

Coexpression of P2X₃ and P2X₂ Receptor Subunits in Varying Amounts Generates Heterogeneous Populations of P2X Receptors That Evoke a Spectrum of Agonist Responses Comparable to That Seen in Sensory Neurons

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ABSTRACT

Using voltage-clamp procedures on *Xenopus* oocytes, agonist-evoked ionic currents by P2X receptors resulting from the coexpression of P2X₂ and P2X₃ subunits were compared against the agonist responses of homomeric P2X₂ and P2X₃ receptors. With the quantity of P2X₃ mRNA kept constant and quantity of P2X₂ mRNA progressively increased, expressed P2X receptors changed from a P2X₃-like receptor to a P2X₂-like receptor. In all cases, however, agonist-evoked responses comprised biphasic (fast and slow) currents—the former showing the properties of P2X₃ receptors and latter consistent with the presence of P2X₂ and P2X_{2/3} receptors. Using desensitization procedures, the P2X₃-like fast current was selectively removed to allow the slow current to be studied in isolation. P2X_{2/3} receptors were then characterized by slowly inactivating inward currents that were reproducible within 30 s of washout and whose pharma-

logical profile [*selective agonists*, Ap₅A > α,β -methylene ATP >> β,γ -methylene ATP > UTP; *antagonists*, TNP-ATP >> suramin \geq Reactive blue-2 (RB-2)] contrasted with the profile of P2X₂ receptors (Ap₅A, α,β -methylene ATP, β,γ -methylene ATP, and UTP inactive; *antagonists*, RB-2 > TNP-ATP > suramin). Thus, our experiments reveal that coexpression of two P2X subunits, which of themselves can generate functional homomeric receptors, results in a complex population of heterogeneous P2X receptors—in this case P2X₂, P2X₃, and P2X_{2/3} receptors. Depending on the relative levels of P2X subunit coexpression, the operational profile of the resultant P2X receptors can change from one phenotype to another. This spectrum may explain the variability of agonist responses in small sensory neurons that also express P2X₂ and P2X₃ subunits in different amounts.

P2X receptors represent a family of ATP-gated ion channels that play a significant role in fast excitation and synaptic transmission in many excitable tissues (Ralevic and Burnstock, 1998). As far as sensory neurons are concerned, P2X receptors have been implicated in the direct excitation of primary afferent nerve fibers (Bland-Ward and Humphrey, 1997; Cook et al., 1997; Dowd et al., 1998; Burgard et al., 1999; Hamilton and McMahon, 2000), in ascending excitation of second order sensory neurons in the dorsal horn of the spinal cord and dorsomedial nuclei of the brainstem (Li and Perl, 1995; Bardoni et al., 1997; Gu and MacDermott, 1997; Scislo et al., 1997; Li et al., 1998; Tsuda et al., 1999)

and in the excitation of higher order neurons in the central nervous system (Dreissen et al., 1998; Ralevic et al., 1999).

Thus far, cDNA sequences for seven P2X subunit proteins (P2X₁₋₇) have been cloned from mammalian tissues. These P2X subunit proteins can combine to form ion channels, as homomeric and heteromeric assemblies of three, possibly four, P2X subunits (Kim et al., 1997; Nicke et al., 1998; Torres et al., 1999). Transcripts for six subunits (P2X₁₋₆) have been found in sensory neurons (Chen et al., 1995; Collo et al., 1996; Cook et al., 1997). However, immunohistochemical studies reveal a predominance of P2X₃-like protein in the cell bodies of sensory ganglia and associated sensory nerve endings in skin and lamina II of the spinal cord. P2X₃-like immunoreactivity frequently colocalizes with P2X₂-like immunoreactivity in sensory neurons (Cook et al., 1997; Vulchanova et al., 1997; Bradbury et al., 1998; Xiang et al., 1998).

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ABBREVIATIONS: DRG, dorsal root ganglion; ATP γ S, adenosine-5'-O-(3-thio)triphosphate; Ap₅A, P¹,P⁵-diadenosine pentaphosphate; cRNA, capped ribonucleic acid; Ip₅I, diinosine pentaphosphate; α,β -meATP, α,β -methylene ATP; β,γ -meATP, β,γ -methylene ATP; 2-MeSATP, 2-methylthioATP; RB-2, Reactive blue 2; PPADS, pyridoxal 5-phosphate 6-azophenyl-2',4'-disulfonic acid; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)-ATP; V_h, holding potential; C/R, concentration/response curve.

Small sensory DRG neurons containing P2X₃-immunopositive material also contain specific biochemical markers for nociceptor cells (Chen et al., 1995; Bradbury et al., 1998).

It is well known that sensory neurons show a marked variability in the time course of responses to P2X receptor agonists. Agonist-evoked ion currents in many rat DRG and trigeminal ganglion neurons are known to activate and inactivate rapidly, in a manner similar to agonist responses of homomeric P2X₃ receptors (Chen et al., 1995; Cook et al., 1997; Rae et al., 1998). Such fast agonist responses are absent in sensory neurons of P2X₃-null (-/-) animals (Cockayne et al., 2000). Rat nodose ganglia and bullfrog DRG neurons often respond to P2X agonists with slowly inactivating ionic currents that are similar to the responses of heteromeric P2X_{2/3} receptors (Bean, 1990; Khakh et al., 1995; Lewis et al., 1995). Furthermore, some DRG neurons produce composite responses with both rapidly and slowly decaying ion currents (Burgard et al., 1999; Grubb and Evans, 1999).

These differences in the operational profile of native P2X receptors may be due to different levels of expression of P2X₂, P2X₃, and P2X_{2/3} receptors in individual sensory neurons (Burgard et al., 1999). Here, we have investigated the operational profiles of the recombinant P2X receptors generated by coexpressing varying amounts of P2X₂ and P2X₃ subunits in *Xenopus* oocytes. The resultant spectrum of operational profiles was attributed to a heterogeneous mixture of homomeric and heteromeric P2X receptors, and this mixture might conveniently explain the range of operational profiles of sensory neurons that also express P2X₂ and P2X₃ receptor subunits in varying amounts.

Materials and Methods

Preparation of Oocytes and Expression of Recombinant P2X Receptors. *Xenopus laevis* were anesthetized with Tricaine (0.2%, w/v) and killed by decapitation. The preparation of defolliculated *Xenopus* oocytes has been described previously (King et al., 1997). Defolliculated oocytes (stages V and VI) were injected (40 nl) cytosolically with capped ribonucleic acid (cRNA) encoding either rat P2X₂ or rat P2X₃ receptor subunits. In coexpression experiments, oocytes were injected with a mixture of cRNAs prepared at four concentration ratios (1:500, 1:100, 1:50, and 1:20), by mixing P2X₂ cRNA (2, 10, 20, and 50 $\mu\text{g ml}^{-1}$) with an equal volume of P2X₃ cRNA (1 mg ml^{-1}). Injected oocytes were incubated at 18°C in Barth's solution (pH 7.5) containing (mM): NaCl 110, KCl 1, NaHCO₃ 2.4, Tris-HCl 7.5, Ca(NO₃)₂ 0.33, CaCl₂ 0.41, MgSO₄ 0.82, supplemented with gentamycin sulfate 50 $\mu\text{g l}^{-1}$ for 48 h to allow full receptor expression and then stored at 4°C for up to 12 days.

Solutions and Electrical Recording of cRNA-Injected Oocytes. Nucleotide-evoked membrane currents were recorded from cRNA-injected oocytes studied under voltage-clamp conditions using a twin-electrode amplifier (Axoclamp 2B, Foster City, CA). Intracellular microelectrodes had a resistance of 1 to 2 M Ω when filled with 3 M KCl. Oocytes were perfused constantly (at 5 ml min^{-1}) with Ringer's solution containing (mM): NaCl 110, KCl 2.5, HEPES 5, BaCl₂ 1.8, pH 7.4 to 7.5. All recordings were made at room temperature (18°C) at a holding potential of -50 mV (unless stated otherwise). Electrophysiological data were recorded on a chart recorder (Gould 2200S, Ilford, UK).

Solutions were delivered by gravity flow from independent reservoirs placed above the recording chamber. Applications of agonists were separated by a 20-min interval, unless otherwise stated. The P2 receptor antagonists TNP-ATP (0.003–1 μM), suramin (0.01–100 μM), PPADS (0.3–100 μM), Reactive blue 2 (RB-2; 0.03–100 μM), and diinosine pentaphosphate (Ip₅I; 0.01–100 μM) were applied for 1

min before, and during, agonist application. When constructing concentration-response curves for P2X agonists, data were normalized to the agonist response evoked by 100 μM α,β -meATP. EC₅₀ values for P2X agonists were taken from Hill plots, where the transform $\log(I/I_{\text{max}} - I)$ was used (I being the current evoked by each concentration of agonist). The Hill coefficient was taken from the slope of Hill plots. Concentration-response curves and inhibition curves were fitted by nonlinear regression analysis using commercial software (Prism v2.0, GraphPad, San Diego, CA). Data are presented as mean \pm S.E. for the given number of observations (n). The Student's unpaired t test was used and p values ≤ 0.05 were considered significant.

Compounds and Salts Used. All common salts were AnalaR grade (Aldrich Chemicals, Gillingham, UK). Adenosine 5'-triphosphate disodium salt (ATP) was purchased from Boehringer (Mannheim, Germany). Adenosine-5'-*O*-(3-trio)triphosphate (ATP γ S), P¹,P⁶-diadenosine pentaphosphate (Ap₅A), α,β -methylene ATP (α,β -meATP), β,γ -methylene ATP (β,γ -meATP), uridine 5'-triphosphate (UTP), and Tricaine (3-aminobenzoic acid ethyl ester) were purchased from Sigma Chemical Co. (Poole, UK), pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) from RBI-Sigma (Natick, NJ) and 2',3'-*O*-trinitrophenyl-ATP (TNP-ATP) from Molecular Probes (Cambridge, UK). Suramin was a gift from Bayer plc (Newbury, UK) and Ip₅I a gift from Dr. Jesus Pintor (University of Complutense, Madrid, Spain). Solutions of agonists and antagonists were prepared daily from a stock solution (10 or 100 mM, stored frozen) made up in extracellular bathing solution.

Results

Three groups of cRNA-injected defolliculated oocytes were tested. The first and second groups comprised oocytes injected with P2X₂ or P2X₃ cRNAs, respectively. The third group of oocytes was injected with a fixed amount of P2X₃ cRNA and varying amounts of P2X₂ cRNA. The first and second groups behaved as previously observed for homomeric P2X₂ and P2X₃ receptors (King, 1998). The third group of oocytes showed a spectrum of operational profiles that ranged from predominantly P2X₃-like to predominantly P2X₂-like.

Use of α,β -meATP to Distinguish Types of P2X Receptors. α,β -meATP reliably distinguishes homomeric P2X₂ receptors from homomeric P2X₃ receptors (Fig. 1A). α,β -meATP (100 μM) did not activate any detectable current at P2X₂ receptors, whereas a rapidly activating and rapidly inactivating current was evoked at P2X₃ receptors. Sham-injected control oocytes did not produce any detectable responses to α,β -meATP (100 μM).

Application of α,β -meATP to oocytes coinjected with mixed cRNAs gave rise to biphasic responses comprising an initial rapidly activating and rapidly inactivating current (I_1) followed by a slowly rising, slowly inactivating component (I_2). The relative amplitudes of fast (I_1) and slow (I_2) currents varied with the concentration ratio of the P2X cRNAs injected (Fig. 1B). As the amount of P2X₂ cRNA was elevated (from a ratio of 1:500–1:20), there was a steady increase in the relative fraction of the slowly inactivating component (I_2) at the expense of the rapidly inactivating component (I_1), which was progressively incorporated into, and ultimately obscured by, the slower event.

Some homomeric P2X receptor ion channels possess binary permeability properties, which can result in biphasic ion currents under certain conditions (Khakh et al., 1999). Other heteromeric P2X receptors (P2X_{1/5} and P2X_{2/6}) also produce

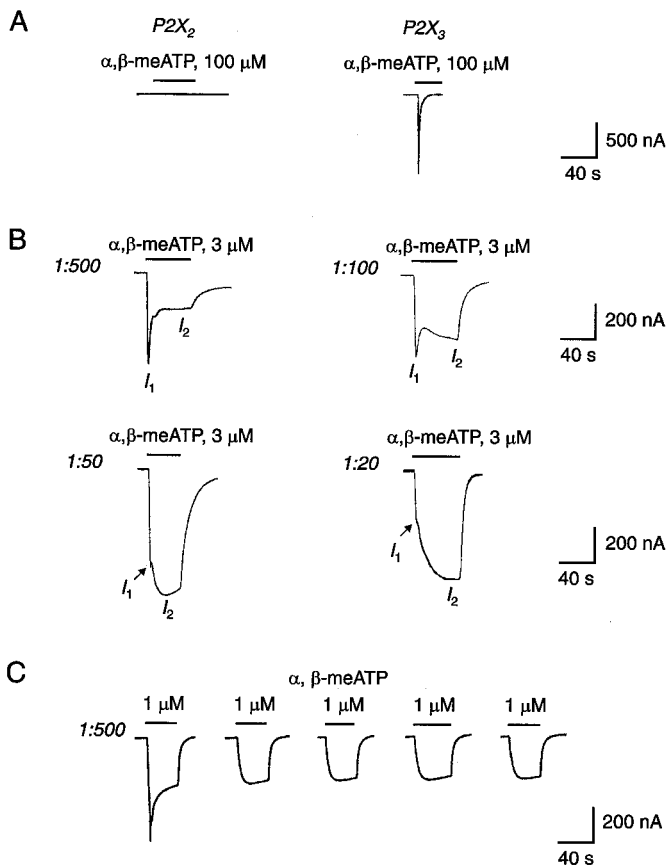


Fig. 1. A, α,β -meATP ($100\ \mu\text{M}$) evoked whole-cell inward currents at homomeric P2X_3 receptors but not homomeric P2X_2 receptors. B, whole-cell currents evoked by α,β -meATP ($3\ \mu\text{M}$) in oocytes injected with P2X_2 and P2X_3 cRNAs (at concentration ratios of 1:500, 1:100, 1:50, and 1:20). Each recording consisted of fast (I_1) and slow (I_2) inward currents. C, consecutive applications of α,β -meATP ($1\ \mu\text{M}$, applied 40 s apart) evoked whole-cell inward currents where the initial fast current became desensitized after the first application of the agonist, whereas the amplitude of slow current was unchanged. A through C, $V_h = -50\ \text{mV}$.

biphasic ion currents that involve complex kinetics of channel inactivation (Haines et al., 1999; King et al., 2000). However, the fast (I_1) and slow (I_2) components of α,β -meATP-evoked currents appeared to be mediated by two different sets of P2X receptors, since the former could be selectively desensitized by repetitive applications of α,β -meATP (40 s apart) without any significant change in the latter component (Fig. 1C). This desensitization procedure was used later to study the slow current in isolation. Homomeric P2X_3 receptors also were found to desensitize fully with repeated applications of α,β -meATP (40 s apart) (data not shown).

Potency and Efficacy of α,β -meATP at P2X Receptors. Concentration/response (C/R) curves for the fast current (I_1) evoked by α,β -meATP were reliably determined for those oocytes injected with mixed cRNAs ratios of 1:500 and 1:100 (Fig. 2A). Here, EC_{50} values (and Hill coefficients) for agonism were $1.7 \pm 0.3\ \mu\text{M}$ (0.8 ± 0.1 ; $n = 7$) and $1.6 \pm 0.4\ \mu\text{M}$ (0.8 ± 0.2 ; $n = 5$), respectively. For oocytes injected with mixed cRNAs ratios of 1:50 and 1:20, the amplitude of the fast current to α,β -meATP could not be easily resolved from the slow current (I_2) over the full range of agonist concentrations used and, accordingly, it was not possible to determine EC_{50} values here. In contrast, α,β -meATP yielded an EC_{50}

value of $1.9 \pm 0.3\ \mu\text{M}$ (0.8 ± 0.1 ; $n = 6$) at homomeric P2X_3 receptors (Fig. 2A).

Slow currents (I_2) evoked by α,β -meATP were more easily monitored in oocytes injected with each of the four ratios of mixed P2X cRNAs. Here, EC_{50} values (and Hill coefficients) for agonism were: $8.6 \pm 1.2\ \mu\text{M}$ (0.9 ± 0.1 ; $n = 7$) at 1:500 ratio; $9.5 \pm 1.0\ \mu\text{M}$ (0.8 ± 0.1 ; $n = 6$) at 1:100 ratio; $10.2 \pm 1.1\ \mu\text{M}$ (1.0 ± 0.1 ; $n = 3$) at 1:50 ratio; and $10.3 \pm 1.1\ \mu\text{M}$ (0.8 ± 0.1 , $n = 3$) at 1:20 ratio (Fig. 2B). None of the EC_{50} values for slow responses were significantly different for each mixture of cRNAs used, but were 5-fold higher than corresponding EC_{50} values for the fast response. This difference again supports the notion that two P2X receptors mediated the fast and slow responses to α,β -meATP—most likely homomeric P2X_3 and heteromeric $\text{P2X}_{2/3}$ receptors, respectively. Under the experimental conditions used, homomeric P2X_3 did not give rise to slow responses to α,β -meATP, whereas homomeric P2X_2 receptors were not activated by this synthetic nucleotide (see Fig. 1A).

The above data revealed that EC_{50} values for either fast or slow currents to α,β -meATP are unaffected by altering the mixture of injected P2X cRNAs. However, closer inspection of the amplitude of fast and slow currents revealed a marked difference in agonist efficacy. For a test concentration of $3\ \mu\text{M}$ α,β -meATP (applied at a constant holding potential of $-50\ \text{mV}$), the mean amplitude of the fast current (I_1) was approximately 800 nA when a small amount of P2X_2 cRNA was injected into oocytes (1:500 ratio), and was significantly lower, approximately 250 nA, when more P2X_2 cRNA was injected (1:20 ratio) (Fig. 2C). The converse occurred for the amplitude of the slow current (I_2) (Fig. 2C). Since the amount of injected P2X_3 cRNA was kept constant in these experiments and only the amount of P2X_2 cRNA was changed, the observed differences in α,β -meATP efficacy appeared to involve a decrease in the number of fast-activating P2X_3 ion channels and concomitant increase in the number of slowly activating $\text{P2X}_{2/3}$ ion channels, presumably as more P2X_2 subunits competed for P2X_3 subunits to generate heteromeric $\text{P2X}_{2/3}$ receptors.

Slow Currents to ATP and α,β -meATP Involve Different P2X Receptors. ATP and other nucleotides were tested on oocytes coinjected with mixed cRNAs (1:500 ratio), then tested again on homomeric P2X_2 receptors. EC_{50} values for these nucleotides at different P2X receptors are given in Table 1. ATP, 2-methylthioATP (2-MeSATP), and $\text{ATP}\gamma\text{S}$ evoked slow currents at homomeric P2X_2 receptors and produced slow ion currents in oocytes coexpressing P2X_2 and P2X_3 subunits. The potency of ATP and 2-MeSATP, but not $\text{ATP}\gamma\text{S}$, was lower at homomeric P2X_2 receptors than at the P2X receptor population formed by P2X_2 and P2X_3 subunit coexpression. Furthermore, four nucleotides (α,β -meATP, β,γ -meATP, Ap_5A , and UTP) were inactive at homomeric P2X_2 receptors but were agonists at presumptive $\text{P2X}_{2/3}$ receptors (Table 1).

Inspection of C/R curves for the above nucleotides showed that ATP, 2-MeSATP, and $\text{ATP}\gamma\text{S}$ had an apparent greater efficacy than α,β -meATP at presumptive heteromeric $\text{P2X}_{2/3}$ receptors. In contrast, β,γ -meATP, Ap_5A , and UTP showed similar or lower efficacy than α,β -meATP (Fig. 3, A and B). Thus, only those nucleotides able to stimulate homomeric P2X_2 receptors seemed to have a greater efficacy at heteromeric $\text{P2X}_{2/3}$ receptors. Otherwise, nucleotides that did not

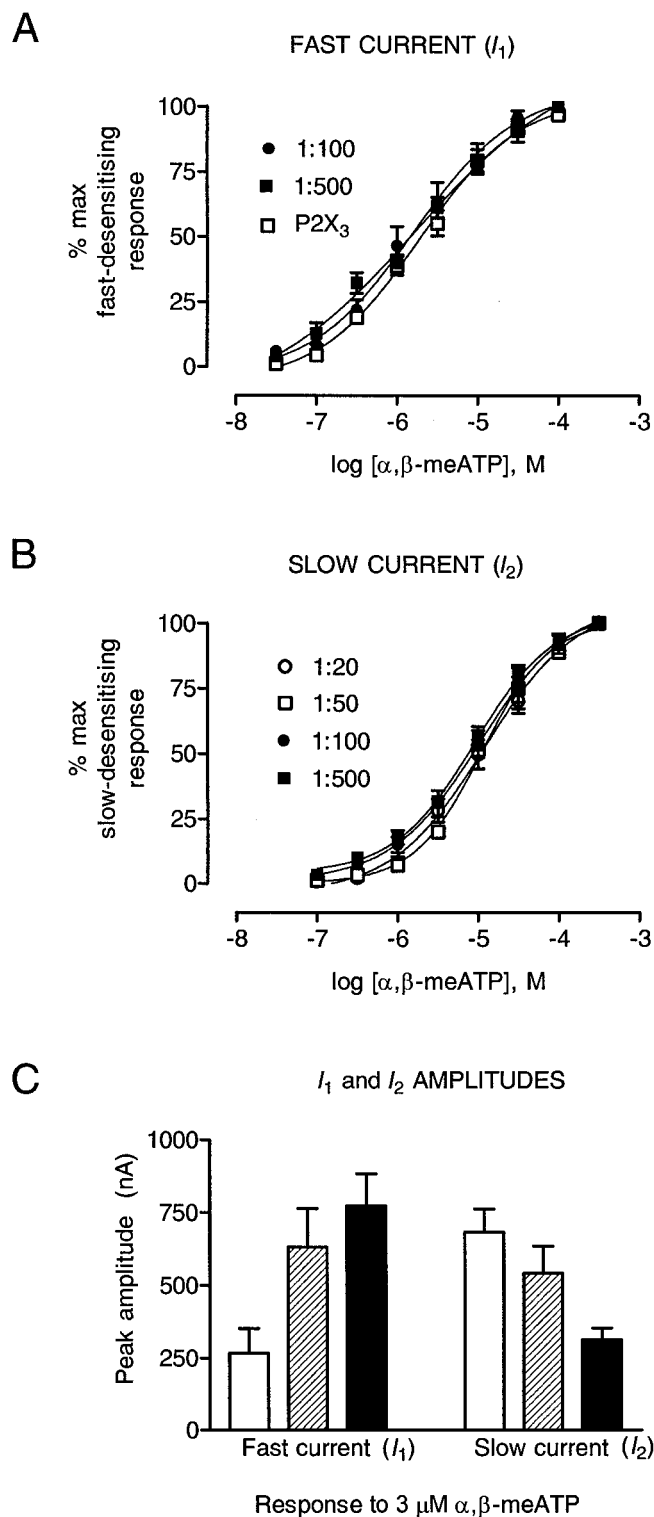


Fig. 2. A, C/R curves are shown for fast currents (I_1) activated by α,β -meATP in oocytes injected with cRNA ratios of 1:500 and 1:100, and oocytes expressing homomeric P2X₃ receptors. Here, the agonist was equipotent for fast responses (I_1) and responses of P2X₃ receptors. B, C/R curves are shown for slow currents (I_2) activated by α,β -meATP in oocytes injected with cRNA ratios of 1:500, 1:100, 1:50, and 1:20. Changing the amounts of cRNAs injected failed to influence agonist potency for slow responses (I_2). C, the mean amplitudes of fast (I_1) and slow (I_2) currents are shown for oocytes injected with cRNAs at 1:20, 1:100, and 1:500 ratios. Here, agonist efficacy for I_1 and I_2 responses was highly dependent on the relative amounts of cRNA injected. Data are expressed as mean \pm S.E. of five to seven cells. cRNA (P2X₂:P2X₃): □, 1:20; ▨, 1:100; ■, 1:500.

TABLE 1

Agonist potency at homomeric and heteromeric P2X receptors
Agonist potency given in terms of EC₅₀ value (μ M) (mean \pm S.E., $n = 4-7$).

Nucleotide	P2X ₂ ^a	P2X ₃ ^b	P2X ₂ + P2X ₃ (I_2) ^c
ATP	4.3 \pm 0.8	1.4 \pm 0.3	1.2 \pm 0.3
2-MeSATP	6.9 \pm 1.2	0.2 \pm 0.03	0.8 \pm 0.2
ATP γ S	8.4 \pm 1.9	1.5 \pm 0.4	9.4 \pm 1.3
α,β -meATP	Inactive	1.9 \pm 0.3	8.6 \pm 1.2
β,γ -meATP	>300	9.2 \pm 2.4	>100
Ap ₅ A	Inactive	0.6 \pm 0.1	1.1 \pm 0.3
UTP	Inactive	>100	>100

^a Determined from the slow responses in oocytes expressing P2X₂ subunits alone, at pH 7.4.

^b Determined from the fast responses in oocytes expressing P2X₃ subunits alone.

^c Determined from peak of slow responses (I_2) in oocytes coexpressing P2X₂ and P2X₃ subunits.

activate P2X₂ receptors showed no better efficacy than α,β -meATP at presumptive P2X_{2/3} receptors. These findings could be explained if P2X₂ and P2X_{2/3} receptors were simultaneously generated when P2X₂ and P2X₃ subunits were coexpressed in oocytes, and if both receptor subtypes contributed toward the slow ion currents evoked by agonists common to each subtype (i.e., ATP, 2-MeSATP, and ATP γ S).

The presence of two distinct P2X receptors were confirmed in desensitization experiments where ATP and α,β -meATP were applied successively to generate two slow currents. Saturating concentrations of α,β -meATP (100 μ M), applied until the evoked slow response had completely desensitized, were always followed by a second slow current when oocytes were challenged with a saturating concentration of ATP (100 μ M) (Fig. 3C). Where ATP was applied first, however, a successive application of α,β -meATP failed to activate an additional slow response (Fig. 3C). These results are compatible with α,β -meATP being an agonist of heteromeric P2X_{2/3} receptors alone, whereas ATP acts as an agonist of both homomeric P2X₂ and heteromeric P2X_{2/3} receptors. Thus, the residual slow response to ATP following desensitization of the slow response to α,β -meATP was mediated by P2X₂ receptors alone, whereas the failure of α,β -meATP to generate an additional current after ATP was due to the latter activating then inactivating P2X_{2/3} receptors.

Antagonism of the Heteromeric P2X_{2/3} Receptor. A way was devised to study P2X_{2/3} receptors in isolation, using α,β -meATP to activate P2X_{2/3} receptors and employing a desensitizing procedure of two agonist pulses (40 s apart) to inactivate all homomeric P2X₃ receptors present (as shown in Fig. 1C). The blocking activity of a series of P2 receptor antagonists was tested on the residual slow response evoked by the second pulse of α,β -meATP. Suramin (0.01–100 μ M), RB-2 (0.03–100 μ M), PPADS (0.03–100 μ M), and TNP-ATP (0.001–1 μ M) caused a concentration-dependent inhibition of P2X_{2/3} receptors, whereas Ip₅I (0.01–100 μ M) was inactive. IC₅₀ values are given in Table 2. The heteromeric P2X_{2/3} receptor was remarkably sensitive to TNP-ATP (IC₅₀, 11 nM), a feature shared with the homomeric P2X₃ receptor (IC₅₀, 0.3 nM). Except for suramin, all antagonists, including TNP-ATP appeared to work in noncompetitive manner, although their blocking actions were slowly reversible after washout. For suramin, a pA₂ value of 5.9 \pm 0.4 was determined at the heteromeric P2X_{2/3} receptor.

Modulation of the Heteromeric P2X_{2/3} Receptor. The same procedure of α,β -meATP agonism and twin agonist pulses (40 s apart) were used again to study P2X_{2/3} receptors

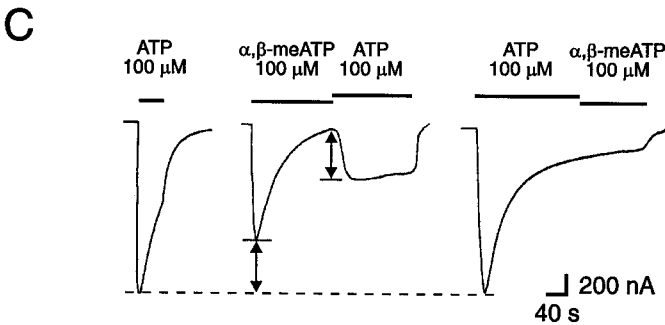
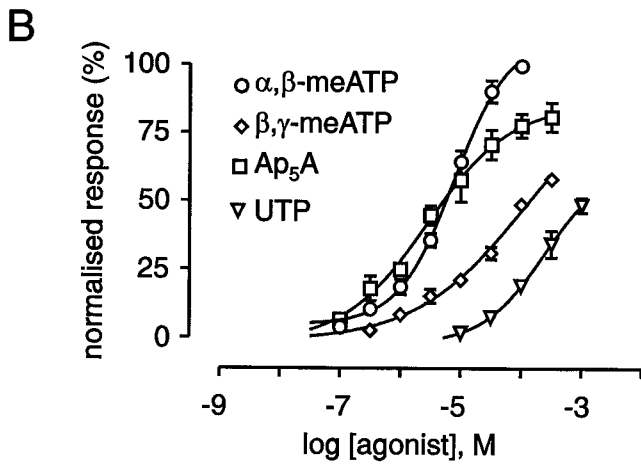
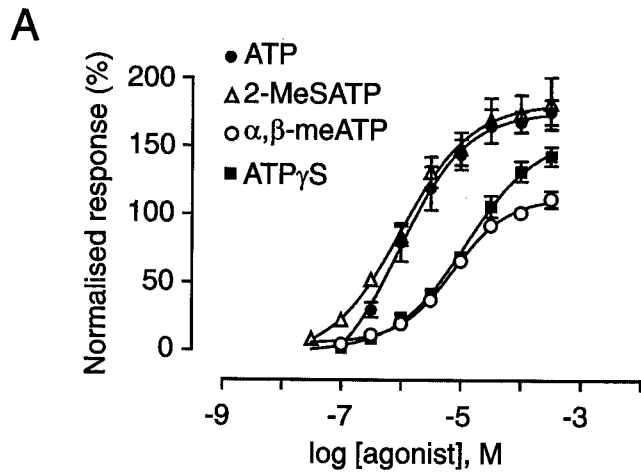


Fig. 3. A, C/R curves are shown for slow currents (I_2) activated by a series of nucleotides at P2X receptors formed by P2X₂ and P2X₃ subunit coexpression. The maximum response (or efficacy) to ATP, 2-MeSATP, and ATP γ S was markedly greater than that of α,β -meATP. B, C/R curves for slow currents activated by a second series of nucleotides at P2X receptors formed by P2X₂ and P2X₃ subunit coexpression. The efficacy of Ap₅A, β,γ -meATP, and UTP was similar to, or less than, that of α,β -meATP. C, the successive application of α,β -meATP and ATP activated two independent slow currents. The sum of these two slow currents (see arrows) equaled the amplitude of an earlier response to ATP, where α,β -meATP desensitization had not yet occurred.

in isolation and to assess the modulatory effects of extracellular H⁺ and Zn²⁺ ions. Under these circumstances, alteration in the extracellular pH (pH 8.0–6.5) caused only a

TABLE 2

Antagonist potency at homomeric and heteromeric P2X receptors
Antagonist potency given in terms of IC₅₀ value (μ M) (mean \pm S.E., $n = 4-7$).

Antagonist	P2X ₂ ^a	P2X ₃ ^b	P2X ₂ + P2X ₃ (I_2) ^c
PPADS	1.6 \pm 0.1	0.22 \pm 0.05	2.3 \pm 0.4
RB-2	0.4 \pm 0.1	45 \pm 11	5.2 \pm 0.9
Suramin	10.4 \pm 1.2	4.1 \pm 0.7	2.4 \pm 0.4
TNP-ATP	1.1 \pm 0.2	0.29 \pm 0.04 ($\times 10^{-3}$)	11.2 \pm 2.5 ($\times 10^{-3}$)
Ip ₅ I	Inactive	2.8 \pm 0.7	Inactive

^a Determined against slow responses to ATP (10 μ M).

^b Determined against fast responses to α,β -meATP (10 μ M), except Ip₅I, which was tested against ATP (3 μ M).

^c Determined against slow responses to α,β -meATP (10 μ M) at P2X_{2/3} receptors.

modest change in agonist potency at P2X_{2/3} receptors (Fig. 4A). These findings contrasted with the strong effect of pH on agonist potency at P2X₂ receptors and weak inhibitory effect of H⁺ on agonism of P2X₃ receptors (see Table 3).

Extracellular Zn²⁺ (1–100 μ M) applied simultaneously

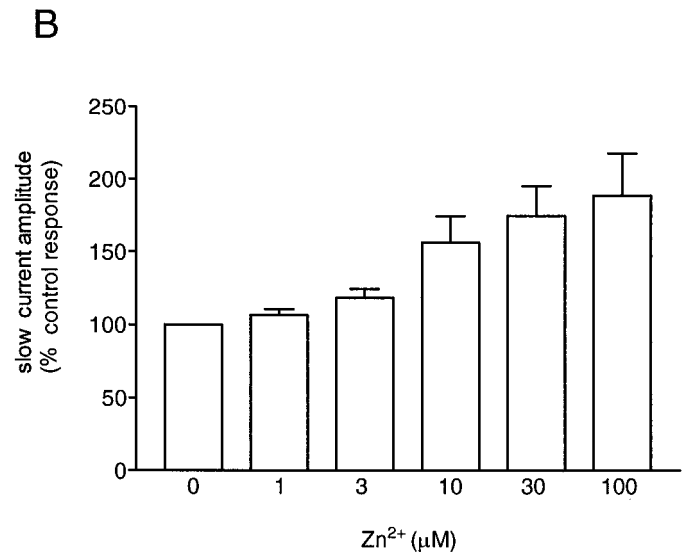
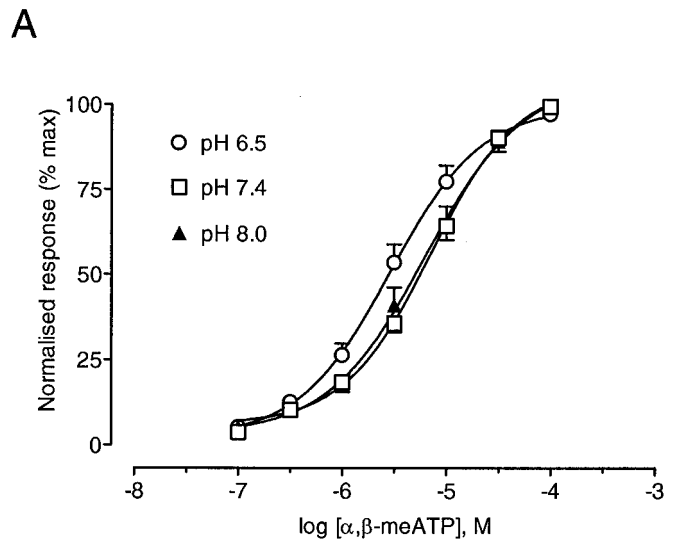


Fig. 4. A, C/R curves are shown for slow currents (I_2) activated by α,β -meATP at P2X_{2/3} receptors for the given extracellular pH levels. B, effect of extracellular Zn²⁺ ions on the amplitude of slow currents activated by α,β -meATP (1 μ M) at P2X_{2/3} receptors.

TABLE 3

Effect of pH on agonist potency at P2X receptors

Agonist potency given in terms of EC₅₀ value (μ M) (mean \pm S.E., $n = 4-6$) for the stated extracellular pH values.

Receptor	pH 6.5	pH 7.5	pH 8.0
P2X ₂ ^a	1.3 \pm 0.2	16.2 \pm 1.4	28 \pm 2
P2X ₃ ^b	2.1 \pm 0.5	1.4 \pm 0.3	1.4 \pm 0.2
P2X _{2/3} ^c	1.8 \pm 0.4	8.6 \pm 1.2	8.3 \pm 1.1

^a Determined from slow responses to ATP at P2X₂ receptors.^b Determined from fast responses to ATP at P2X₃ receptors.^c Determined from slow responses to α,β -meATP at P2X_{2/3} receptors.

with α,β -meATP (1 μ M) caused a modest potentiation of slow responses (Fig. 4B), with a maximal effect of $89 \pm 29\%$ ($n = 4$) above control responses. This finding contrasted with the strong potentiation by Zn²⁺ ions (100 μ M) of ATP responses at P2X₂ receptors by some $1320 \pm 90\%$ ($n = 4$) and its weak inhibitory action against P2X₃ receptors in reducing control responses by $17 \pm 2\%$ ($n = 6$).

Discussion

The present study was carried out for two reasons. First, we wished to characterize the pharmacological profile of the P2X_{2/3} receptor, about which surprisingly little is known apart from α,β -meATP agonism (Lewis et al., 1995) and TNP-ATP antagonism (Thomas et al., 1998). Second, we wished to shed further light on the variability of P2X responses in sensory neurons, in which P2X₂ and P2X₃ subunits commonly occur and where P2X_{2/3} receptors may play a role in sensory transmission.

Sensory neurons show a marked variability in the time course of responses to P2X receptor agonists. Grubb and Evans (1999) reported that the majority (80%) of adult rat DRG neurons responded to nucleotides with a P2X₃-like phenotype, and the remainder possessed noninactivating P2X receptors of undetermined phenotype. Burgard and colleagues (1999) reported similar findings in adult rat DRG neurons, where 70% of IB4-labeled nociceptors showed fast-desensitizing P2X₃-like agonist responses and the remaining 30% showed either mixed (fast and slow responses) or slow responses. To exemplify these phenotypes, we carried out a brief survey of ATP responses in rat neonatal DRG neurons and found qualitatively and quantitatively similar results (Fig. 5). In this exploratory survey, 50% of small DRG neurons produced fast P2X₃-like ATP responses, 30% gave slowly

desensitizing responses, and 20% gave mixed responses. Burgard and colleagues (1999) have attributed the last phenotype to mixed P2X receptors resulting from the coexpression of P2X₂ and P2X₃ subunits, and this observation motivated us to study the pharmacological properties of heteromeric P2X_{2/3} receptors as fully as possible.

Coexpression of P2X₂ and P2X₃ subunits in approximately equal amounts in HEK 293 cells was reported, in the first study of its kind, to generate a hybrid P2X_{2/3} receptor that showed the agonist profile of P2X₃ receptors and inactivation properties of P2X₂ receptors (Lewis et al., 1995). However, we have found that the outcome of P2X subunit coexpression is not straightforward. Where P2X₃ expression was kept constant and the level of P2X₂ expression gradually increased, the resultant P2X receptors changed their phenotype from predominantly P2X₃-like to a mixed phenotype of P2X₂, P2X₃ and P2X_{2/3} receptors. Thus, coexpression of two P2X subunits, where each can independently form functional homomeric receptors, seems to generate a heterogeneous population of P2X receptors—in this case P2X₂, P2X₃, and P2X_{2/3} receptors. This finding suggests that the process of P2X receptor assembly occurs in a stochastic manner, the probability of forming either homomeric or heteromeric receptors being dependent on the relative numbers of the two types of P2X subunit available for receptor assembly.

For each of the four levels of P2X₂/P2X₃ subunit coexpression studied, the resultant population of recombinant P2X receptors gave rise to complex agonist responses. These responses involved an initial fast current that, we believe, was mediated by a small population of homomeric P2X₃ receptors. This conclusion was based in part on the finding that α,β -meATP was equipotent for the P2X₃-like fast current and homomeric P2X₃ receptor itself, and α,β -meATP caused prolonged desensitization in both cases. Also, other P2X₃ receptor agonists (e.g., Ap₅A) evoked fast currents in those oocytes coexpressing P2X₂ and P2X₃ subunits. Additionally, we have already shown that Ip₅I blocks the P2X₃-like fast current, blocks P2X₃ receptors, and also blocks the fast current of DRG sensory neurons with similar IC₅₀ values (King et al., 1999; Dunn et al., 2000). Lastly, the amplitude of the P2X₃-like fast current was reduced as more P2X₂ subunits were expressed—a consequence, we believe, of the reduction in the numbers of P2X₃ receptors as their constituent subunits were incorporated instead into P2X_{2/3} receptors.

The initial P2X₃-like fast current was followed by a slow current that, in all probability, involved P2X_{2/3} receptors when α,β -meATP was the agonist, and both P2X₂ and P2X_{2/3} receptors when ATP was the agonist. Significantly, other studies of P2X₂ and P2X₃ coexpression have also alluded to the dual formation of P2X₂ and P2X_{2/3} receptors (Thomas et al., 1998; Ueno et al., 1998). A slow current to α,β -meATP is a recognized signature of P2X_{2/3} receptors (Lewis et al., 1995; Ueno et al., 1999), but surprisingly little else can be said with certainty about its agonist profile. Agonism of P2X_{2/3} receptors was reassessed recently (Bianchi et al., 1999), although these investigators may not have taken into account the dual formation of P2X₂ and P2X_{2/3} receptors in their expression system.

The P2X_{2/3} receptor was further characterized in our experiments by an agonist potency order of Ap₅A > α,β -meATP > β,γ -meATP > UTP, which took into consideration that none of these nucleotides can activate P2X₂ receptors. In all

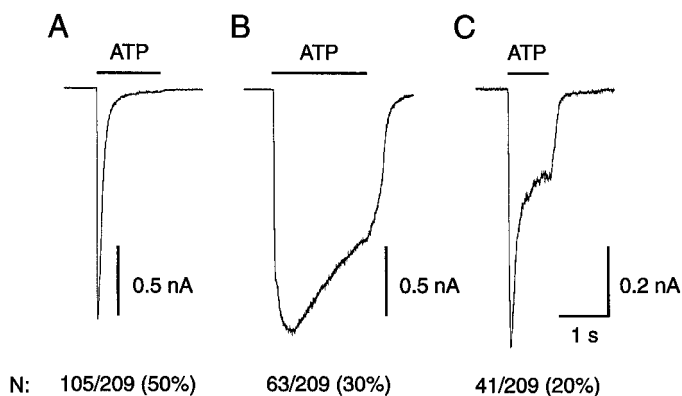


Fig. 5. Whole-cell inward currents activated by ATP (10 μ M) in rat neonatal DRG neurons. Three phenotypes were observed in 209 cells studied under patch-clamp conditions ($V_h = -60$ mV) [for methods, see Dunn et al. (2000)].

probability, ATP, 2-MeSATP, and ATP γ S are agonists at P2X_{2/3} receptors also, but a true assessment of their potency will require the discovery of selective P2X₂ receptor antagonists to strip away the complication of coactivating P2X₂ and P2X_{2/3} receptors. The agonist potency order for homomeric P2X₃ receptors was Ap₅A > α , β -meATP > β , γ -meATP > UTP (setting aside data for ATP, 2-MeSATP, and ATP γ S), which matched the rank order for heteromeric P2X_{2/3} receptors, although the EC₅₀ values for these two P2X subtypes are not identical (see Table 1).

The antagonist potency order at P2X_{2/3} receptors was TNP-ATP > PPADS = suramin > RB-2, with Ip₅I inactive. These antagonists were tested only on slow currents to α , β -meATP and, therefore, any interaction of these blocking agents with P2X₂ and P2X₃ receptors can be discounted. TNP-ATP was potent in the near nanomolar concentration range, in agreement with an earlier study (Thomas et al., 1998). However, our analysis of the inhibition curve ($n_H \sim 1$) suggested only a single population of heteromeric P2X_{2/3} receptors. PPADS, suramin, and RB-2 also blocked the P2X_{2/3} receptor in micromolar concentrations, where the potency order (PPADS = suramin > RB-2) differed from that for homomeric P2X₂ receptors (RB-2 > PPADS > suramin) and P2X₃ receptors (PPADS > suramin > RB-2). Regarding our negative results with Ip₅I, it is of note that slow currents to α , β -meATP in sensory neurons (nodose and DRG) are similarly unaffected by this dinucleotide (Dunn et al., 2000) and provide indirect proof that such slow responses probably involve native P2X_{2/3} receptors.

The potentiating effect of extracellular H⁺ and Zn²⁺ was modest at recombinant P2X_{2/3} receptors compared with their reported facilitatory actions on P2X₂ receptors (Wildman et al., 1998). Regarding the effects of H⁺ ions, our results revealed a 4-fold increase in agonist potency at P2X_{2/3} receptors in changing from pH 8.0 to 6.5, and a 21-fold increase for P2X₂ receptors. Regarding the effects of Zn²⁺ ions, agonist activity was enhanced about 2-fold at P2X_{2/3} receptors, but 14-fold at P2X₂ receptors. Both H⁺ and Zn²⁺ ions potentiate ATP-activated slow currents in nodose ganglia, which express P2X₂ and P2X₃ subunits (Li et al., 1997). From our present data, it is likely that any H⁺- and Zn²⁺-based potentiation of ATP-activated responses in sensory ganglia will involve both P2X₂ and P2X_{2/3} receptors.

In summary, we have carried out a sequential pharmacological survey of the possible homomeric and heteromeric P2X receptors that could be generated by coexpression of P2X₂ and P2X₃ subunits in the same cell. Our experiments indicate that, where cell types naturally express more than one P2X subunit, it is likely that a mixture of homomeric and heteromeric receptors will be present in these cells. Where the P2X_{2/3} receptor was studied in isolation, we found that the pharmacological profile is comparatively unique and does not seem to be governed by either of the constituent P2X₂ and P2X₃ subunits. Thus, α , β -meATP (but not the structurally related β , γ -meATP) is a potent agonist at P2X_{2/3} receptors—a profile that does not fit either homomeric P2X₂ or P2X₃ receptors. Also, the potency of the antagonists tested here on P2X_{2/3} receptors, particularly TNP-ATP, RB-2, and the inactive Ip₅I, did not mirror our findings with homomeric P2X₂ and P2X₃ receptors. These pharmacological findings indicate that the therapeutic value of P2X subunit-selective agonists and antagonists (a popular objective at this point in

time) might not be entirely appropriate and that selective agonists and antagonists for those P2X heteromultimers found in tissues need also be sought.

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