Heteromultimeric P2X$_{1/2}$ Receptors Show a Novel Sensitivity to Extracellular pH

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Abstract

Rat P2X$_1$ and P2X$_2$ subunits were coexpressed in defolliculated Xenopus oocytes and the resultant P2X receptors studied under voltage-clamp conditions. Extracellular ATP elicited biphasic inward currents, involving an initially rapidly inactivating (P2X$_1$-like) component and a later slowly inactivating (P2X$_2$-like) component. The maximum amplitude of P2X$_2$-like ATP responses was increased in some cells by lowering extracellular pH (from 7.5 to 6.5), whereas P2X$_1$-like responses and those of homomeric P2X$_2$, and P2X$_6$ receptors were not changed by this treatment. Concentration-response (C/R) curves for ATP for pH-enhanced P2X$_2$-like responses were biphasic, and clearly distinct from monophasic ATP C/R curves for homomeric P2X$_2$, and ATP C/R curves for P2X$_1$-like responses showed increases in agonist potency and efficacy, compared with data at pH 7.5, but the same was not true of homomeric P2X$_2$, or P2X$_6$ receptors. ATP C/R curves for P2X$_2$-like responses overlay C/R curves for homomeric P2X$_2$, receptors, and determinations of agonist potency and efficacy were identical for P2X$_2$-like and P2X$_6$ responses at all pH levels tested. Our results show that P2X$_2$-like responses possessed the kinetics of homomeric P2X$_1$ receptors but an acid sensitivity different from homomeric P2X$_2$, and P2X$_6$ receptors. In contrast, the P2X$_2$-like responses exactly matched the profile expected of homomeric P2X$_2$ receptors. Thus, coexpression of P2X$_1$ and P2X$_2$ subunits yielded a mixed population of homomeric and heteromeric P2X$_{1/2}$ receptors, with a subpopulation of novel pH-sensitive P2X$_{1/2}$ receptors showing identifiably unique properties that indicated the formation of heteromeric P2X$_{1/2}$ ion channels.

ATP acts as a fast excitatory transmitter in the central, peripheral, and enteric nervous systems (Ralevic and Burnstock, 1998). Here, extracellular ATP exerts its effects through two classes of P2 receptors: the P2X and P2Y families (Burnstock and King, 1996). Seven subunits of the P2X receptor class (P2X$_1$–7) have been cloned thus far. P2X subunits have two membrane-spanning domains connected by a large cysteine-rich extracellular loop, with three, or possibly four, subunits assembling to form ligand-gated cation channels.

AABBREVIATIONS: cRNA, capped RNA; C/R, concentration-response; Ap$_6$A, P$_1$P$_6$-diadenosine hexaphosphate ammonium salt; α,β-meATP, α,β-methylene ATP lithium salt; pH$_e$, extracellular pH.
coexist in respiratory centers in the rat brainstem and these two subunits form heteromeric P2X$_{2/6}$ receptors with distinct properties (King et al., 2000).

Biochemical evidence, from coimmunoprecipitation experiments, has supported the possible association of P2X$_1$ and P2X$_2$ subunits and formation of a heteroreceptor (Torres et al., 1999). However, supporting evidence for in vivo formation of P2X$_{1/2}$ heteromers rests solely with the colocalization of P2X$_1$ and P2X$_2$ transcripts and their proteins. Overlapping expression of P2X$_2$ and P2X$_3$ transcripts is seen in sensory and auditory nerves and in regions of the developing rat brain (Kidd et al., 1995; Xiang et al., 1998, 1999). Furthermore, positive immunoreactivity is seen for P2X$_1$ and P2X$_2$ subunits in the dorsal horn of the spinal cord and selected regions of the adult rat brain (Kanjan et al., 1996; Vulchanova et al., 1996; Loesch and Burnstock, 1998).

Where homomeric and heteromeric P2X receptors have been studied and compared, it has been difficult to clearly distinguish one receptor subtype from another solely on the basis of their agonist profiles. However, another way to differentiate P2X subtypes is to monitor their reaction to changes in extracellular pH. Past studies have revealed homomeric P2X$_2$ receptors show an increase in agonist potency, without changing the maximum response, when the bathing solution is made more acidic (pH < 7.5) and a decrease in agonist potency, without changing the maximum response, under more alkaline conditions (pH > 7.5) (King et al., 1996c, 1997; Stoop et al., 1997). Other studies have revealed homomeric P2X$_1$ receptors show a different pattern of pH sensitivity: a decrease in agonist potency, without change in the maximum response, under acidic conditions and no effect on agonist potency and efficacy under alkaline conditions (Stoop et al., 1997; Wildman et al., 1999). Where either P2X$_1$ or P2X$_2$ subunits have been coexpressed with other P2X subunits (e.g., P2X$_3$, P2X$_5$, or P2X$_6$), the resultant heteromeric P2X receptors show a pH sensitivity that is different from the phenotype expected of homomeric P2X$_1$ and P2X$_2$ receptors (King et al., 2000; Surprenant et al., 2000; Liu et al., 2001).

In the present study, the possibility of heteromeric assemblies of P2X$_1$ and P2X$_2$ subunits was examined by comparing, at different extracellular pH levels, the pharmacological and kinetic profiles of recombinant P2X receptors formed by coexpression of these two P2X subunits in defolliculated Xenopus oocytes. The results indicate the presence of a novel pH-sensitive P2X receptor phenotype and highlight the increased complexity in ATP-mediated excitatory transmission through heteropolymerization of P2X subunits.

**Materials and Methods**

**Preparation and Injection of Oocytes with Capped RNA (cRNA).** Xenopus laevis were anesthetized with Tricaine (3-aminobenzoic acid ethyl ester; 0.2%; w/v; Sigma Chemical, Poole, Dorset, UK) and killed by decapitation. Mature oocytes (stages V and VI) were harvested and prepared for injection as described in detail previously (King et al., 1997). Defolliculated oocytes do not possess native P1 or P2 receptors that might otherwise complicate the analysis of agonist activity (King et al., 1996a, b). Defolliculated oocytes were injected cytosolically with a mixture of cRNAs. This cRNA mixture consisted of 20 nl of cRNA encoding rat P2X$_2$ (1 µg/µl; Valera et al., 1994) and 20 nl of cRNA encoding rat P2X$_3$ (0.002 µg/µl; Brake et al., 1994). Some batches of oocytes were injected with 40 nl of cRNA for either rat P2X$_1$ or rat P2X$_2$ alone. Injected oocytes were incubated at 18°C in Barth’s solution, pH 7.5, containing 110 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 7.5 mM Tris-HCl, 0.33 mM Ca(NO$_3$)$_2$, 0.41 mM MgCl$_2$, 0.82 mM MgSO$_4$, supplemented with 50 µM 1'-gamentan sulfate for 24 h and then stored at 4°C for up to 10 days.

**Electrophysiology.** Membrane currents were recorded under voltage-clamp conditions by using a twin-electrode amplifier (Axoc-lamp 2B; Axon Instruments, Union City, CA). Intracellular micro-electrodes were filled with 3 M KCl and showed 1 to 2 MΩ resistance. Oocytes were placed in a Perspex recording chamber and perfused at a constant rate of 5 ml/min $^{-1}$ with Ringer’s solution containing 110 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1.8 mM BaCl$_2$, adjusted to pH 7.5. The pH level of all drugs solutions stated in the text was adjusted by adding either 1 N HCl or 1 N NaOH. Solutions were delivered by a gravity flow system from separate reservoirs placed above the recording chamber. All drugs were prepared in nominally Ca$^{2+}$-free Ringer’s solution at the concentrations stated in the text. Agonists were perfused for 30 s or until the evoked current reached a maximum. Applications of agonists were separated by intervals of 20 min. All recordings were made at room temperature (18°C) and at a holding potential of between −60 and −90 mV. Electrophysiological data were recorded using the software package Acqknowledge III (Biopac Systems; Goleta, CA).

**Data Analysis.** EC$_{50}$ values for agonists were taken from Hill plots by using the transformation log (I/I$_{max}$ − I), where I is the current evoked by each concentration of agonist. Hill coefficients were also taken from the slope of these plots. Concentration/response (C/R) curves were fitted by nonlinear regression analysis by using commercial software (Prism version 2.0; GraphPad Software, San Diego, CA). Data are presented as mean ± S.E.M. of four or more determinations. Significant differences were determined by either unpaired Student's t test or one-way analysis of variance followed by Dunnett’s test, by using commercially available software (Instat version 2.05a; GraphPad Software).

**Chemicals.** All common salts were AnalR-grade (Alrdich Chemical, Gillingham, UK). ATP disodium salt was purchased from Roche Molecular Biochemicals (Mannheim, Germany) and Sigma/RBI (Natick, MA). P$_1$, P$_6$-Diadenosine hexaphosphate (Ap$_6$A, ammonium salt), α,β-methylene ATP (α,β-meatP, lithium salt) were purchased from Sigma Chemical. Agonist solutions were prepared daily, made up in extracellular bathing medium, and the pH adjusted to the desired level.

**Results**

**Use of Acidic Conditions to Distinguish Types of P2X Receptors.** The coexpression of two P2X subunits, individually capable of forming homomeric P2X receptors (e.g., P2X$_1$ and P2X$_2$), has been shown to generate a mixed population of homomeric and heteromeric assemblies (Liu et al., 2001). This finding also appeared to be true for P2X$_1$/P2X$_2$ cRNA-coexpressed oocytes. Here, ATP (100 µM) evoked biphasic inward currents that comprised an initial rapidly inactivating (P2X$_1$-like) component followed by a second slowly inactivating (P2X$_2$-like) component (Fig. 1). Thus, it was necessary to find a way to separate the agonist responses of homomeric rP2X$_1$ and rP2X$_2$ receptors from those mediated by putative heteromeric P2X$_{1/2}$ receptors.

The extracellular pH (pH$_e$) of bathing solutions is known to influence ATP responses at homomeric rP2X$_1$ and rP2X$_2$ receptors in opposite ways (King et al., 1996c, 1997; King et al., 1996c, 1997; Stoop et al., 1997; Wildman et al., 1999). Thus, lowering pH (from 7.5 to 6.5) inhibits submaximal ATP responses at P2X$_1$ receptors and potentiates submaximal ATP responses at P2X$_2$ receptors, but fails to alter the amplitude of the maximum response for each P2X subtype.
Therefore, coinjected oocytes were tested at two pH levels (7.5 and 6.5) in the hope of revealing differences between ATP responses at homomeric and heteromeric P2X assemblies (Fig. 1, A and B).

In most cells tested (from an initial sample of 73 of 87 coinjected oocytes), the P2X$_1$-like response to ATP (100 µM) was inhibited at pH 6.5 and the P2X$_{2/3}$-like response unaffected or slightly potentiated (Fig. 1A). In a smaller subset of tested cells (14 of 87 coinjected oocytes; 16%), the rapidly inactivating P2X$_1$-like response was significantly potentiated at pH 6.5 and this finding indicated a novel P2X receptor might be involved (Fig. 1B). Further batches of P2X$_1$-P2X$_{2/3}$ cRNA-coinjected oocytes were prepared and surveyed to explore the properties of rapidly inactivating P2X$_1$-like responses potentiated under acidic conditions.

**Potency of Agonists Mediating pH-Sensitive Inward (P2X$_1$-Like) Currents.** Recombinant P2X receptors in coinjected oocytes reacted to very low concentrations of ATP, with a threshold below 10 nM, and were activated maximally at high ATP concentrations (100–300 µM). The amplitude of these rapidly inactivating P2X$_1$-like responses grew incrementally over this extended concentration range (10 nM–300 µM) (Fig. 2A), whereas the slower P2X$_{2/3}$-like responses were evident only over a limited concentration range (approximately 3–300 µM). The C/R curve for ATP activated P2X$_1$-like responses is shown in Fig. 3A. The apparent EC$_{50}$ value (and Hill coefficient) was 0.56 ± 0.09 µM ($n_H = 0.37$) ($n = 9$), but the C/R curve was shallow and appeared to be biphasic. Resolving for each phase, mean EC$_{50}$ values were 54 nM ($n_H = 1.05$) and 3.28 µM ($n_H = 0.82$). This first EC$_{50}$ value matches a determination for ATP potency at homomeric hP2X$_1$ receptors (mean EC$_{50}$ of 56 nM; Bianchi et al., 1999), but is statistically lower ($p < 0.05$) than our present determination for homomeric rP2X$_1$ receptors (mean EC$_{50}$ of 98 nM; $n_H = 0.80$) (Fig. 3B). The second EC$_{50}$ value is unrelated to any determination for ATP potency at homomeric P2X$_1$ receptors.

Ap$_6$A and α,β-meaATP also evoked rapidly inactivating P2X$_1$-like responses in P2X$_1$-P2X$_{2/3}$ cRNA-coinjected oocytes (Fig. 2B). Ap$_6$A is inactive at homomeric rP2X$_2$ receptors (Jacobson et al., 2000) and, accordingly, failed to evoke slow inward currents in our experiments (Fig. 2B). α,β-meaATP is a weak agonist at homomeric P2X$_2$ receptors (Jiang et al., 2001) and only evoked very small P2X$_{2/3}$-like responses (Fig. 2B). The resultant agonist C/R curves for P2X$_1$-like responses were shallow and extended over a wide concentration range (3 nM–100 µM) (Fig. 3A) to give EC$_{50}$ values for Ap$_6$A of 0.74 ± 0.10 µM ($n_H = 0.50$) ($n = 6$); and for α,β-meaATP of 0.43 ± 0.05 µM ($n_H = 0.89$) ($n = 8$). The C/R curve for Ap$_6$A appeared to be biphasic, with mean EC$_{50}$ values of 49 nM ($n_H = 1.37$) and 2.02 µM ($n_H = 1.25$). However, we were unable to dissect the C/R curve for α,β-meaATP into two phases even though the C/R curve was shallow. Agonist potency data are summarized in Table 1.

**Effects of Extracellular pH on Agonist Efficacy at P2X$_1$-Like Responses.** The C/R relationship for rapidly inactivating P2X$_1$-like ATP responses was reexamined at four different levels of pH$_e$ (Fig. 4, A–D). At the four levels tested...
Fig. 3. Agonist potency at P2X subunit assemblies. A, C/R curves for rapidly inactivating P2X$_1$-like responses evoked by ATP, Ap$_6$A and $\alpha$,$\beta$-meATP in oocytes coexpressing rP2X$_1$ and rP2X$_2$ subunits. Data given as mean ± S.E.M. (6–9 sets of observations). B, C/R curves for ATP, Ap$_6$A and $\alpha$,$\beta$-meATP in oocytes expressing homomeric P2X$_1$ receptors ($n$ = 4–8). Where missing, error bars are occluded by symbols. Curves fitted to the Hill equation, by using Prism version 2.0 (GraphPad Software).

(pH 8.5, 7.5, 6.5, and 5.5), the resultant C/R curves extended over 5 log$_{10}$ units of agonist concentration (1 nM-100 μM) and the slopes of these curves were shallow ($n_H$ ≤ 0.5). It was difficult to dissect some ATP C/R curves into first and second phases, particularly at pH 5.5, where agonist sensitivity was heightened. However, it was clear that the amplitude of P2X$_1$-like responses was consistently greater under acidic conditions at all ATP concentrations tested (especially at pH 6.5 and 5.5) (Fig. 4, C and D). Compared with control data at pH 7.5, the relative amplitude of the maximum response ($I_{max}$) was 134 ± 8% (at pH 6.5; $n$ = 6) and 284 ± 18% (at pH 5.5; $n$ = 4). Thus, one effect of acidic pH$_a$ was an increase in agonist efficacy.

Under alkaline conditions (pH 8.5), the amplitude of P2X$_1$-like responses at low concentrations (ATP, 1–100 nM) was not significantly different from P2X$_1$-like responses obtained at pH 7.5 (Fig. 4B). At higher ATP concentrations (300 nM-100 μM), the amplitude of P2X$_1$-like responses was greater at pH 8.5 than pH 7.5 (Fig. 4B). Compared with control data, the relative amplitude of the maximum response ($I_{max}$) was 154 ± 16% at pH 8.5 ($n$ = 5). Thus, the amplitude of P2X$_1$-like responses and agonist efficacy were enhanced under both alkaline (pH 8.5) and acidic (pH 6.5 and 5.5) conditions. It is not unknown for alkaline and acidic conditions to exert the same, rather than opposing, effects at P2X receptors. At heteromeric P2X$_{1,5}$ receptors, for example, acidic and alkaline bathing solutions exert the same effect on agonist efficacy although, in this case, it is reduced (Surprenant et al., 2000).

Effects of Extracellular pH on Agonist Potency at P2X$_1$-Like Responses. The effects of extracellular pH on agonist potency were assessed in one of two ways. Where there was no clear boundary between two phases of the C/R curve (particularly at pH 6.5 and 5.5), agonist potency was assessed as the EC$_{50}$ value over the full concentration range (1 nM-100 μM) (Fig. 4). Where possible, C/R curves were analyzed over first and second phases of the curve and changes in EC$_{50}$ values noted. This was only possible for C/R curves defined at pH 7.5 and pH 8.5 (Fig. 4).

Where C/R curves were analyzed over the full concentration range, apparent EC$_{50}$ values were as follows for P2X$_1$-like responses: pH 8.5, 0.39 ± 0.13 μM ($n$ = 5); pH 7.5, 0.55 ± 0.09 μM ($n$ = 9); pH 6.5, 0.08 ± 0.02 μM ($n$ = 6); and pH 5.5, 0.12 ± 0.05 μM ($n$ = 4). Thus, ATP potency was not significantly different at pH 8.5 and 7.5, yet was enhanced by 5- to 7-fold at pH 6.5 and 5.5. These results contrast with data for homomeric rP2X$_1$ receptors where it is known that ATP potency is reduced under acidic conditions (Stoop et al., 1997; Wildman et al., 1999). The effects of pH on P2X$_1$-like responses and responses by homomeric rP2X$_1$ receptors are summarized in Table 2.

Data were reanalyzed to take into account the biphasic nature of C/R curves defined at pH 7.5 and pH 8.5 (Fig. 4B). For the first phase, mean EC$_{50}$ values were 48 nM (pH 7.5) and 45 nM (pH 8.5) and, for the second phase, 1.59 and 1.42 μM, respectively. Thus, ATP potency was not affected under alkaline conditions at either phase of these complex C/R relationships.

Potency of Agonists Mediating Slow Inward (P2X$_{2,}$-Like) Currents. Slowly inactivating P2X$_{2,}$-like responses were evoked at ATP concentrations in excess of 3 μM and maximal at high concentrations (300 μM) (Fig. 2A). It was not possible to determine the threshold concentration required to elicit these slow responses, because the initial P2X$_1$-like responses showed deactivating tail currents that obscured the smallest of P2X$_{2,}$-like responses. The C/R curve for ATP-mediated P2X$_{2,}$-like responses is shown in Fig. 5. At pH 7.5, the apparent EC$_{50}$ value (and Hill coefficient) was 7.7 ± 0.6 μM ($n_H$ = 1.00) for the P2X$_{2,}$-like response, which was not significantly different from the determination for homomeric rP2X$_2$ receptors (5.6 ± 0.5 μM; $n_H$ = 1.21) (Fig. 5). At pH 6.5, the EC$_{50}$ value for the P2X$_{2,}$-like response was 0.71 ± 0.06 μM ($n_H$ = 1.95), similar to the determination for homomeric rP2X$_2$ receptors (1.09 ± 0.12 μM; $n_H$ = 1.81) (Fig. 5). The ATP C/R curves for P2X$_{2,}$-like responses and responses by homomeric rP2X$_2$ receptors appeared to be monophasic at the pH levels tested. Also, the maximum amplitude for P2X$_{2,}$-like responses, as for homomeric rP2X$_2$ receptors (King et al., 1996c), was not significantly different at the pH levels tested (Fig. 5B). Furthermore, Ap$_6$A and $\alpha$,$\beta$-meATP (both 30 μM) were ineffective at eliciting P2X$_{2,}$-like responses (Fig. 2B) or activating homomeric rP2X$_2$ receptors (Jacobson et al., 2000).
In the present study, heterologous coexpression of P2X1 and P2X2 subunits in defolliculated Xenopus oocytes resulted in the formation of a complex population of P2X receptors. Activation of these P2X receptors with extracellular ATP resulted in biphasic inwards currents that involved rapidly and slowly inactivating components and, at first glance, these results could be explained by the successive activation of homomeric P2X1 and P2X2 receptors. This conclusion has been already stated in a preliminary report on the coexpression of P2X1 and P2X2 subunits (Lewis et al., 1995). In the intervening time, however, much more has been learned about the operational profiles of homomeric rP2X1 and rP2X2 receptors, not least, the influence of extracellular pH on agonist activity (King et al., 1996c, 1997, 2000; Stoop et al., 1997; Stoop and Quayle, 1998; Wildman et al., 1997, 1999; Ding and Sachs, 1999). Also, further recent biochemical evidence has suggested that P2X1 and P2X2 subunits can heteropolymerize (Torres et al., 1999).

By lowering extracellular pH (from pH 7.5 to 6.5), we noted that a relatively small sample (14 of 87 cells) of P2X1/P2X2 cRNA-coinjected oocytes responded to ATP with rapidly inactivating inward currents (P2X1-like responses) that were potentiated under acidic conditions. This behavior was atypical of fast inward currents carried by homomeric rP2X1 receptors, which are inhibited under acidic conditions by a mechanism that decreases ATP potency (Stoop et al., 1997; Wildman et al., 1999). On the other hand, P2X1-like responses in a sample of 73 of 87 cells were decreased under acidic conditions and, here, we have assumed that homomeric rP2X1 receptors were in abundance. Thus, we believe the cellular conditions in oocytes may favor the assembly of homomeric P2X1 receptors over heteromeric P2X1/2 receptors, although these heteromeric assemblies appear to be abundant in approximately one in six cells. In this limited population of cells, the outcome of lowering extracellular pH seemed more in keeping with homomeric P2X2 receptors, at which the amplitude of submaximal ATP responses is enhanced under acidic conditions by a mechanism increasing ATP potency (King et al., 1996c, 1997, 2000; Stoop et al., 1997; Ding and Sachs, 1999). Thus, P2X1-like responses possessed the kinetics of homomeric P2X1 receptors and, to an extent, the acid sensitivity of homomeric P2X2 receptors. It is therefore possible that a significant part of P2X1-like re-

### Table 1

<table>
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<tr>
<th>Receptor</th>
<th>ATP EC\textsubscript{50} (nM)</th>
<th>ATP n\textsubscript{H}</th>
<th>Ap\textsubscript{50} EC\textsubscript{50} (nM)</th>
<th>Ap\textsubscript{50} n\textsubscript{H}</th>
<th>(\alpha,\beta)-meATP EC\textsubscript{50} (nM)</th>
<th>(\alpha,\beta)-meATP n\textsubscript{H}</th>
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<tr>
<td>Homomeric rP2X1</td>
<td>98 ± 11</td>
<td>0.80</td>
<td>2598 ± 721</td>
<td>0.74</td>
<td>3301 ± 492</td>
<td>0.77</td>
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<td>1st phase</td>
<td>54 ± 19</td>
<td>1.05</td>
<td>49 ± 10</td>
<td>1.37</td>
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<tr>
<td>2nd phase</td>
<td>3276 ± 309</td>
<td>0.82</td>
<td>2020 ± 163</td>
<td>1.25</td>
<td>Not determined</td>
<td></td>
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<tr>
<td>Full range</td>
<td>555 ± 84</td>
<td>0.37</td>
<td>741 ± 98</td>
<td>0.50</td>
<td>427 ± 50</td>
<td>0.89</td>
</tr>
<tr>
<td>Homomeric P2X2</td>
<td>5623 ± 488</td>
<td>1.21</td>
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<td></td>
<td>Inactive</td>
<td></td>
</tr>
<tr>
<td>P2X1-like</td>
<td>7728 ± 569</td>
<td>1\textsuperscript{a}</td>
<td>Inactive</td>
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<td>Inactive</td>
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\(\textsuperscript{a}\) The Hill coefficient for the P2X2-like response was constrained to a value of 1 because of limited C/R data at low agonist concentrations (<10 \textmu M).

### Discussion

In the present study, heterologous coexpression of P2X1 and P2X2 subunits in defolliculated Xenopus oocytes resulted in the formation of a complex population of P2X receptors. Activation of these P2X receptors with extracellular ATP resulted in biphasic inwards currents that involved rapidly and slowly inactivating components and, at first glance, these results could be explained by the successive activation of homomeric P2X1 and P2X2 receptors. This conclusion has been already stated in a preliminary report on the coexpression of P2X1 and P2X2 subunits (Lewis et al., 1995). In the intervening time, however, much more has been learned about the operational profiles of homomeric rP2X1 and rP2X2 receptors, not least, the influence of extracellular pH on agonist activity (King et al., 1996c, 1997, 2000; Stoop et al., 1997; Stoop and Quayle, 1998; Wildman et al., 1997, 1999; Ding and Sachs, 1999). Also, further recent biochemical evidence has suggested that P2X1 and P2X2 subunits can heteropolymerize (Torres et al., 1999).

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### Fig. 4

Effects of extracellular pH of ATP activity at P2X1-like responses. A, C/R curves for rapidly inactivating P2X1-like responses evoked by ATP at four levels of extracellular pH (8.7, 7.5, 6.5, and 5.5) Data given as mean ± S.E.M. (4–9 sets of observations). B to D, C/R curves are redrawn to compare the effects of test pH levels (B, pH 8.5; C, pH 6.5; D, pH 5.5) against control data (pH 7.5). Curves fitted to the Hill equation, by using Prism version 2.0 (GraphPad Software). Data compared by Student's unpaired t test (N.S., not significantly different; *, p < 0.05; **, p < 0.01).
sponses was mediated by heteromeric P2X_{1/2} receptors sharing some of the properties of their constituent P2X subunits.

Alternatively, it could be argued that, in those cells showing a potentiation of P2X_{1}-like responses under acidic conditions, this effect was no more than the relaxation of receptor desensitization for a significant proportion of the available homomeric P2X_{1} receptor population. However, several lines of evidence disprove this argument. First, the amplitude of P2X_{1}-like responses under control conditions was constant for successive agonist applications, consistently potentiating under acidic conditions, and returned to control values when pH levels were restored (Fig. 6). Second, the potentiation of P2X_{1}-like responses under acidic conditions was due to an increase in ATP potency, an effect unrelated to the number of P2X receptors available for activation. Third, P2X_{1}-like responses could be evoked by very low agonist concentrations, not only ATP but also Ap_{6}A and α,β-meATP, which are inert at rP2X_{2} receptors (Table 1). Also, elevation of the second phase of the ATP C/R curve at pH 8.5 (Fig. 4B) was inconsistent with the actions of alkaline bathing solutions at homomeric rP2X_{2} receptors (King et al., 1997). Furthermore, the elevation of ATP C/R curves for

![Image](308x496)

**Fig. 5.** Effects of extracellular pH of ATP activity at P2X_{1-like} responses. A, C/R curves for slowly inactivating inward currents evoked by ATP in oocytes either coexpressing P2X_{1} and P2X_{2} subunits or expressing homomeric rP2X_{2} receptors. ATP activity was assessed at pH 7.5 and again at pH 6.5. Curves fitted to the Hill equation, by using Prism version 2.0 (GraphPad Software). B, relative amplitude of maximum P2X_{1-like} responses to ATP (100 μM), at three test pH levels (8.5, 6.5, and 5.5), compared with control data (pH 7.5), in oocytes coexpressing P2X_{1} and P2X_{2} subunits. Data given as mean ± S.E.M. (4–9 observations). Data compared by Student’s unpaired t test (N.S., not significantly different).
P2X₁-like responses under acidic conditions (Fig. 4, C and D) was equally incompatible with the involvement of homomer rP2X₂ receptors (King et al., 1997). EC₅₀ values for ATP and Ap₆A fell in the region of 50 nM for the first phase of C/R curves for P2X₁-like responses, significantly different (p < 0.05) from agonist EC₅₀ values at homomeric rP2X₁ receptors (Fig. 3; Table 1). It has been reported that rapidly inactivating inward currents elicited by heteromeric P2X₁/µ receptors are also evoked by very low ATP concentrations (mean EC₅₀ of 55 nM) (Surprenant et al., 2000). Thus, a trend is emerging that P2X heteromers comprising P2X₁ subunits are extremely sensitive to ATP and, conceivably, this represents a useful adaptation to enhance purinergic signaling at sites where homomeric P2X₁ receptors are also used. EC₅₀ values for the second phase were in the low micromolar (~2–3 µM) concentration range and these values were unrelated to EC₅₀ values for homomeric P2X₁ or P2X₂ receptors (Table 1). Where EC₇₀ values were determined over the full range of data points for each C/R curve, the agonist potency order was α,β-meATP > ATP > Ap₆A, which, again, was unrelated to data for homomeric rP2X₁ receptors (ATP > Ap₆A > α,β-meATP) and homomeric rP2X₂ receptors (ATP active, α,β-meATP, a weak agonist, and Ap₆A inactive) (Table 1).

A thorough study of ATP potency and efficacy at different extracellular pH levels provided further evidence that P2X₁-like responses were mediated by novel pH-sensitive heteromeric P2X receptors. Here, we found that lowering pH caused an increase in the maximum ATP response and displaced the C/R curve in a leftwards manner. In contrast, acidic conditions decrease ATP potency without altering the maximum response at homomeric rP2X₁ receptors (Stoop et al., 1997; Wildman et al., 1999) (Table 2), or increase ATP potency without altering the maximum response at homomeric P2X₂ receptors (King et al., 1996c, 1997; Stoop et al., 1997) (Fig. 5). In the present study, we observed that raising pH increased the maximum ATP response without altering agonist potency for P2X₁-like currents, whereas, in contrast, alkaline conditions have no effect on ATP responses at homomeric P2X₁ receptors (Wildman et al., 1999), or decrease ATP potency without changing the maximum response at homomeric P2X₂ receptor (King et al., 1996c, 1997).

It seemed unlikely that P2X₂-like responses were mediated by homomeric P2X₂ receptors, for a number of reasons. The P2X₂-like responses were rapidly inactivating, evoked by Ap₆A and α,β-meATP, and their maximal amplitude potentiated by both acidic and alkaline conditions. None of these features match the profile of homomeric rP2X₂ receptors (King et al., 1997; Jacobson et al., 2000). Instead, there appeared to be a major role for homomeric rP2X₂ receptors in the later P2X₂-like responses that showed the appropriate sensitivity to ATP at all pH levels tested.

To the best of our knowledge, there are no reports of native P2X receptors in neural systems that are similar to the findings in this study, although our attention was drawn to a report on the guinea pig vas deferens where P2X₁-like responses are potentiated under acidic conditions (Nakanishi et al., 1999). However, the molecular characterization of the guinea pig P2X₁ receptor is required before further conclusions can be drawn. The recent report of phenotypically altered rat P2X₁ receptors generated by alternative splicing (Greco et al., 2001) further complicates the comparison of native and recombinant P2X₂ receptors. We are left with the conclusion that heteromeric assemblies of rP2X₁ and rP2X₂ subunits would best explain the unique pH sensitivity and unusual pharmacological activity of agonists at P2X₁-like responses. P2X receptors are now viewed as either trimeric or tetrameric assemblies (Kim et al., 1997; Nicke et al., 1998) and, hence, expression of heteromeric P2X₁/2 receptors could involve from one to three P2X₂ subunits. Perhaps such differences in subunit composition of heteromeric P2X₁/2 receptors can help explain the complex C/R curves observed in our study, but that is a matter of conjecture. We envision naturally occurring P2X₁/2 receptors to be activated by very low concentrations of released ATP and Ap₆A, and that purinergic transmission would be facilitated under the acidic environment associated with exocytosis of neurotransmitters or with tissue inflammation (King et al., 1997).

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References


Kanjan B, Humphrey P, and Surprenant A (1995) Electrophysiological properties of...


