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Coexpression of Rat $P2X_2$ and $P2X_6$ Subunits in *Xenopus* Oocytes

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Transcripts for $P2X_2$ and $P2X_6$ subunits are present in rat CNS and frequently colocalize in the same brainstem nuclei. When rat $P2X_2$ ($rP2X_2$) and rat $P2X_6$ ($rP2X_6$) receptors were expressed individually in *Xenopus* oocytes and studied under voltage-clamp conditions, only homomeric $rP2X_2$ receptors were fully functional and gave rise to large inward currents (2–3 μA) to extracellular ATP. Coexpression of $rP2X_2$ and $rP2X_6$ subunits in *Xenopus* oocytes resulted in a heteromeric $rP2X_{2/6}$ receptor, which showed a significantly different phenotype from the wild-type $rP2X_2$ receptor. Differences included reduction in agonist potencies and, in some cases (e.g., Ap_4A), significant loss of agonist activity. ATP-evoked inward currents were biphasic at the heteromeric $rP2X_{2/6}$ receptor, particularly when Zn^{2+} ions were present or extracellular pH was lowered. The pH range was narrower for H^+ enhancement of ATP responses at the heteromeric $rP2X_{2/6}$ receptor. Also, H^+ ions inhibited ATP responses at low pH levels (<pH 6.3). The pH-dependent blocking activity of suramin was changed at this heteromeric receptor, although the potentiating effect of Zn^{2+} on ATP responses was unchanged. Thus, the $rP2X_{2/6}$ receptor is a functionally modified $P2X_2$-like receptor with a distinct pattern of pH modulation of ATP activation and suramin blockade. Although homomeric $P2X_6$ receptors function poorly, the $P2X_6$ subunit can contribute to functional heteromeric P2X channels and may influence the phenotype of native P2X receptors in those cells in which it is expressed.

Key words: P2X receptor; ionotropic receptor; heteromer; ATP; purinergic; oocyte

P2X receptors are ligand-gated cation channels that when activated by extracellular ATP mediate fast excitation in various cells, including central and peripheral neurons (Burnstock, 1997). Neuronal P2X receptors show considerable differences in their sensitivity to naturally occurring agonists, P2 receptor antagonists, and allosteric modulators and, furthermore, show differences in kinetics of receptor activation and inactivation (Khakh et al., 1995; King, 1998). Such diversity in the operational profiles of ATP-gated ion channels may be attributable to the subunit composition of native P2X receptors, because other classes of ionotropic receptors show differing phenotypes that depend on subunit composition (Barnard et al., 1998). Seven P2X receptor subunits ($P2X_{1-7}$) have been cloned, each of which is believed to form functional homomeric assemblies (Buell et al., 1996). They can also coassemble with other P2X subunits to form heteromeric P2X receptors of three, or possibly four, protein subunits per ATP-gated ion channel (Kim et al., 1997; Nicke et al., 1998; Torres et al., 1999). Three functional heteromeric P2X receptors have been reported: $P2X_{2/3}$ (Lewis et al., 1995; Radford et al., 1997), $P2X_{4/6}$ (Lê et al., 1998), and $P2X_{1/5}$ (Torres et al., 1998; Haines et al., 1999; Lê et al., 1999). Heteromeric channels composed of splice variants of the same P2X subunit (e.g., $mP2X_4$ and $mP2X_{4a}$) can also generate a different phenotypic form of the wild-type P2X receptor (Townsend-Nicholson et al., 1999).

The potential for heteropolymerization among $P2X_{1-7}$ receptor subunits was recently investigated using coimmunoprecipitation procedures (Torres et al., 1999). For P2X subunits concentrated in the CNS (namely $P2X_2$, $P2X_4$, and $P2X_6$) (Collo et al., 1996), epitope-tagged $P2X_2$ and $P2X_6$ subunits or $P2X_4$ and $P2X_6$ subunits (but not $P2X_2$ and $P2X_4$ subunits) were shown to form immunopositive heteromeric assemblies. The functional properties of heteromeric $P2X_{4/6}$ receptors have been established (Lê et al., 1998), but not yet the phenotype of heteromeric $P2X_{2/6}$ receptors. The result of $P2X_2$ and $P2X_6$ subunit coexpression is of considerable interest because of (1) the distinct pH modulation of ATP responses at the homomeric $P2X_2$ receptor (King, 1998), (2) a growing belief that the $P2X_6$ subunit might only contribute to functional channels when other P2X subunits are present (Torres et al., 1999), and (3) the recent identification of a pH-modulated ATP receptor in those nuclei of rat brainstem where $P2X_2$ and $P2X_6$ transcripts have been detected (Thomas et al., 1999; Thomas and Spyer, 2000).

Thus, it was of interest to examine the contribution of the $P2X_6$ subunit, when coexpressed with the pH-modulated $P2X_2$ subunit, to the operational profile of the resultant heteromeric $P2X_{2/6}$ receptor expressed in defolliculated *Xenopus* oocytes. Differences in the ways heteromeric $P2X_{2/6}$ and homomeric $P2X_2$ receptors respond to nucleotidic agonists, suramin, pH, and Zn^{2+} ions were investigated in the oocyte expression system. The results establish the $P2X_{2/6}$ receptor as the fourth example of a heteropolymeric ATP-gated ion channel that, in this case, possesses a pattern of pH modulation of ATP responses distinct from other known homomeric and heteromeric P2X receptors.

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MATERIALS AND METHODS

Oocyte preparation. Xenopus laevis frogs were killed by immersion in a lethal dose in Tricaine (0.4% w/v, in tap water) and then decapitated, and ovarian lobes were removed by blunt dissection. *Xenopus* oocytes (stages V and VI) were defolliculated by a two-step process involving (1) collagenase treatment (Type IA, 2 mg/ml in Ca^{2+}-free Ringer's solution, for 2–3 hr) and (2) stripping away the follicle cell layer with fine forceps. Defolliculated oocytes do not possess native P1 and P2 receptors (King et al., 1996a,b) and are largely devoid of ecto-ATPases (Ziganshin et al., 1995). Oocytes were stored in Barth's solution (pH 7.5, at 4°C) containing (in mM): NaCl 110, KCl 1, $NaHCO_3$ 2.4, Tris HCl 7.5, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, supplemented with gentamycin sulfate, 50 μg/l. Cells were injected cytosolically with cRNA for $rP2X_2$ (40 nl, 0.002 μg/μl) or $rP2X_6$ (40 nl, 1 μg/μl) or both $rP2X_2$ and $rP2X_6$ (40 nl of each) and incubated for 48 hr at 18°C in Barth's solution. Thereafter, injected oocytes were kept at 4°C for up to 12 d until they were used in electrophysiological experiments.

Electrophysiology. Membrane currents were recorded from cRNA-injected oocytes using a twin-electrode voltage-clamp amplifier (Axoclamp 2A). The holding potential (V_h) was −50 mV, unless stated otherwise. The voltage-recording and current-recording microelectrodes (1–5 MΩ tip resistance) were filled with 3.0 M KCl. Oocytes were placed in an electrophysiological chamber (volume, 0.5 ml) and superfused with Ringer's solution (5 ml/min, at 18°C) containing (in mM): NaCl 110, KCl 2.5, HEPES 5, $CaCl_2$ 1.8, adjusted to pH 7.5. Extracellular pH (pH_e) was adjusted with HCl (1.0N) or NaOH (1.0N) to reach the desired level. Electrophysiological data were stored on magnetic tape using a DAT recorder (Sony 1000ES) and displayed using a pen recorder (Gould 2200S).

Drug solutions. ATP and other nucleotides were prepared in Ringer's solution, and the pH of stock solutions was readjusted to the desired level. Agonists were superfused, at the concentrations given in the text, by a gravity-feed continuous flow system allowing the rapid addition and washout of drugs. ATP was added for 120 sec or until the current reached a peak, then washed off with Ringer's solution for a period of 5 min. Where used, antagonists were applied for 5 min before and during the application of agonists.

Agonist responses were normalized to the maximum inward current (I_{max}) evoked by ATP at pH 7.5, including agonist responses recorded at lower pH levels. At pH 7.5, maximum responses were evoked by 300–1000 μM ATP. The agonist concentration required to evoke 50% of the maximum response (EC_{50}) was taken from Hill plots, using the transform $\log (I/I_{max} - I)$, where I is the peak current evoked by each concentration of ATP.

The potentiating effects of extracellular Zn^{2+} ions on agonist activity were investigated in two ways. Zn^{2+} ions were either applied simultaneously with ATP or added to the Ringer's solution for 5 min before ATP was applied (with Zn^{2+} present).

Statistics and graphs. Data are presented as mean ± SEM of four to seven sets of data from different oocyte batches. Concentration–response curves and inhibition curves were fitted by nonlinear regression analysis using Prism v2.0 (GraphPad). Significant differences were determined by unpaired Student's *t* test or one-way ANOVA followed by Dunnett's *post hoc* test, again using Prism v2.0 (GraphPad).

Drugs and reagents. All common salts and reagents were AnalaR grade (Aldrich Chemicals, Poole, UK). ATP and ATPαS were purchased from Boehringer (Mannheim, Germany). 2-Methylthio ATP (2-MeSATP) was obtained from RBI (Natick, MA), and other nucleotides [ATPγS, ADP, AMP, adenosine, UTP, UDP, UMP, uridine, CTP, GTP, ITP, diadenosine polyphosphates (Ap_nA; n = 2–6), α,β-meATP, β,γ-meATP, and 2′- and 3′-*O*-(4-benzoyl-benzoyl)ATP (BzATP)] came from Sigma (Poole, UK). Suramin was a gift from Bayer (Newbury, UK).

RESULTS

ATP responses of P2X receptors

In initial experiments, the functionality of homomeric $rP2X_2$ and $rP2X_6$ receptors expressed in *Xenopus* oocytes was tested against a near-saturating concentration of ATP (100 μM), according to available pharmacological data on homomeric P2X receptors (King, 1998). At a holding potential of −50 mV, ATP-activated $rP2X_2$ receptors produced fast-activating and slowly inactivating inward currents (1993 ± 147 nA; n = 6) (Fig. 1*A*,*C*). $rP2X_6$ receptors failed to respond to ATP at a holding potential −50 mV, but where increased to −90 mV, the agonist did evoke low-amplitude slowly activating inward currents (4.57 ± 1.31 nA, n = 7) (Fig. 1*A*,*C*). Control (water-injected) oocytes failed to respond to ATP, at either −50 or −90 mV.

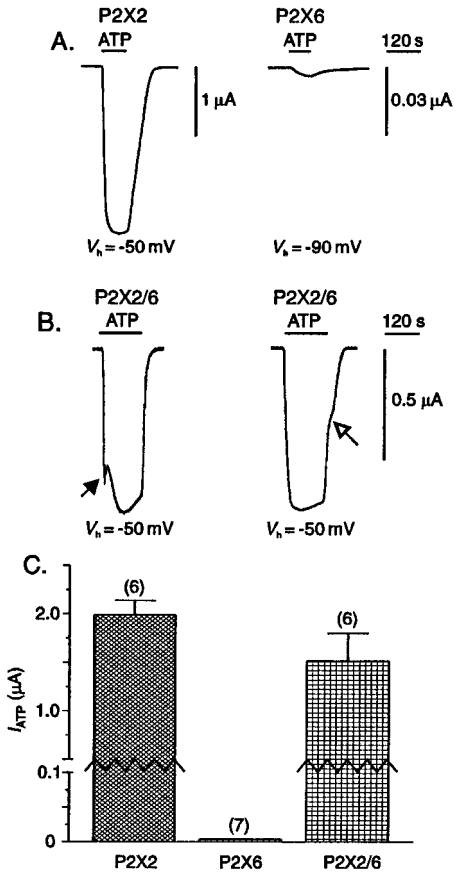

Figure 1. Expression of homomeric and heteromeric P2X receptors. *A*, Whole-cell inward currents by homomeric $rP2X_2$ and $rP2X_6$ receptors activated by a near-saturating ATP concentration (100 μM, for 60 sec), at the given holding potentials (V_h). *B*, Whole-cell inward currents by ATP-activated heteromeric $rP2X_{2/6}$ receptors. ATP responses were often biphasic, showing a transient component (*filled arrow*) followed by a sustained current. The deactivation of inward current occasionally showed two phases of current decay (*open arrow*). *C*, Averaged whole-cell inward currents by homomeric $rP2X_2$, $rP2X_6$, and heteromeric $rP2X_{2/6}$ receptors activated by ATP (100 μM). The *y*-axis of the histogram has been truncated to help reveal the small responses by $rP2X_6$ receptors. Data are expressed as mean ± SEM for six to seven cells per determination.

In further experiments, coexpression of $rP2X_2$ and $rP2X_6$ subunits resulted in fast-activating and slowly inactivating inward currents (1516 ± 286 nA, n = 6) (Fig. 1*B*,*C*), which were broadly similar in their time course to the ATP responses produced by homomeric $rP2X_2$ receptors. However, ATP-activated heteromeric $rP2X_{2/6}$ receptors uniquely showed biphasic (transient and sustained) components to the evoked inward currents (Fig. 1*B*, *closed arrow*). Such biphasic responses were seen in all cRNA-injected oocytes tested (n = 175), although the amplitude of each component of biphasic currents was variable from response to response. Furthermore, evoked responses would change in an unpredictable manner from biphasic to monophasic currents (and back again) over several successive ATP applications. However, the reproducibility of biphasic inward currents by $rP2X_{2/6}$ recep-

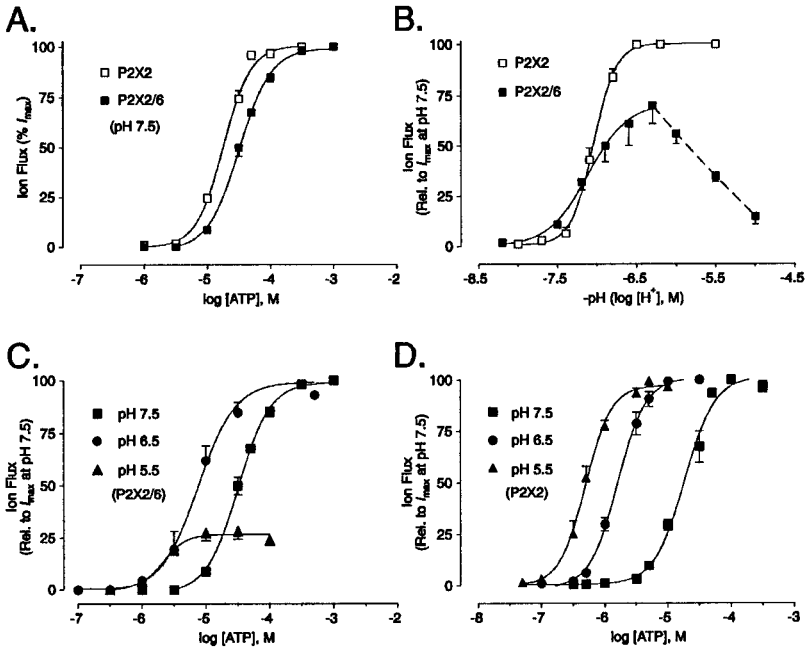

Figure 2. ATP activity at homomeric and heteromeric P2X receptors. *A*, C–R relationship for ATP-activated inward currents at $rP2X_2$ and $rP2X_{2/6}$ receptors, at pH 7.5. *B*, The relationship between the amplitude of ATP responses ($rP2X_2$, 3 μM; $rP2X_{2/6}$, 10 μM; each producing 5% of the maximum response) and the extracellular pH level (range, pH 8.3–5.0) at homomeric and heteromeric P2X receptors. *C*, The C–R curves for ATP activation of $rP2X_{2/6}$ receptors at the pH_e levels indicated. ATP efficacy was markedly reduced at pH 5.5. *D*, The C–R curves for ATP activation of $rP2X_2$ receptors. ATP efficacy was not altered at pH 5.5. Curves were fitted by the Hill equation in *A–D* (*solid lines*) and by a single exponential function in *B* (*dashed line*). Data given as mean ± SEM for four to six cells per curve.

tors was enhanced when pH_e was lowered or Zn^{2+} ions were present in the bathing solution (see Fig. 5). Deactivation of $rP2X_{2/6}$ receptors frequently comprised two phases of current decay (Fig. 1*B*, *open arrow*). Neither biphasic inward currents nor biphasic current decays were seen at $rP2X_2$ receptors.

The concentration–response (C–R) relationship was studied for ATP responses at $rP2X_2$ and $rP2X_{2/6}$ receptors, at pH 7.5 (Fig. 2*A*). ATP was more potent (approximately twofold) at $rP2X_2$ receptors (EC_{50}, 18 ± 2.1 μM; n_H = 2.0 ± 0.2) than $rP2X_{2/6}$ receptors (EC_{50}, 32 ± 1.6 μM; n_H = 1.7 ± 0.2) ($p < 0.05$, unpaired *t* test). Since the potency of agonists at $rP2X_2$ receptors is strongly affected by pH_e, the above differences in ATP activity could potentially be attributed to incorrect pH_e measurements. However, $rP2X_2$ and $rP2X_{2/6}$ receptors responded in different ways to changes in pH_e (Fig. 2*B*). The amplitude of ATP responses at $rP2X_2$ receptors increased over the range of pH 8.0 to 6.3 and was maintained at lower pH_e levels (up to pH 5.0). ATP responses at $rP2X_{2/6}$ receptors initially increased in size over the range of pH 8.0 to 6.3, then decreased in amplitude as pH_e levels were lowered further. The pK_a value for the potentiating phase of the H^+ effect was 7.04 ± 0.05 (n = 4) at $P2X_{2/6}$ receptors, a value not significantly different from that of $P2X_2$ receptors (7.05 ± 0.05; n = 4). However, the slopes of the curves describing the potentiating H^+ effect were significantly different ($P2X_{2/6}$, 1.83 ± 0.31; $P2X_2$, 3.04 ± 0.22; $p < 0.05$).

The C–R relationship for ATP was reexamined at different pH_e levels for $rP2X_{2/6}$ receptors. ATP potency was increased fourfold at pH 6.5 and 15-fold at pH 5.5 (Table 1; see EC_{50} values). The maximum response to ATP was unchanged at pH 6.5, but agonist efficacy was significantly reduced (by 76 ± 3%) at pH 5.5 (Fig. 2*C*). At $rP2X_2$ receptors, acidification of the bathing solution shifted the ATP C–R curve to the left without a reduction in the maximum (Fig. 2*D*). ATP potency was increased 12-fold at pH 6.5 and 30-fold at pH 5.5 at $rP2X_2$ receptors (Table 1; see EC_{50} values). The effects of lowering pH_e were reversed on restoration to pH 7.5 for both $rP2X_2$ and $rP2X_{2/6}$ receptors.

Agonist activity at P2X receptors

ATP, ATPαS, ATPγS, and 2-meSATP are known to be full agonists at $rP2X_2$ receptors (King et al., 1997), and consequently their ability to activate $rP2X_{2/6}$ receptors was investigated. Each nucleotide (30 μM) elicited large, slowly inactivating inward currents at $rP2X_{2/6}$ receptors, with an apparent potency order of (estimated EC_{50} value) ATP (29.9 μM) = ATPγS (30.8 μM) > 2-MeSATP (34.8 μM) > ATPαS (40.6 μM) (Fig. 3*A*,*C*). BzATP was a weak agonist at $rP2X_{2/6}$ receptors (EC_{50}, 399 μM) (Fig. 3*A*,*C*). $P2X_{2/6}$ receptors did not respond to ADP, AMP, adenosine, UTP, UDP, UMP, uridine, CTP, GTP, ITP, α,β-meATP, and β,γ-meATP (each tested at 30 and 100 μM) (data not shown). Of the diadenosine polyphosphates tested (Ap_nA, n = 2–6), Ap_4A alone showed activity but proved to be a weak agonist (EC_{50}, >1 mM) (Fig. 3*B*,*C*). This weak activity contrasted with results from $rP2X_2$ receptors, at which Ap_4A is a full agonist (EC_{50}, 15.2 μM) (Pintor et al., 1996).

Suramin blockade at P2X receptors

Suramin is an effective antagonist at $rP2X_2$ receptors, at which its potency is enhanced when pH_e levels are lowered (King et al., 1997). Similar results were obtained for $P2X_{2/6}$ receptors, with suramin reducing ATP responses in a concentration-dependent

Table 1. Effect of extracellular pH on ATP potency

pH_e	$rP2X_2$ receptor	$rP2X_{2/6}$ receptor
pH 7.5	16.2 ± 1.4	32.0 ± 1.6*
	(1.8 ± 0.2)	(1.7 ± 0.2)
pH 6.5	1.3 ± 0.2	7.5 ± 1.1*
	(2.0 ± 0.2)	(1.5 ± 0.3)
pH 5.5	0.55 ± 0.04	2.2 ± 0.7*
	(2.1 ± 0.3)	(2.5 ± 0.3)

EC_{50} values (μM) and Hill slopes (n_H, in brackets) for ATP activation of $rP2X_2$ and $rP2X_{2/6}$ receptors at the given extracellular pH (pH_e) levels are shown. At each pH_e level tested, EC_{50} values were significantly different (*$p < 0.05$, by unpaired *t* test). Data are expressed as mean ± SEM (n = 4).

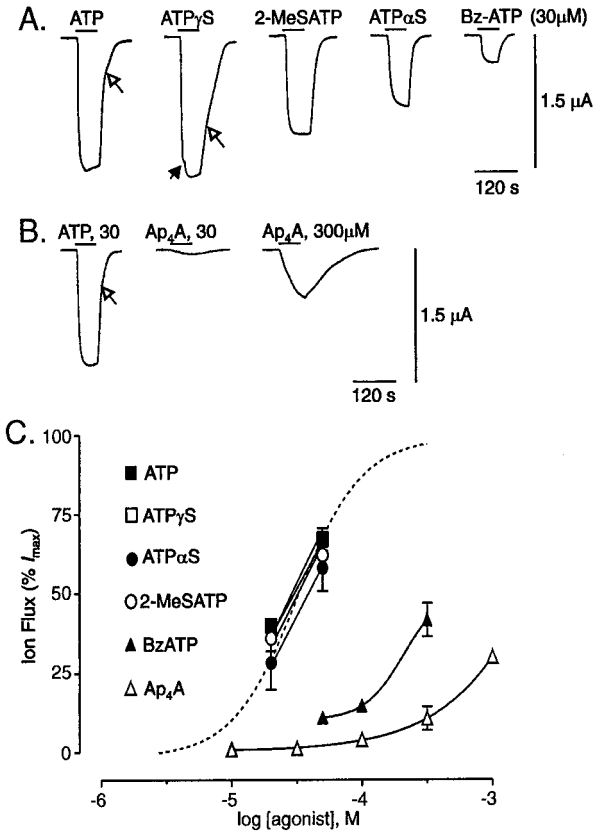

Figure 3. Nucleotide activation of $rP2X_{2/6}$ receptors. In *A*, whole-cell inward currents at the heteromeric $rP2X_{2/6}$ receptor were evoked by ATP, ATPγS, 2-MeSATP, ATPαS, and BzATP (30 μM), each of which is a known agonist of $rP2X_2$ receptors (King et al., 1997). In *B*, the $rP2X_{2/6}$ receptor was activated weakly by Ap_4A (30 and 300 μM), and the kinetics of activation and deactivation were considerably slower than ATP responses. *C*, C–R relationship for agonist activation of $rP2X_{2/6}$ receptors at pH 7.5. Estimates of EC_{50} values (micromolar concentration) were made using the "2 + 2 assay" method of Arunlakshana and Schild (1959): ATP, 29.9 ± 1.9; ATPγS, 30.8 ± 2.9; ATPαS, 40.6 ± 8.0; 2-MeSATP, 34.8 ± 5.1; BzATP, 399 ± 66; Ap_4A, >1000 (n = 4–6). The *dashed line* shows the position of the full C–R curve for ATP (redrawn from Fig. 2*A*). *Open* and *filled arrows* (in *A* and *B*) draw attention to biphasic components of receptor activation and deactivation. Data are given as mean ± SEM for four to six cells per determination.

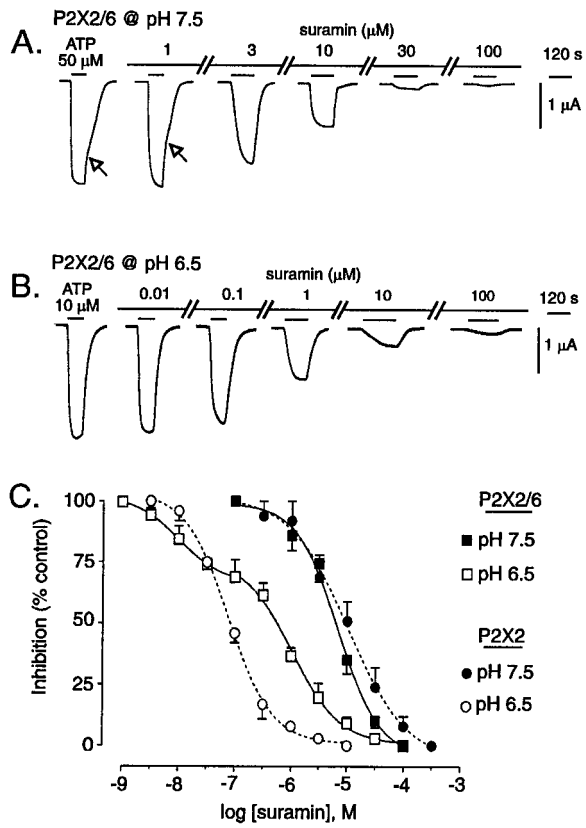

Figure 4. Suramin antagonism of $rP2X_{2/6}$ receptors. Shown is antagonism of ATP responses (V_h = −50 mV) at heteromeric $rP2X_{2/6}$ receptors by suramin at pH 7.5 (*A*) and pH 6.5 (*B*). Suramin was effective at micromolar concentrations at pH 7.5, but the concentration range for suramin blockade was extended at pH 6.5. *C*, Inhibition curves for suramin blockade of ATP responses at $rP2X_2$ and $rP2X_{2/6}$ receptors at the given pH levels. At pH 6.5, the inhibition curve for $rP2X_{2/6}$ was fitted best by a biphasic curve. IC_{50} values are given in Table 2. *Open arrows* draw attention to biphasic current decays. Data are expressed as mean ± SEM for four to eight cells per curve. The biphasic curve for $rP2X_{2/6}$ was constructed from eight sets of data, using the results from the first two log_{10} units of concentration (suramin, 0.001–0.01 μM) to represent the first component of the inhibition curve.

manner and its potency enhanced with acidification of the bathing solution (Fig. 4*A*,*B*). At pH 7.5, suramin was equipotent at $rP2X_2$ and $rP2X_{2/6}$ receptors (Table 2; see IC_{50} values). Differences in blocking activity were only observed at lower pH_e levels where, at pH 6.5, the inhibition curve for suramin was biphasic for $rP2X_{2/6}$ receptors and monophasic for $rP2X_2$ receptors (Fig. 4*C*). Comparison of IC_{50} values at pH 6.5 revealed that activity indices for each of the two phases of $P2X_{2/6}$ receptor blockade was significantly different ($p < 0.05$, unpaired t test) compared with the IC_{50} value for $rP2X_2$ receptors (Table 2). The blocking activity of suramin at $rP2X_2$ and $rP2X_{2/6}$ receptors was reversed on washout, at all pH_e levels studied.

Actions of Zn^{2+} ions at P2X receptors

Extracellular Zn^{2+} is known to potentiate ATP responses at $P2X_2$ receptors (Wildman et al., 1998), although the degree of potentiation depends on whether Zn^{2+} is applied before, or simultaneously with, the agonist. When applied 5 min before ATP, Zn^{2+} ions (1–30 μM) progressively increased ATP responses at $rP2X_{2/6}$ receptors, by 6- to 14-fold (averaging 9.82 ± 2.29, n = 6), whereas higher concentrations (30–300 μM) progressively decreased and abolished ATP responses in a concentration-dependent manner (Fig. 5*A*). The potentiating and inhibitory effects were reversed on washout. Zn^{2+} preincubation also affected ATP responses by clearly increasing the incidence of biphasic inward currents, a phenomenon also seen when pH_e levels were lowered (Fig. 5*A*,*C*). Where applied simultaneously with ATP, Zn^{2+} ions (1–300 μM) only caused a concentration-dependent increase (8- to 25-fold; averaging 16.35 ± 4.28, n = 5) in the amplitude of ATP responses at $P2X_{2/6}$ receptors (Fig. 5*B*). Without Zn^{2+} preincubation, the above inhibitory Zn^{2+} effect was not seen, and the incidence of biphasic ATP responses was inconsistent and infrequent for each oocyte tested. EC_{50} values for the potentiating effects of Zn^{2+} ions at $rP2X_2$ and $rP2X_{2/6}$ receptors were similar (Fig. 5, see legend).

DISCUSSION

In the present study, expression of homomeric $rP2X_6$ receptors in defolliculated *Xenopus* oocytes resulted in functional P2X receptors that, even under heightened conditions for channel activa-

Table 2. Blockade by suramin of P2X receptors

pH_e	$rP2X_2$ receptor	$rP2X_{2/6}$ receptor
pH 7.5	10.4 ± 1.2	6.06 ± 1.22
	(−0.83 ± 0.12)	(−1.19 ± 0.14)
pH 6.5	0.078 ± 0.005	0.013 ± 0.003 (I_1)*
	(−1.19 ± 0.08)	(−1.28 ± 0.15)
		1.61 ± 0.28 (I_2)*
		(−0.96 ± 0.16)

IC_{50} values (μM) and Hill slopes (n_H, in brackets) for suramin blockade of $rP2X_2$ and $rP2X_{2/6}$ receptors at the given extracellular pH (pH_e) levels are shown. The inhibition curve for suramin blockade of $rP2X_{2/6}$ receptors was biphasic at pH 6.5, showing high-affinity (I_1) and low-affinity (I_2) components of blockade of ATP responses (Fig. 4C). The IC_{50} values of $rP2X_2$ and $rP2X_{2/6}$ receptors, at pH 6.5, were significantly different (*$p < 0.05$, by unpaired t test). Data are expressed as mean ± SEM ($n = 4$ for $rP2X_2$ at pH 7.5 and 6.5, and for $rP2X_{2/6}$ at pH 7.5; $n = 8$ for $rP2X_{2/6}$ at pH 6.5).

tion, only managed to produce low-amplitude responses. Such weak ATP responses were attributed to the activation of just a small number of functional $rP2X_6$ receptors, because defolliculated oocytes do not possess native P1 or P2 receptors to complicate the analysis of agonist actions (King et al., 1996a,b). Homomeric $rP2X_6$ receptors have thus far been reported to function well in human embryonic kidney (HEK) 293 cells (Buell et al., 1996; Collo et al., 1996), to be silent in HEK 293 cells (Torres et al., 1999), or not to function at all in *Xenopus* oocytes (Soto et al., 1996; Lê et al., 1998). Our initial experiments thus confirmed that there are difficulties associated with $rP2X_6$ receptor expression in *Xenopus* oocytes and, in all probability, in other cell systems. It is possible that *Xenopus* oocytes and occasionally HEK 293 cells fail to produce an essential protein necessary to insert $P2X_6$ subunits into the cell membrane. One plausible candidate for this protein is another P2X subunit, perhaps the $P2X_4$ subunit, on the grounds that a $P2X_4$-like cDNA (AF012903) has been isolated from HEK 293 cells (direct submission GenBank by Chang and Chang in 1996) and the $P2X_4$ protein is present at low levels in these cells (Worthington et al., 1999). The heteromeric $rP2X_{4/6}$ receptor is similar in its functional properties to the operational profile of homomeric $rP2X_6$ receptors (Lê et al., 1998). Because <5% of HEK 293 cells transfected with $rP2X_6$ cDNA go on to assemble a functional $P2X_6$ (or possibly $P2X_{4/6}$-like) receptor (Collo et al., 1996), the $P2X_4$ subunit may not be present in all HEK 293 cells.

Where coexpression of $P2X_2$ and $P2X_6$ subunits in *Xenopus* oocytes was concerned, our experiments were based on a comparison of the operational profiles of wild-type $rP2X_2$ receptors and heteromeric $rP2X_{2/6}$ receptors. The $rP2X_2$ receptor has already been characterized in our laboratory in an extensive survey of agonists, antagonists, and modulators at this ATP-gated ion channel (King et al., 1996c, 1997; Pintor et al., 1996; Wildman et al., 1997, 1998, 1999a,b,c). Torres and colleagues (1999) have demonstrated that epitope-tagged $rP2X_2$ and $rP2X_6$ subunits will coprecipitate when expressed in a heterologous expression system. Thus, our present results confirm that functional heteromeric $P2X_{2/6}$ receptors are indeed formed and inserted into the membrane of *Xenopus* oocytes. Several key observations were made on this new heteromeric P2X receptor, particularly (1) the nature of the evoked inward currents, (2) the potency of agonists, and (3) the effect of pH on ATP responses and suramin blockade.

ATP-evoked inward currents at heteromeric $rP2X_{2/6}$ receptors were sometimes biphasic in nature, involving transient and sustained components that varied in amplitude from response to

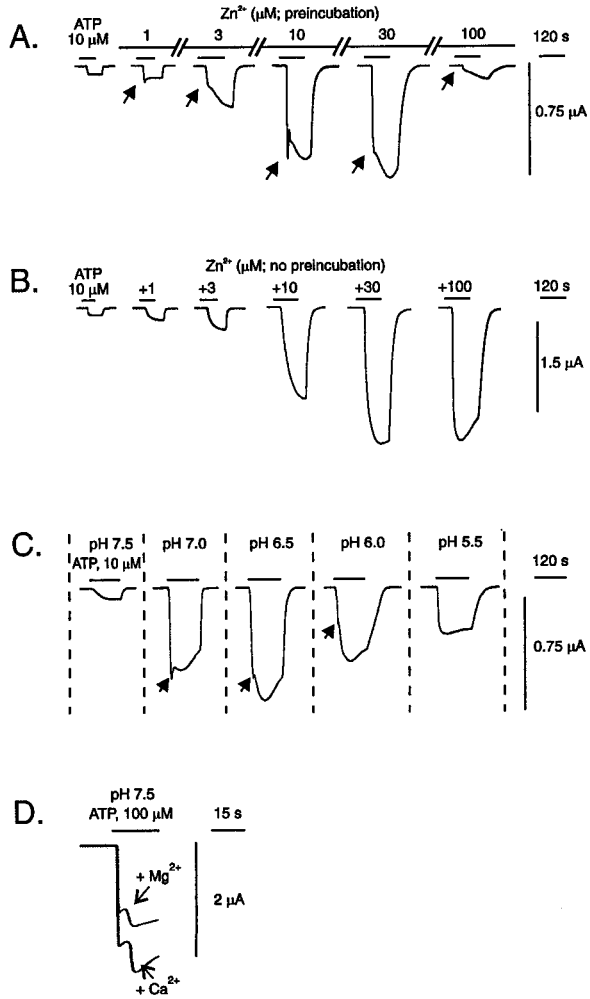

Figure 5. Modulation of ATP responses by Zn^{2+} and H^+ at $rP2X_{2/6}$ receptors. *A*, Concentration-dependent potentiation and inhibition of agonist-evoked inward currents by extracellular Zn^{2+} (1–100 μM) given 5 min before and during ATP application at $rP2X_{2/6}$ receptors, at pH 7.5. EC_{50} values (micromolar concentration) for Zn^{2+} potentiation of ATP responses was $rP2X_{2/6}$, 6.8 ± 1.0 versus $rP2X_2$, 6.9 ± 1.1 ($n = 4$). *B*, Concentration-dependent potentiation of ATP-evoked inward currents by extracellular Zn^{2+} (1–100 μM) applied simultaneously with the agonist. Under these circumstances, biphasic currents were rarely seen, and the inhibitory action of Zn^{2+} was lost. EC_{50} values (micromolar concentration) for Zn^{2+} potentiation of ATP responses were $rP2X_{2/6}$, 8.2 ± 0.5 versus $rP2X_2$, 11.7 ± 2.8 ($n = 6$). *C*, Concentration-dependent potentiation and inhibition of ATP-evoked inward currents by extracellular H^+ ions (pH 7.0–5.5) at $rP2X_{2/6}$ receptors. pK_a values ($-\log_{10}[H^+]$ causing 50% potentiation) were $rP2X_{2/6}$, 7.04 ± 0.05 versus $rP2X_2$, 7.05 ± 0.05 ($n = 4$). *D*, Paired biphasic inward currents evoked by ATP (100 μM, at pH 7.5) at $rP2X_{2/6}$ receptors with either Mg^{2+} or Ca^{2+} (1.8 mM) present in the bathing solution. Substitution of Ca^{2+} with Mg^{2+} resulted in a reduction of ATP potency [as shown for $rP2X_2$ receptors (King *et al.*, 1997)] without significantly altering the appearance of biphasic currents. *Filled arrows* draw attention to transient component of ATP-evoked inward currents (*A*, *C*). Data are expressed as mean ± SEM for four to six cells per determination.

response in the same cell. However, the incidence and reproducibility of biphasic responses in each oocyte varied in an unpredictable manner. The incidence of biphasic currents was greater and reproducibility more consistent when extracellular Zn^{2+} was present (Fig. 5*A*) or extracellular pH was lowered (Fig. 5*C*). Biphasic currents have already been reported at homomeric

$rP2X_4$ receptors, at which time-dependent changes in channel permeability were observed and shifts in the reversal potential for ATP-evoked currents noted (Khakh et al., 1999; Virginio et al., 1999). The binary permeability properties of $rP2X_4$ receptors were seen only when extracellular Ca^{2+} levels were lowered or zero $Ca^{2+}{}_o$ conditions imposed (Khakh et al., 1999). Therefore, we explored this possibility and found that biphasic responses at heteromeric $rP2X_{2/6}$ receptors were not enhanced when Ca^{2+} was replaced with equimolar Mg^{2+} (Fig. 5*D*). Ca^{2+}-independent binary permeability properties have been reported for homomeric $rP2X_2$ receptors, although the time- and concentration-dependent changes in permeability do not result in biphasic currents to ATP (Khakh et al., 1999; Virginio et al., 1999). Others have reported, however, that $rP2X_2$ receptor ion channels do not show significant changes in unitary conductance or reversal potential of whole-cell currents (Ding and Sachs, 1999b). This inconsistency with the $P2X_2$ receptor is reminiscent of the variability of agonist responses (monophasic and biphasic) at the heteromeric $P2X_{2/6}$ receptor. Currently, there is no satisfactory explanation for biphasic ATP responses at heteromeric $P2X_{2/6}$ receptors.

The potency of ATP was lower at heteromeric $rP2X_{2/6}$ receptors than homomeric $rP2X_2$ receptors, regardless of the pH level studied (Table 1). Although ATP potency was decreased overall, the rank potency order for mononucleotidic agonists at the heteromeric receptor remained the same as at the $rP2X_2$ receptor, namely ATP = ATPγS > 2-MeSATP > ATPαS > BzATP. One significant difference in agonist activity involved the dinucleotide diadenosine tetraphosphate (Ap_4A), which is a full and potent agonist at $rP2X_2$ receptors (Pintor et al., 1996; Wildman et al., 1999a) and only a weak agonist at $rP2X_{2/6}$ receptors. This difference in Ap_4A activity is potentially important, because this dinucleotide occurs naturally and is released in a Ca^{2+}-dependent manner from central synaptosomes in rat brain (Pintor et al., 1992). Therefore, Ap_4A may subserve a transmitter role at homomeric $rP2X_2$ receptors but not at heteromeric $rP2X_{2/6}$ receptors.

Extracellular pH is known to exert a profound effect on ATP potency at homomeric $rP2X_2$ receptors (King et al., 1996c, 1997; Stoop et al., 1997; Wildman et al., 1997, 1998, 1999b,c; Stoop and Quayle, 1998; Ding and Sachs, 1999a). A secondary inhibitory effect is observed at very low pH levels (e.g., pH 4.2), at which ATP responses rapidly desensitize, yet recover quickly, if pH_e is reversed to levels above pH 5.0, a phenomenon called "fade and rebound" (Stoop and Quayle, 1998). The heteromeric $rP2X_{2/6}$ receptor showed both the potentiating and inhibitory effects of extracellular H^+, and the pH ranges for these two separate effects are compressed when compared with $rP2X_2$ receptors. The inhibitory effect was caused by a reduction in agonist efficacy alone and not a decrease in agonist potency, as evidenced by the lower maximum for the ATP C–R curve at pH 5.5 (Fig. 2*C*). Because some homomeric P2X receptors ($rP2X_1$, $rP2X_3$, $rP2X_4$, and $rP2X_7$) also show a reduction in ATP activity when pH_e is lowered (Virginio et al., 1997; Wildman et al., 1999c), it is conceivable that the observed H^+ inhibitory effect at $rP2X_{2/6}$ receptors is caused as much by an action of H^+ ions at the $rP2X_6$ subunit as at the $rP2X_2$ subunit.

The potency of suramin is progressively enhanced at $rP2X_2$ receptors as pH is lowered, with blockade occurring at nanomolar concentrations at pH 5.5 (King et al., 1997). The present results now show that this is an attribute shared by heteromeric $rP2X_{2/6}$ receptors, although subtle differences were observed at pH 6.5 for suramin blockade of homomeric $rP2X_2$ and heteromeric $rP2X_{2/6}$ receptors. There appeared to be high-affinity (I_1) and low-affinity (I_2) sites for suramin at $rP2X_{2/6}$ receptors, and activity indices for each component failed to match the corresponding IC_{50} value at $rP2X_2$ receptors. The precise cause of this unusual effect is as yet unresolved. However, one possibility may involve differences in the subunit composition of heteromeric $P2X_{2/6}$ receptors, if subpopulations of oligomeric assemblies containing different numbers of $rP2X_6$ subunits were generated. Where shown to be functional, the homomeric $rP2X_6$ receptor (or even the heteromeric $rP2X_{4/6}$ receptor) has been reported to be relatively insensitive to suramin blockade (Collo et al., 1996; Lê et al., 1998). The suramin insensitivity of the $P2X_6$ subunit might help contribute to biphasic inhibition curves seen at pH 6.5 with the heteromeric $P2X_{2/6}$ receptor.

The potentiating effect of extracellular Zn^{2+} was not significantly different at $rP2X_2$ and $rP2X_{2/6}$ receptors. However, one subtle difference was noted when using high concentrations ($\geq$ 100 μM) of this transition metal, which appeared to directly activate the heteromeric $rP2X_{2/6}$ receptors without the need for exogenous ATP (data not shown). It is known that *Xenopus* oocytes continuously extrude small amounts of intracellular ATP via a mechanogated transport pathway (Nakamura and Strittmatter, 1996), and consequently the potency of locally released ATP may be sufficiently elevated by Zn^{2+} ions to explain the apparent Zn^{2+}-activated inward currents. The subsequent inhibition of ATP responses by high concentrations of Zn^{2+} ions may be caused by a gradual desensitization of the receptor pool by locally released ATP.

In conclusion, the heteromeric $P2X_{2/6}$ receptor possesses a significantly different operational profile from the wild-type $P2X_2$ receptor. It is of interest to us that $rP2X_2$ and $rP2X_6$ transcripts are found in rat brainstem (Collo et al., 1996; Comer et al., 1997) in nuclei with demonstrable pH-dependent chemoreceptive inputs (Thomas et al., 1999). The pH modulation of the homomeric $P2X_2$ and heteromeric $rP2X_{2/6}$ receptor forms an interesting basis for examining the recently discovered involvement of ATP receptors in the CO_2-evoked (and pH-dependent) changes in central respiratory drive in rat (Thomas et al., 1999; Thomas and Spyer, 2000). At this point in time, however, the present results establish the $P2X_{2/6}$ receptor as the fourth example of a heteropolymeric ATP-gated ion channel, which in this case possesses a pattern of pH modulation of ATP responses distinct from other known homomeric and heteromeric P2X receptors.

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