\%$, $n = 3$; 30 μ M suramin, $28 \pm 3\%$, $n = 3$). The inhibition of the current that did occur with high concentrations ($>30 \mu$ M)

Table 2. Brain distribution of four P2X receptor RNAs

	P2X ₂	P2X ₄	P2X ₅	P2X ₆
Olfactory bulb				
Mitral cells	-	++	-	++
Granular layer	-	+/-	-	+/-
Tufted cells	-	++	-	++
Anterior olfactory nucleus	-	+	-	++
Cerebral cortex				
Agranular insular cortex	-	++	-	++
Anterior cingulate cortex	+	+	-	++
Ventrolateral orbital cortex	-	++	-	++
Piriform cortex	+	++	-	++
Frontal cortex	+	++	-	++
Parietal cortex	-	+	-	++
Occipital cortex	-	+	-	++
Subcortical telencephalon				
Tenia tecta	-	++	-	++
Clastrum	-	++	-	+
Nucleus diagonal band	-	++	-	+
Bed nucleus stria terminalis	+	+/-	-	+
Medial septa nucleus	++	+	-	+
Lateral septal nucleus	-	+	-	+
Islands of Calleja ^a	-	+/-	-	++
Olfactory tubercle	+	++	-	++
Accumbens nucleus	-	+/-	-	+/-
Caudate/putamen	-	+/-	-	+
Globus pallidum	-	-	-	-
Substantia innominata	-	+	-	+
Entopeduncular nucleus	-	+/-	-	+/-
Lateral olfactory tract nucleus	-	+	-	++
Anterior cortical amygdaloid nucleus	-	+	-	++
Basolateral amygdaloid nucleus	-	++	-	+
Central amygdaloid nucleus	-	++	-	+
Medial amygdaloid nucleus	-	+	-	+
Amygdalohippocampal area	-	+	-	+
Hippocampal fields CA1-CA4	-	++	-	++
Dentate gyrus	-	++	-	++
Epithalamus				
Medial habenular nucleus	-	+	-	+
Lateral habenular nucleus ^a	-	-	-	+
Thalamus				
Anterior nuclei	++	+	-	++
Laterodorsal nucleus, ventrolateral division	+	+	-	+
Mediodorsal nucleus	-	+	-	+
Ventrolateral nucleus	-	+	-	+
Ventromedial nucleus	-	+	-	+
Reticular nucleus	-	++	-	++
Paraventricular nucleus, anterior part	++	++	-	++
Central medial nucleus	-	+	-	+
Xiphoid nucleus	-	++	-	+
Rhomboid nucleus	-	++	-	+
Lateral geniculate nucleus	-	+	-	+
Medial geniculate nucleus	-	+	-	+
Hypothalamus				
Suprachiasmatic nucleus	++	++	-	++
Medial preoptic nucleus	+	+	-	+
Medial preoptic area	-	++	-	+
Supraoptic nucleus	++	++	-	++
Paraventricular nucleus	+	+	-	+
Ventromedial nucleus	++	+	-	+
Arcuate nucleus	++	+	-	+
Tuberomammillary nucleus	-	++	-	++
Midbrain				
Zona incerta	-	+	-	+/-
Medial tuberal nucleus	-	+	-	+
Substantia nigra zona compacta	+	+	-	+
Substantia nigra zona reticulata	-	+	-	++
Supramammillary nucleus	-	++	-	+
Ventral tegmental area	+/-	+	-	+

Table 2. continued

	P2X ₂	P2X ₄	P2X ₅	P2X ₆
Medial terminal nucleus accessory optic tract	-	+	-	+
Oculomotor nucleus	+	++	-	++
Edinger-Westphal nucleus	-	+	-	+
Central gray	-	+	-	+
Superior colliculus, deep gray	-	+	-	++
Superior colliculus, superficial gray	-	+/-	-	+/-
Inferior colliculus	-	+/-	-	+
Interpeduncular nucleus	-	+	-	+
Trochlear nucleus	-	+	-	++
Pedunculopontine tegmental nucleus	-	+	-	+/-
Hindbrain				
Pontine nuclei	-	+	-	+
Raphe pontis nucleus	-	+	-	+
Raphe dorsalis	-	++	-	++
Mesencephalic trigeminal nucleus	-	++	++	+
Dorsomedial tegmental area	-	+	-	+
Locus ceruleus	++	+/-	-	+/-
Subceruleus nucleus	-	+	-	+
Barrington's nucleus	-	+	-	+
Pontine reticular nucleus, ventral part	-	++	-	+
Pontine reticular nucleus, caudal part	-	+	-	++
Red nucleus	+	+	-	+
Motor trigeminal nucleus	-	++	-	+
Superior olivary nucleus	-	+	-	++
Principal sensory trigeminal nucleus	-	+	-	++
Spinal trigeminal nucleus	-	+	-	+
Paragigantocellular nucleus	-	++	-	++
Abducens nucleus	-	++	-	++
Facial nucleus	-	++	-	++
Accessory facial nucleus	-	++	-	++
Ambiguous nucleus	-	++	-	+
Inferior olive	-	++	-	+
Prepositus hypoglossal nucleus	+	++	-	+
Dorsal motor vagus nucleus	++	+	-	+
Hypoglossal nucleus	+	++	-	++
Solitary tract nucleus	-	+	-	+
Lateral reticular nucleus	+	++	-	+
Cuneate fasciculus	-	-	-	+/-
Area postrema	++	+	-	+
Cerebellum				
Purkinje cells	-	+++	-	+++
Granular layer	-	+	-	+
Lateral cerebellar nucleus	-	++	-	++
Interposed cerebellar nucleus, anterior part	-	+	-	+
Cerebral white matter				
Corpus callosum	-	-	-	-
Optic nerve	-	-	-	-
Anterior commissure	-	-	-	-
Internal capsule	-	-	-	-
Ventricular structures				
Ependymal layer ^a	-	-	-	+++
Subfornical organ	-	+	-	+
Choroid plexus	-	-	-	-
Pineal gland ^a	-	++	-	+/-
Lamina I	-	+/-	-	+/-
Lamina II	++	++	-	++
Lamina III	-	-	-	-
Lamina IV	-	+	-	+
Lamina V	+	++	+	+
Lamina VI	+	+	+	+
Lamina VII	+	++	+	++
Lamina VIII	++	+	+	+
Lamina IX	++	++	+	++
Lamina X	+	+	-	+

Signal was scored as absent (-), just detectable (+/-), moderate (+), strong (++), or very strong (+++).

^aIndicates striking differences between P2X₄ and P2X₆.

was rapid and readily reversible with antagonist applications of <15 min, unlike the slow kinetics of PPADS inhibition at P2X₁, P2X₂ (Evans et al., 1995; Buell et al., 1996), and P2X₅ receptors (see below). Higher concentrations (>30 μ M) inhibit other ligand-gated currents nonselectively (Buell et al., 1996).

We have found previously that the lysine at position 246 in the P2X₂ receptor is critical for the slowly reversible component of the inhibition by low concentrations of PPADS (Buell et al., 1996). The P2X₆ receptor has leucine in the equivalent position (Fig. 1). When this was changed to lysine by site-directed mutagenesis (P2X₆[L251K]), PPADS (3–10 μ M) inhibited the ATP-induced currents with slowly reversible kinetics (Fig. 3*B,D*). Suramin remained ineffective at the P2X₆[L251K] receptor (Fig. 3*B*). This mutated receptor also expressed at a low rate (0–12% of cells from five transfections).

P2X₆ receptor RNA was expressed heavily in the CNS (Figs. 4–7; Table 1). The most dense staining was in cerebellar Purkinje cells (Fig. 5*I*) and the ependyma (Fig. 6; Table 2), but other areas with strong hybridization included mitral and tufted cells of the olfactory bulb, the cortex (Fig. 6*B*), all hippocampal fields (Figs. 4*C*, 7), the dentate gyrus, some hypothalamic (Fig. 6*F*) and thalamic nuclei, the mesencephalic nucleus of the trigeminal nerve (Fig. 5*G*), and the cranial nerve motor nuclei (Fig. 6*H*; Table 2). Weak hybridization was observed in the striatum, and no signal was detected in globus pallidus or any white matter. There was a marked loss of hybridizing signal in the CA3 pyramidal cells and CA4 mossy cells in rats that had been treated 48 hr before with kainic acid (Fig. 7), implying a neuronal rather than a glial localization.

In situ hybridization on coronal sections of rat cervical spinal cord (C4–C7 levels) showed RNA in several laminae (Fig. 8; Table 2). The highest expression was in the spinal motoneurons of lamina IX, and the superficial dorsal horn neurons of lamina II were stained more heavily than adjacent laminae I and III. Less intense signals were observed in large and medium-sized neurons of laminae IV, V, VII, and VIII. The lateral spinal nucleus was stained. The P2X₆ receptor RNA was observed in trigeminal and dorsal root ganglia and celiac ganglia. Outside the nervous system,

P2X₆ receptor RNA was prominent in gland cells of the uterus, granulosa cells of the ovary, and bronchial epithelia, but it was absent from salivary epithelia, adrenal medulla, and bladder smooth muscle (Table 3).

P2X₅ receptor

The cDNA (2436 bp) was isolated from a library prepared from rat celiac ganglia. The predicted protein (417 amino acids) has the overall pattern of hydrophobicity and the conserved cysteine and glycine residues of the other members of the family (Fig. 1). ATP-induced currents in HEK cells transfected with P2X₅ receptors had all the same properties described above for the P2X₆ receptor, with two important differences. The similarities were rapid activation, minimal desensitization, reversal at -1 ± 1.8 mV ($n = 9$), and lack of effect of $\alpha\beta$ meATP (Fig. 2). The first difference was that the currents were inhibited readily by suramin and PPADS (1–30 μ M). The inhibition by PPADS was similar to that observed for P2X₁ and P2X₂ receptors; it developed slowly (5–10 min) and was only partially reversible, even with washing for 25 min (Fig. 3). Suramin and PPADS inhibited currents evoked by ATP or 2MeSATP to the same extent. Suramin (3 and 30 μ M) inhibited ATP-evoked currents by $42 \pm 3\%$ and $94 \pm 3\%$ (Fig. 3*D*) and 2MeSATP-evoked currents by $46 \pm 4\%$ and $93 \pm 5\%$ ($n = 4$), respectively. PPADS (10 μ M) inhibited ATP-evoked currents by $81 \pm 3\%$ (Fig. 3*D*) and 2MeSATP-evoked currents by $84 \pm 4\%$ ($n = 4$). The second difference was that the peak currents observed in HEK cells expressing P2X₅ receptors were always small: 5–10% of those observed with any of the other subtypes expressed under similar conditions (Figs. 2, 3) (Evans et al., 1995; Lewis et al., 1995; Buell et al., 1996).

In situ hybridization provided no evidence for P2X₅ RNA in the brain, with the single exception of the mesencephalic nucleus of the trigeminal nerve (Fig. 5*D*; Table 2). These “displaced” cell bodies of primary afferent neurons, serving proprioception from muscles of mastication, are situated immediately lateral to the locus ceruleus. The cervical spinal cord contained P2X₅ RNA in the motoneurons of the ventral horn. Large pseudounipolar neu-

Figure 7. Kainate acid lesion indicates that P2X₆ RNA expression in hippocampus is largely neuronal. *A* and *B* show the hippocampal hilus; *C* and *D* show the CA3 region. Reduced number of cell profiles and reduced staining intensity was seen clearly after kainic acid administration, both among the mossy cells of CA4 field (*B*, arrowhead) and the pyramidal cells of CA3 field (*D*, arrowhead). Scale bar (shown in *A*): 50 μ m.

Figure 8. Rat cervical spinal cord. Spinal cord has all RNAs except P2X₃. High-magnification photomicrographs of spinal motoneurons using probes for P2X₁ (A), P2X₂ (B), P2X₄ (C), P2X₅ (D), P2X₃ (E), and P2X₆ (F) are shown. Hybridization signals for P2X₅ and P2X₄ in the dorsal horn are shown in H and G, respectively. I, Distribution of P2X receptor RNAs through laminae I–X. Different symbols represent different P2X receptors. P2X₁ and P2X₅ RNAs (blue) were localized prevalently in the central and ventral gray matter, whereas P2X₂ RNA (yellow) was also expressed in lamina II. Hybridization signals for P2X₄ and P2X₆ (red) were found in neurons throughout the spinal cord, with stronger signals in laminae II, VII, and IX. Large symbols indicate that P2X RNA was detected in large multipolar neurons; small symbols represent detection in medium-size neurons. Scale bars: A–F, 25 μ m; G, H, 100 μ m.

rons in the trigeminal and dorsal root ganglia also stained for P2X₅ RNA.

P2X₄ receptor

The isolation of this cDNA was reported by Bo et al. (1995), Buell et al. (1996), Seguela et al. (1996), and Soto et al. (in press). The basic electrophysiological properties are summarized for comparison in Table 1. P2X₄ receptor RNA was also expressed abundantly in the CNS, with the pattern of staining closely mirroring that described above for the P2X₆ receptor RNA (Figs. 4–8; Table 2). The most notable difference was that P2X₄ RNA was not observed in the ependyma: the lateral habenula and islands of Cajalla were stained less heavily with P2X₄ than with P2X₆, whereas the pineal gland showed the converse. As for P2X₆ RNA, the intensity of hybridization of P2X₄ RNA was much reduced in hippocampal neurons 48 hr after kainate acid administration.

At the level of the spinal cord, P2X₄ receptor RNA also overlapped almost completely with P2X₆ RNA, being highest in ventral horn motoneurons, lamina II, and lateral spinal nucleus, and less intense in laminae IV, V, VII, and VIII. As for P2X₆ RNA, P2X₄ hybridization was observed in uterine gland cells, ovarian granulosa cells, bronchial epithelia, and testis, and it was not seen in bladder smooth muscle or adrenal gland (Table 3).

Only P2X₄ RNA was expressed by acinar cells of the salivary glands (Buell et al., 1996).

P2X₃ receptor

The P2X₃ receptor was cloned from a dorsal root ganglion cDNA library (Chen et al., 1995; Lewis et al., 1995); the functional properties are summarized in Table 1. In the present study, we sought P2X₃ RNA receptor in brain, spinal cord, and peripheral tissues, but found it only in sensory neurons of trigeminal, dorsal root, and nodose ganglia.

P2X₂ receptor

This cDNA was isolated by Brake et al. (1994) from a library of PC12 cells differentiated with nerve growth factor; the properties of the expressed receptor (Brake et al., 1994; Evans et al., 1995) are summarized in Table 1. The RNA was present in brain but in a much more restricted set of neurons than seen with the P2X₆ and P2X₄ riboprobes (Table 2). Positive cells were observed in the thalamus (paraventricular and anterior nuclear groups), in the arcuate and ventromedial hypothalamic nuclei of the hypothalamus, and in the preoptic area. RNA was also present in some cells of the red nucleus, oculomotor nucleus, locus ceruleus, and dorsal motor nucleus of the vagus, and staining was detectable but weak

Table 3. Summary of distribution of six P2X receptor RNAs

	P2X ₁	P2X ₂	P2X ₃	P2X ₄	P2X ₅	P2X ₆
Brain ^a	–	+	–	+	–	+
Spinal cord	+	+	–	+	+	+
Sensory ganglia ^b	+	+	+	+	+	+
Superior cervical ganglion	+	+	–	+	–	+
Salivary gland ^c	–	–	–	+	–	–
Bronchial epithelium	–	–	–	+	–	+
Adrenal medulla	–	+	–	–	–	–
Smooth muscle ^d	+	–	–	–	–	–

^aP2X₅ is found in brain only in mesencephalic nucleus of the trigeminal nerve.

^bIncludes dorsal root ganglia, trigeminal ganglia, and nodose ganglia.

^cAcinar cells only.

^dVas deferens, bladder, some peripheral arteries.

in some cortical areas (piriform, cingulate, perirhinal), substantia nigra, prepositus hypoglossi, and the area postrema. There was no signal in hippocampus and cerebellum. P2X₂ RNA was present in the large motoneurons of lamina IX as well as the small neurons of lamina II and the medium-sized neurons of laminae V–VIII. P2X₂ receptor RNA was the only RNA observed in the adrenal medulla (Table 3).

P2X₁ receptor

The receptor cDNA was originally isolated from vas deferens (Valera et al., 1994); the functional properties are summarized in Table 1. The present experiments failed to detect RNA in adult rat brain or the major arteries at the base of the brain, although it was observed clearly within cells of the cervical spinal cord where its distribution matched that of the P2X₅ RNA. The RNA was found in dorsal root ganglia, trigeminal ganglia, and celiac ganglia. It was the only RNA visualized in smooth muscle of the bladder; staining was not seen in the adrenal medulla (Table 3).

DISCUSSION

A family of related channel proteins

A comparison of the six amino acid sequences (Fig. 1A) shows that P2X receptor subunits form a homologous group of proteins. Their overall relatedness (36–48% identical) is similar to that seen among the four NR2 subunits of the NMDA receptor, for example. All have a similar pattern of hydrophobicity (Fig. 1C) as well as a conserved predicted secondary structure. The large loop that connects the two hydrophobic domains is thought to be extracellular for two reasons. First, the P2X₁ receptor translated *in vitro* has a MW of 45 kDa in the absence of microsomes and 60 kDa when the translation is carried out in the presence of microsomes (Valera et al., 1995). The mutated receptor P2X₁[N184S] migrates at 45 kDa with or without microsomes, implying that residue 184 is glycosylated and therefore presumably extracellular (G. Buell, unpublished observations). Second, the lysine residue involved in the slowly reversible component of block by PPADS (K249 in P2X₁, Fig. 1) (Buell et al., 1996) must be extracellular. This overall topology is shared with the amiloride-sensitive sodium channels (ENaC) (Canessa et al., 1994), which also have fully conserved cysteine residues (16 in the case of α , β , and γ ENaC, 10 for the P2X receptors) but lack primary sequence homology with any of the P2X receptors. It is noted that the presumed extracellular loop of the P2X receptors has 7 fully conserved lysine residues and 13 conserved glycine residues. Ly-

sine and glycine residues are critical residues in most nucleotide-binding proteins (Koonin, 1993; Traut, 1994). By contrast, the longer loops of the ENaC channels have no conserved lysines and only six conserved glycine residues.

The conservation among P2X receptors is highest immediately preceding the second hydrophobic domain, and this region might contribute to the ionic pore (Brake et al., 1994; Valera et al., 1994). The eight amino acid sequence found here in the P2X₁ receptor that resembles a highly conserved sequence found in potassium channels pores (Valera et al., 1994), however, is not present in the other P2X receptors (Fig. 1A). Although the second hydrophobic domain does not show a high level of absolute conservation, helical wheel plots indicate that in all six receptors there is a row of polar or small amino acids along one face of the helix; this could also line the conducting pore.

Functional expression

A comparison of the properties of all six receptors provides the basis for classifying them into three groups (Table 1). P2X₁ and P2X₃ form one group, by virtue of their very rapid desensitization and sensitivity to $\alpha\beta$ meATP; they are also ~10-fold more sensitive to ATP than the other receptors are (see Surprenant et al., 1995). The other four receptors can be divided into two additional groups. These are P2X₂ and P2X₅ receptors, which are sensitive to the antagonists suramin and PPADS, and P2X₄ and P2X₆ receptors, which are not. These groups do not correlate as yet with any obvious pattern of sequence relatedness, except that the irreversible block by PPADS requires lysine at position 249 (of P2X₁) (Fig. 1A).

The expression of the P2X₅ (small currents) and P2X₆ receptors (small fraction of cells expressing currents) was poor in comparison with the first four members of the family. There is no obvious technical reason for this difference, and it may indicate that they do not readily form homomeric channels in native tissues. Such an interpretation would be consistent with the overlap in the distribution of P2X₄ and P2X₆ RNAs, but additional experiments will be required to test it.

Tissue distribution of RNAs

The distribution of the six receptor RNAs ranges from almost completely overlapping to discrete (Tables 2, 3). The most marked overlap is seen in the sensory ganglia; cells of the dorsal root, trigeminal, and nodose ganglia expressed multiple RNAs. P2X responses were first described in dorsal root ganglion cells (Jahr and Jessel, 1983; Krishtal et al., 1983). The

overlap was apparent also for the cell bodies of proprioceptive afferents in the mesencephalic nucleus of the trigeminal nerve, which express P2X₄, P2X₅, and P2X₆ receptors. These cells are excited by ATP (Salt and Hill, 1983). Both the high percentage of positive cells and the staining observed with different probes on adjacent sections indicate that many of the same neurons express multiple RNAs. The responses to ATP of neurons in the nodose ganglion do not correspond well to the currently known functional categories (Table 3), and it has been suggested that they result from heteropolymeric channel formation between P2X₂ and P2X₃ subunits (Lewis et al., 1995). Clearly, the coexpression of other RNAs raises other possibilities for heteropolymerization, and the precise assignment of P2X receptor subunit expression to primary afferent neurons of known functional modality will be important to our understanding of their role in sensory physiology.

A strongly overlapping distribution was observed also in the spinal cord, which contains all RNAs except that of the P2X₃ receptor. Spinal motoneurons expressed five RNAs, although staining was consistently weaker with P2X₁ and P2X₅ riboprobes. The strong staining of dorsal horn neurons, particularly lamina II, with P2X₂, P2X₄ and P2X₆ riboprobes may be correlated with the observations that ATP opens a ligand-gated ion channel in dissociated rat dorsal horn cells (Jahr and Jessell, 1983). It is consistent with a role for ATP in primary afferent transmission.

The overlap in the localization of the two predominant central forms of the P2X receptor (P2X₆ and P2X₄) is a striking finding of the present work (Table 2). The pattern of staining was coincident in more than 100 discrete brain regions, implying that these subunits are expressed by the same neurons. For example, all neurons in adjacent sections of the supraoptic nucleus were stained with both riboprobes. It has been suggested that ATP mediates a synaptic input to supraoptic neurons (Day et al., 1993). The excitation by ATP, however, was mimicked by $\alpha\beta$ meATP and blocked by suramin, and neither the P2X₄ nor the P2X₆ receptor corresponds in these properties (Table 1). Other regions of the CNS in which functional responses have been attributed to activation of P2X receptors also express P2X₄ and P2X₆ RNA, but in these cases also (medial habenula, Edwards et al., 1992; nucleus tractus solitarius, Ueno et al., 1992; tuberomammillary nucleus, Furukawa et al., 1994), the simple coexpression of P2X₄ and P2X₆ seems unlikely to account for the functional responses observed. The most obvious difference in each case is that the effects of ATP are reported to be blocked by the antagonist suramin. The present work shows that when expressed as homopolymers, the P2X₄ and P2X₆ receptors are most notable for their insensitivity to suramin. P2X₂ receptors are sensitive to suramin and PPADS, but this RNA is found at much lower abundance in the brain.

The few differences that do occur in the distributions of P2X₄ and P2X₆ RNAs are both quantitative and qualitative. A stronger hybridization signal for P2X₆ than for P2X₄ was found in the islands of Calleja and the lateral habenula, whereas the P2X₄ hybridization signal was stronger than the P2X₆ signal in the pineal gland. The ependymocyte showed intense expression of P2X₆ receptor RNA, but not P2X₄; the ependymal layer was stained throughout the ventricular cavities of the brain, including the central canal of the spinal cord. The disparity in distribution of these two RNAs was greatest in non-neuronal tissues. For example, P2X₄ RNA was found in the acinar cells

of the salivary gland in which P2X₆ RNA is absent (Table 3) (Buell et al., 1996).

The six P2X receptors are a homologous family of mammalian channel proteins that form a third structural class distinct from the nicotinic and glutamate families (North, 1996). Their RNAs are distributed extensively in neurons of the brain (particularly P2X₆ and P2X₄), spinal cord (all except P2X₃), and sensory ganglia (all six). The functional properties of the six (homomeric) receptors correspond imprecisely with responses in some neurons. This suggests that the family identified currently may be destined for further growth.

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