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Heteromultimeric $P2X_{1/2}$ Receptors Show a Novel Sensitivity to Extracellular pH

SEAN G. BROWN, ANDREA TOWNSEND-NICHOLSON, KENNETH A. JACOBSON, GEOFFREY BURNSTOCK, and BRIAN F. KING

Autonomic Neuroscience Institute, Royal Free and University College Medical School, Royal Free Campus, Hampstead, United Kingdom (S.G.B., G.B., B.F.K.); Department of Biochemistry and Molecular Biology, University College London, London, United Kingdom (A.T.N.); and Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland (K.A.J.)

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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 initial rapidly inactivating ($P2X_1$-like) component and a later slowly inactivating ($P2X_2$-like) component. The maximum amplitude of $P2X_1$-like ATP responses was increased in some cells by lowering extracellular pH (from 7.5 to 6.5), whereas $P2X_2$-like responses and those of homomeric $rP2X_1$ and $rP2X_2$ receptors were not changed by this treatment. Concentration-response (C/R) curves for ATP for pH-enhanced $P2X_1$-like responses were biphasic, and clearly distinct from monophasic ATP C/R curves for homomeric $rP2X_1$ and $rP2X_2$ receptors. Under acidic (pH 5.5 and 6.5) and alkaline (pH 8.5) conditions, 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 $rP2X_1$ and $rP2X_2$ receptors. ATP C/R curves for $P2X_2$-like responses overlay C/R curves for homomeric $rP2X_2$ receptors, and determinations of agonist potency and efficacy were identical for $P2X_2$-like and $P2X_2$ responses at all pH levels tested. Our results show that $P2X_1$-like responses possessed the kinetics of homomeric $P2X_1$ receptors but an acid sensitivity different from homomeric $P2X_1$ and $P2X_2$ 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 receptors, with a subpopulation of novel pH-sensitive P2X 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 selective for ATP (Brake et al., 1994; Valera et al., 1994; Bo et al., 1995; Chen et al., 1995; Collo et al., 1996; Surprenant et al., 1996; Kim et al., 1997; Nicke et al., 1998).

Transcripts for all but the $P2X_7$ subunit have been found in sensory, sympathetic, and auditory nerves (Collo et al., 1996; Xiang et al., 1999). It has been suggested that this overlap allows for the coassembly of P2X receptor subunits into heteromeric complexes with distinct phenotypic properties. Indeed, coexpression of $P2X_2$ and $P2X_3$ subunits results in the formation of a heteromer that shows pharmacological properties distinct from homomeric $P2X_2$ or $P2X_3$ receptors (Lewis et al., 1995; Liu et al., 2001). The formation of heteromeric $P2X_{2/3}$ receptors has, in part, helped explain the pharmacological properties of some P2X receptors in sensory and sympathetic nerves (Khakh et al., 1995; Lewis et al., 1995; Radford et al., 1997; Zhong et al., 2000). Similar work has been conducted with $P2X_4$ and $P2X_6$ subunits, the transcripts of which show an overlapping expression in regions of adult rat brain, with the translated proteins generating a heteromeric P2X ion channel (Lê et al., 1998). $P2X_1$ and $P2X_5$ transcripts show an overlapping expression in the ventral horn of the spinal cord (Collo et al., 1996) and the coexpression of these subunits results in yet another P2X receptor phenotype (Torres et al., 1998). $P2X_2$ and $P2X_6$ transcripts

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ABBREVIATIONS: cRNA, capped RNA; C/R, concentration-response; Ap_6A, 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 heteromeric receptor (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 $P2X_1$ and $P2X_2$ 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 (Kanjhan 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 $P2X_1$ (1 μg/μl; Valera et al., 1994) and 20 nl of cRNA encoding rat $P2X_2$ (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 $CaCl_2$, 0.82 mM $MgSO_4$, supplemented with 50 $\mu g\,l^{-1}$ gentamycin 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 (Axoclamp 2B; Axon Instruments, Union City, CA). Intracellular microelectrodes 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 $ml\cdot min^{-1}$ 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 AnalaR grade (Aldrich 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_6A, 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., $P2X_2$ and $P2X_3$), 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-coinjected 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.

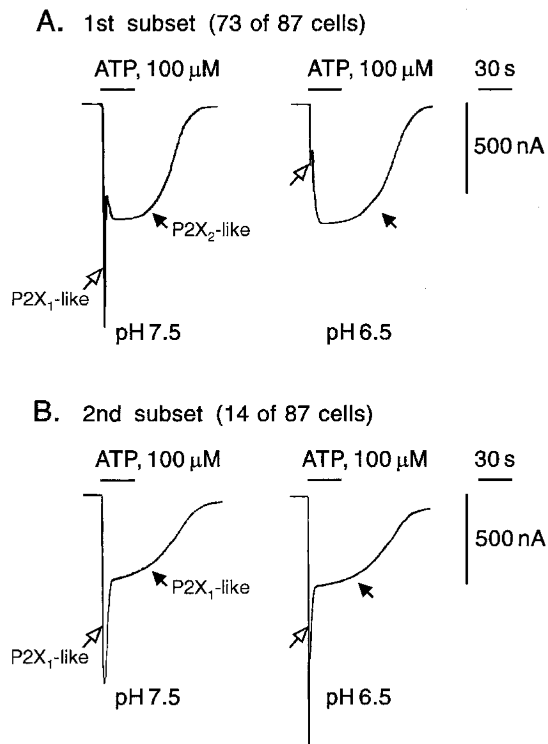

Fig. 1. Effect of extracellular pH on evoked biphasic responses. In A, example of a subset of $rP2X_1/rP2X_2$ cRNA-coinjected oocytes (73 of 87 cells sampled) that responded to extracellular ATP (100 μM) with biphasic inward currents, where the initial $P2X_1$-like response (open arrows) was inhibited by lowering extracellular pH from 7.5 to 6.5, and the later $P2X_2$-like response (closed arrows) largely unaffected. Both records in A from the same cell (V_h = −60 mV). B, example of another subset of coinjected oocytes (14 of 87 cells sampled) where the $P2X_1$-like response was potentiated by lowering extracellular pH from 7.5 to 6.5, and later $P2X_2$-like response unaffected. Both records in B from another cell, but same batch as oocyte A (V_h = −60 mV).

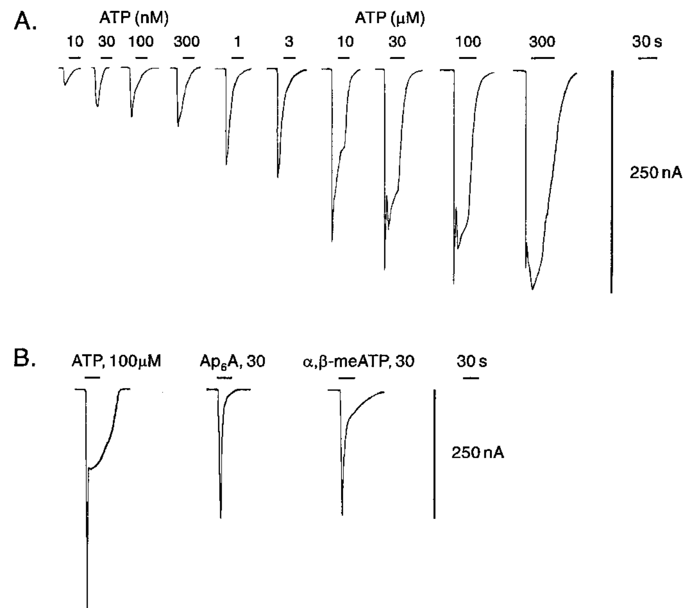

Fig. 2. Agonist responses in oocytes expressing pH-sensitive P2X assemblies. A, concentration-dependent inward currents to ATP (10 nM-300 μM, for 30 s, and 20 min apart) recorded from a single $rP2X_1/rP2X_2$ cRNA-coinjected oocyte (V_h = −60 mV). Monophasic $P2X_1$-like responses were evoked by low ATP concentrations (<3 μM), and biphasic ($P2X_1$-like and $P2X_2$-like) responses by higher ATP concentrations (3–300 μM). B, inward currents evoked by a saturating concentration of ATP (100 μM) and by known homomeric $rP2X_1$ receptor agonists Ap_6A (30 μM) and α,β-meATP (30 μM) from a single $rP2X_1/rP2X_2$ cRNA-coinjected oocyte. Records in B from the same cell (V_h = −60 mV).

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 $P2X_2$-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 $P2X_1/P2X_2$ 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 $P2X_2$-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_6A and α,β-meATP also evoked rapidly inactivating $P2X_1$-like responses in $P2X_1/P2X_2$ cRNA-coinjected oocytes (Fig. 2B). Ap_6A 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-meATP is a weak agonist at homomeric $P2X_2$ receptors (Jiang et al., 2001) and only evoked very small $P2X_2$-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_6A of 0.74 ± 0.10 μM (n_H = 0.50) (n = 6); and for α,β-meATP of 0.43 ± 0.05 μM (n_H = 0.89) (n = 8). The C/R curve for Ap_6A 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-meATP 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

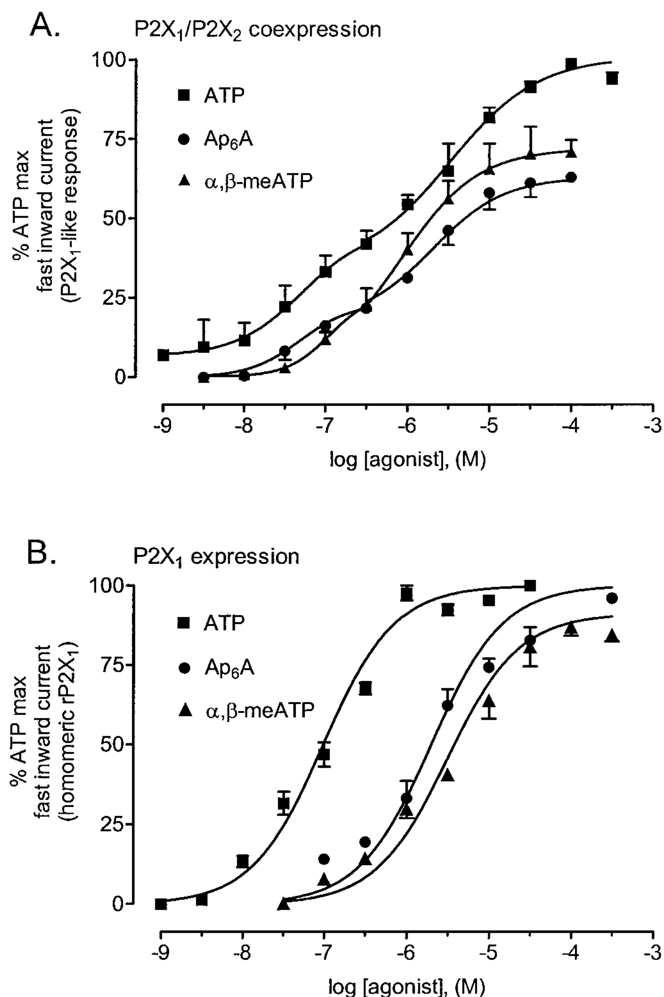

Fig. 3. Agonist potency at P2X subunit assemblies. A, C/R curves for rapidly inactivating $P2X_1$-like responses evoked by ATP, Ap_6A and α,β-meATP in oocytes coexpressing $rP2X_1$ and $rP2X_2$ subunits. Data given as mean ± S.E.M. (6–9 sets of observations). B, C/R curves for ATP, Ap_6A and α,β-meATP 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 ($n_H \leq 0.5$). 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_e 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_6A and α,β-meATP (both 30 μM) were ineffective at eliciting $P2X_2$-like responses (Fig. 2B) or activating homomeric $rP2X_2$ receptors (Jacobson et al., 2000).

TABLE 1

Agonist potency at P2X receptor assemblies

Determinations of EC_{50} values and Hill coefficients (n_H) for ATP, Ap_6A, and α,β-meATP at homomeric $rP2X_1$ and $rP2X_2$ receptors and P2X receptors responsible for the $P2X_1$-like and $P2X_2$-like responses. Data for $P2X_1$-like responses were analyzed for both phases of biphasic C/R curves and reanalyzed for full range of data points.

Receptor	ATP		Ap_6A		α,β-meATP	
	EC_{50}	n_H	EC_{50}	n_H	EC_{50}	n_H
	nM		*nM*		*nM*	
Homomeric $rP2X_1$	98 ± 11	0.80	2598 ± 721	0.74	3301 ± 492	0.77
$P2X_1$-like						
1st phase	54 ± 19	1.05	49 ± 10	1.37	Not determined	
2nd phase	3276 ± 309	0.82	2020 ± 163	1.25	Not determined	
Full range	555 ± 84	0.37	741 ± 98	0.50	427 ± 50	0.89
Homomeric $P2X_2$	5623 ± 488	1.21	Inactive		Inactive	
$P2X_2$-like	7726 ± 560	1[a]	Inactive		Inactive	

[a] The Hill coefficient for the $P2X_2$-like response was constrained to a value of 1 because of limited C/R data at low agonist concentrations (<10 μM).

Discussion

In the present study, heterologous coexpression of $P2X_1$ and $P2X_2$ 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 $P2X_1$ and $P2X_2$ receptors. This conclusion has been already stated in a preliminary report on the coexpression of $P2X_1$ and $P2X_2$ subunits (Lewis et al., 1995). In the intervening time, however, much more has been learned about the operational profiles of homomeric $rP2X_1$ and $rP2X_2$ 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 $P2X_1$ and $P2X_2$ 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 $P2X_1/P2X_2$ cRNA-coinjected oocytes responded to ATP with rapidly inactivating inward currents ($P2X_1$-like responses) that were potentiated under acidic conditions. This behavior was atypical of fast inward currents carried by homomeric $rP2X_1$ 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, $P2X_1$-like responses in a sample of 73 of 87 cells were decreased under acidic conditions and, here, we have assumed that homomeric $rP2X_1$ receptors were in abundance. Thus, we believe the cellular conditions in oocytes may favor the assembly of homomeric $P2X_1$ receptors over heteromeric $P2X_{1/2}$ 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 $P2X_2$ 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, $P2X_1$-like responses possessed the kinetics of homomeric $P2X_1$ receptors and, to an extent, the acid sensitivity of homomeric $P2X_2$ receptors. It is therefore possible that a significant part of $P2X_1$-like re-

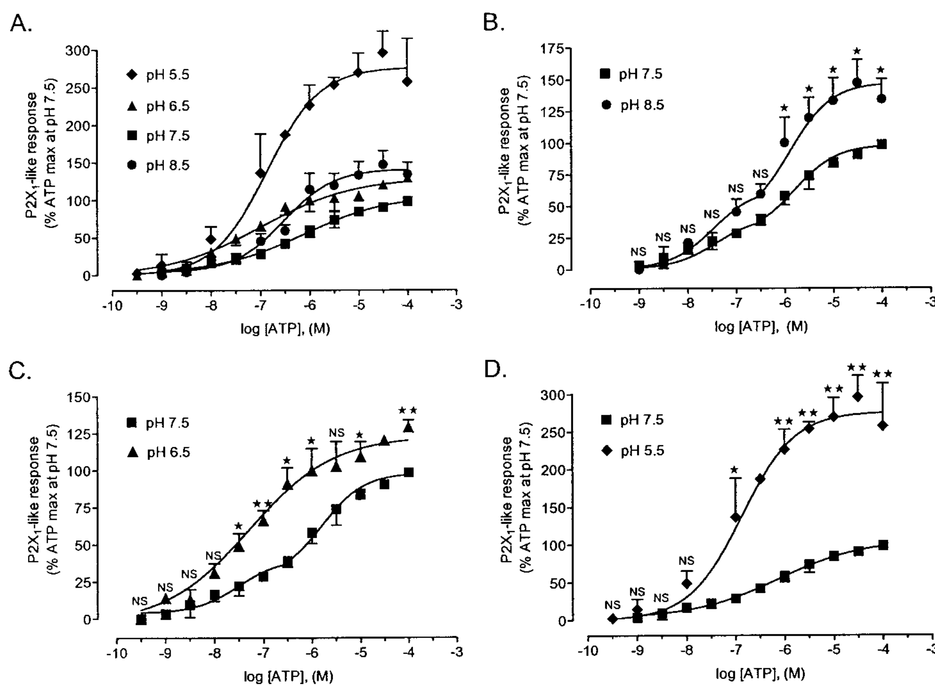

Fig. 4. Effects of extracellular pH of ATP activity at $P2X_1$-like responses. A, C/R curves for rapidly inactivating $P2X_1$-like responses evoked by ATP at four levels of extracellular pH (8.7, 7.5, 6.5, and 5.5) Data given as mean ± S.E.M. (4–9 sets of observations). B to D, C/R curves are redrawn 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; ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$).

TABLE 2

Effect of extracellular pH at P2X receptor assemblies

Determination of EC_{50} values and maximum response (I_{max}) for ATP at different extracellular pH levels, at homomeric $rP2X_1$ receptors, and P2X receptors responsible for $P2X_1$-like responses. The given I_{max} values were normalized to the maximum response to ATP at pH 7.5.

Agonist	Homomeric $rP2X_1$		Heteromeric $rP2X_{1/2}$ ($P2X_1$-like response)	
	EC_{50} Value	I_{max}	EC_{50} Value	I_{max}
	μM	%	μM	%
ATP				
pH 8.5	0.31 ± 0.02[a]	98 ± 2[a]	0.39 ± 0.13	154 ± 16
pH 7.5	0.10 ± 0.01	100	0.56 ± 0.09	100
pH 6.5	0.60 ± 0.05[a]	99 ± 1[a]	0.08 ± 0.02	134 ± 8
pH 5.5	1.70 ± 0.32[a]	96 ± 4[a]	0.12 ± 0.05	284 ± 18

[a] Data taken from Wildman et al. (1999).

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 potentiated 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_6A and α,β-meATP, in contrast to parallel experiments where homomeric $P2X_1$ receptors were studied separately. None of these observations are consistent with the behavior of desensitized homomeric $P2X_1$ receptors.

Only one in every six $P2X_1/P2X_2$ cRNA-coinjected oocytes showed pH-potentiated $P2X_1$-like responses and, accordingly, it was difficult to carry out extensive pharmacological investigations. We altered the concentrations of injected cRNAs in an attempt to increase the expression of heteromeric P2X assemblies, as carried out in our experiments on heteromeric $P2X_{2/3}$ receptors (Liu et al., 2001), but this procedure only resulted in cells with P2X receptors showing the expected properties of homomeric $P2X_1$ and $P2X_2$ receptors (data not shown). This outcome may explain, in part, why a previous attempt using human embryonic kidney 293 cells to coexpress $P2X_1$ and $P2X_2$ subunits failed to reveal the presence of heteromeric $P2X_{1/2}$ assemblies (Lewis et al., 1995). Also, these investigators were faced with the problem of finding a way of pharmacologically dissecting heteromeric and homomeric P2X receptors present when, at that time, the discriminating effects of extracellular pH were unknown.

Where $P2X_1/P2X_2$ cRNA-coinjected cells were studied, agonist activation of the mixed P2X receptor population resulted in complex C/R curves for pH-potentiated $P2X_1$-like responses. C/R curves extended over a large concentration range and, at pH 7.5, were clearly biphasic for ATP and Ap_6A. It seems likely that biphasic C/R curves resulted from the separate activation of different populations of P2X assemblies capable of generating rapidly inactivating $P2X_1$-like responses. It is less likely that the second phase of complex C/R curves was caused by a simultaneous activation of homomeric $P2X_1$ and $P2X_2$ ion channels and the summation of inward currents, particularly in the case of Ap_6A or 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

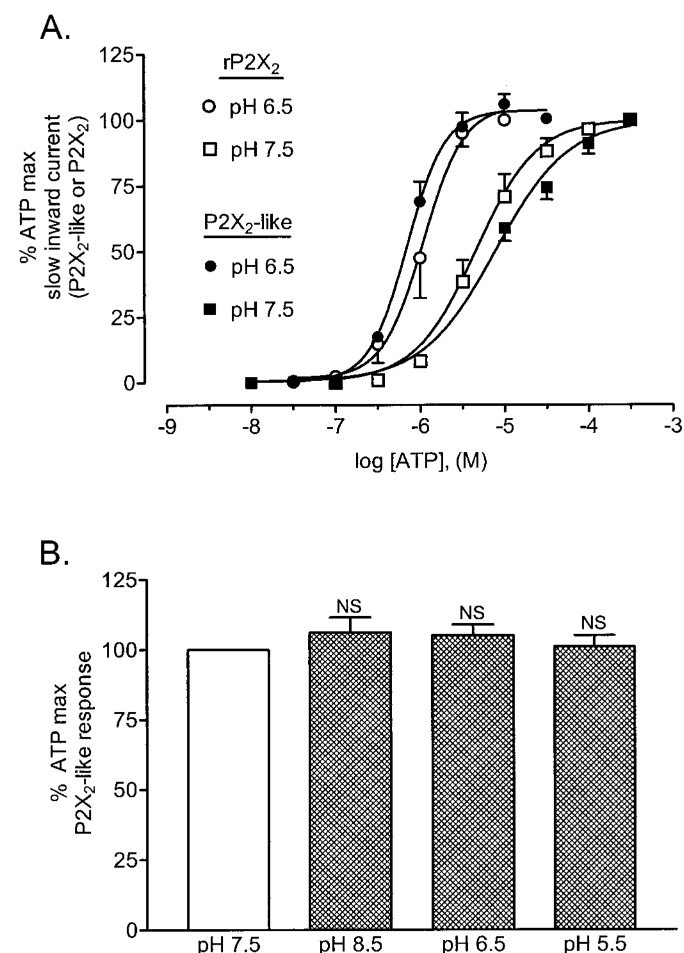

Fig. 5. Effects of extracellular pH of ATP activity at $P2X_2$-like responses. A, C/R curves for slowly inactivating inward currents evoked by ATP in oocytes either coexpressing $P2X_1$ and $P2X_2$ subunits or expressing homomeric $rP2X_2$ receptors. ATP activity was assessed at pH 7.5 and again at pH 6.5. Curves fitted to the Hill equation, by using Prism version 2.0 (GraphPad Software). B, relative amplitude of maximum $P2X_2$-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).

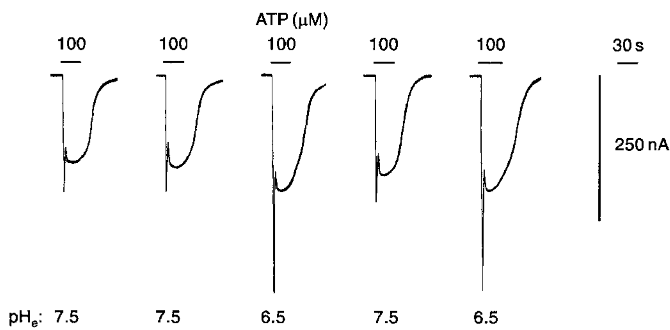

Fig. 6. Reproducibility of $P2X_1$-like and $P2X_1$-like responses. ATP (100 μM, for 30 s, and 20 min apart) evoked biphasic inward currents of consistent amplitude, at either pH 7.5 or pH 6.5, in oocytes coexpressing $rP2X_1$ and $rP2X_2$ subunits. The reproducibility of $P2X_1$-like responses at each pH_e level supported the conclusion that pH potentiation was not due to a relaxation of $rP2X_1$ receptor desensitization; the maximum amplitude of $P2X_2$-like responses was not significantly affected by extracellular pH (Fig. 5B).

$P2X_1$-like responses under acidic conditions (Fig. 4, C and D) was equally incompatible with the involvement of homomeric $rP2X_2$ receptors (King et al., 1997).

EC_{50} values for ATP and Ap_6A fell in the region of 50 nM for the first phase of C/R curves for $P2X_1$-like responses, significantly different ($p < 0.05$) from agonist EC_{50} values at homomeric $rP2X_1$ receptors (Fig. 3; Table 1). It has been reported that rapidly inactivating inward currents elicited by heteromeric $P2X_{1/5}$ receptors are also evoked by very low ATP concentrations (mean EC_{50} of 55 nM) (Surprenant et al., 2000). Thus, a trend is emerging that P2X heteromers comprising $P2X_1$ subunits are extremely sensitive to ATP and, conceivably, this represents a useful adaptation to enhance purinergic signaling at sites where homomeric $P2X_1$ receptors are also used. EC_{50} values for the second phase were in the low micromolar (~2–3 μM) concentration range and these values were unrelated to EC_{50} values for homomeric $P2X_1$ or $P2X_2$ receptors (Table 1). Where EC_{50} values were determined over the full range of data points for each C/R curve, the agonist potency order was α,β-meATP > ATP > Ap_6A, which, again, was unrelated to data for homomeric $rP2X_1$ receptors (ATP > Ap_6A > α,β-meATP) and homomeric $rP2X_2$ receptors (ATP active, α,β-meATP, a weak agonist, and Ap_6A inactive) (Table 1).

A thorough study of ATP potency and efficacy at different extracellular pH levels provided further evidence that $P2X_1$-like responses were mediated by novel pH-sensitive heteromeric P2X receptors. Here, we found that lowering pH_e caused an increase 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 $rP2X_1$ receptors (Stoop et al., 1997; Wildman et al., 1999) (Table 2), or increase ATP potency without altering the maximum response at homomeric $P2X_2$ receptors (King et al., 1996c, 1997; Stoop et al., 1997) (Fig. 5). In the present study, we observed that raising pH_e increased the maximum ATP response without altering agonist potency for $P2X_1$-like currents, whereas, in contrast, alkaline conditions have no effect on ATP responses at homomeric $P2X_1$ receptors (Wildman et al., 1999), or decrease ATP potency without changing the maximum response at homomeric $P2X_2$ receptor (King et al., 1996c, 1997).

It seemed unlikely that $P2X_1$-like responses were mediated by homomeric $P2X_2$ receptors, for a number of reasons. The $P2X_1$-like responses were rapidly inactivating, evoked by Ap_6A and α,β-meATP, and their maximal amplitude potentiated by both acidic and alkaline conditions. None of these features match the profile of homomeric $rP2X_2$ receptors (King et al., 1997; Jacobson et al., 2000). Instead, there appeared to be a major role for homomeric $rP2X_2$ receptors in the later $P2X_2$-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 $P2X_1$-like responses are potentiated under acidic conditions (Nakanishi et al., 1999). However, the molecular characterization of the guinea pig $P2X_1$ receptor is required before further conclusions can be drawn. The recent report of phenotypically altered rat $P2X_1$ receptors generated by alternative splicing (Greco et al., 2001) further complicates the comparison of native and recombinant P2X receptor responses. We are left with the conclusion that heteromeric assemblies of $rP2X_1$ and $rP2X_2$ subunits would best explain the unique pH sensitivity and unusual pharmacological activity of agonists at $P2X_1$-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 $P2X_{1/2}$ receptors could involve from one to three $P2X_1$ subunits. Perhaps such differences in subunit composition of heteromeric $P2X_{1/2}$ receptors can help explain the complex C/R curves observed in our study, but that is a matter of conjecture. We envision naturally occurring $P2X_{1/2}$ receptors to be activated by very low concentrations of released ATP and Ap_6A, 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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Address correspondence to: Brian F. King, Ph.D., Autonomic Neuroscience Institute, Royal Free and University College Medical School, Royal Free Campus, Rowland Hill St., Hampstead, London NW3 2PF, UK. E-mail: b.king@ucl.ac.uk