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

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Abstract

Rat P2X₁ and P2X₂ 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 (P2X₁-like) component and a later slowly inactivating (P2X₂-like) component. The maximum amplitude of P2X₁-like ATP responses was increased in some cells by lowering extracellular pH (from 7.5 to 6.5), whereas P2X₂-like responses and those of homomeric rP2X₁ and rP2X₂ receptors were not changed by this treatment. Concentration-response (C/R) curves for ATP for pH-enhanced P2X₁-like responses were biphasic, and clearly distinct from monophasic ATP C/R curves for homomeric rP2X₁ and rP2X₂ receptors. Under acidic (pH 5.5 and 6.5) and alkaline (pH 8.5) conditions, ATP C/R curves for P2X₁-like responses showed increases in agonist potency and efficacy, compared with data at pH 7.5, but the same was not true of homomeric rP2X₁ and rP2X₂ receptors. ATP C/R curves for P2X₂-like responses overlay C/R curves for homomeric rP2X₂ receptors, and determinations of agonist potency and efficacy were identical for P2X₂-like and P2X₂ responses at all pH levels tested. Our results show that P2X₁-like responses possessed the kinetics of homomeric P2X₁ receptors but an acid sensitivity different from homomeric P2X₁ and P2X₂ receptors. In contrast, the P2X₂-like responses exactly matched the profile expected of homomeric P2X₂ receptors. Thus,

coexpression of P2X₁ and P2X₂ 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 (P2X₁₋₇) 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 P2X₇ 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 P2X₂ and P2X₃ subunits results in the formation of a heteromer that shows pharmacological properties distinct from homomeric P2X₂ or P2X₃ 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 P2X₄ and P2X₆ 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). P2X₁ and P2X₅ 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). P2X₂ and P2X₆ transcripts 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 P2X₁ and P2X₂ 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 P2X₁ and P2X₂ transcripts and their proteins. Overlapping expression of P2X₁ and P2X₂ 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 P2X₁ and P2X₂ 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 P2X₂ 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 ($\text{pH} > 7.5$) (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 co-expression 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 ($1 \mu\text{g}/\mu\text{l}$; Valera et al., 1994) and 20 nl of cRNA encoding rat P2X_2 ($0.002 \mu\text{g}/\mu\text{l}$; 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 $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 , 0.82 mM MgSO_4 , supplemented with $50 \mu\text{g l}^{-1}$ 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 M Ω resistance. Oocytes were placed in a Perspex recording chamber and perfused at a constant rate of $5 \text{ ml} \cdot \text{min}^{-1}$ 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

EC₅₀ 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 $\pm$ 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). P¹,P⁶-Diadenosine hexaphosphate (Ap₆A, 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., P2X₂ and P2X₃), 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 P2X₁/P2X₂ cRNA-coinjecting oocytes. Here, ATP (100 μ M) evoked biphasic inward currents that comprised an initial rapidly inactivating (P2X₁-like) component followed by a second slowly inactivating (P2X₂-like) component (Fig. 1). Thus, it was necessary to find a way to separate the agonist responses of homomeric rP2X₁ and rP2X₂ 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 rP2X₁ and rP2X₂ 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 P2X₁ receptors and potentiates submaximal ATP responses at P2X₂ 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 P2X₁-like response to ATP (100 μ M) was inhibited at pH 6.5 and the P2X₂-like response unaffected or

slightly potentiated (Fig. 1A). In a smaller subset of tested cells (14 of 87 coinjected oocytes; 16%), the rapidly inactivating P2X₁-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 P2X₁/P2X₂ cRNA-coinjected oocytes were prepared and surveyed to explore the properties of rapidly inactivating P2X₁-like responses potentiated under acidic conditions.

Potency of Agonists Mediating pH-Sensitive Inward (P2X₁-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 μ M). The amplitude of these rapidly inactivating P2X₁-like responses grew incrementally over this extended concentration range (10 nM–300 μ M) (Fig. 2A), whereas the slower P2X₂-like responses were evident only over a limited concentration range (approximately 3–300 μ M). The C/R curve for ATP activated P2X₁-like responses is shown in Fig. 3A. The apparent EC₅₀ value (and Hill coefficient) was $0.56 \pm 0.09 \mu$ M ($n_H = 0.37$) ($n = 9$), but the C/R curve was shallow and appeared to be biphasic. Resolving for each phase, mean EC₅₀ values were 54 nM ($n_H = 1.05$) and 3.28 μ M ($n_H = 0.82$). This first EC₅₀ value matches a determination for ATP potency at homomeric hP2X₁ receptors (mean EC₅₀ of 56 nM; Bianchi et al., 1999), but is statistically lower ($p < 0.05$) than our present determination for homomeric rP2X₁ receptors (mean EC₅₀ of 98 nM; $n_H = 0.80$) (Fig. 3B). The second EC₅₀ value is unrelated to any determination for ATP potency at homomeric P2X₁ receptors.

Ap₆A and α,β -meATP also evoked rapidly inactivating P2X₁-like responses in P2X₁/P2X₂ cRNA-coinjected oocytes (Fig. 2B). Ap₆A is inactive at homomeric rP2X₂ 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 P2X₂ receptors (Jiang et al., 2001) and only evoked very small P2X₂-like responses (Fig. 2B). The resultant agonist C/R curves for P2X₁-like responses were shallow and extended over a wide concentration range (3 nM–100 μ M) (Fig. 3A) to give EC₅₀ values for Ap₆A of 0.74 ± 0.10 M ($n_H = 0.50$) ($n = 6$); and for α,β -meATP of $0.43 \pm 0.05 \mu$ M ($n_H = 0.89$) ($n = 8$). The C/R curve for Ap₆A appeared to be biphasic, with mean EC₅₀ values of 49 nM ($n_H = 1.37$) and 2.02 μ M ($n_H = 1.25$). 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 P2X₁-Like Responses

The C/R relationship for rapidly inactivating P2X₁-like ATP responses was reexamined at four different levels of pH_e (Fig. 4, A–D). At the four levels tested (pH 8.5, 7.5, 6.5, and 5.5), the resultant C/R curves extended over 5 log₁₀ units of agonist concentration (1 nM–100 μ M) and the slopes of these curves were shallow ($n_H \approx 0.5$). 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 P2X₁-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 $134 \pm 8\%$ (at pH 6.5; $n = 6$) and $284 \pm 18\%$ (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 P2X₁-like responses at low concentrations (ATP, 1–100 nM) was not significantly different from P2X₁-like responses obtained at pH 7.5 (Fig. 4B). At higher ATP concentrations (300 nM–100 μ M), the amplitude of P2X₁-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 $154 \pm 16\%$ at pH 8.5 ($n = 5$). Thus, the amplitude of P2X₁-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 P2X₁-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 EC₅₀ value over the full concentration range (1 nM–100 μ M) (Fig. 4). Where possible, C/R curves were analyzed over first and second phases of the curve and changes in EC₅₀ 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 EC₅₀ values were as follows for P2X₁-like responses: pH 8.5, $0.39 \pm 0.13 \mu\text{M}$ ($n = 5$); pH 7.5, $0.55 \pm 0.09 \mu\text{M}$ ($n = 9$); pH 6.5, $0.08 \pm 0.02 \mu\text{M}$ ($n = 6$); and pH 5.5, $0.12 \pm 0.05 \mu\text{M}$ ($n = 4$). 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 rP2X₁ 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 P2X₁-like responses and responses by homomeric rP2X₁ 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 EC₅₀ 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 (P2X₂-Like) Currents

Slowly inactivating P2X₂-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 P2X₁-like responses showed deactivating tail currents that obscured the smallest of P2X₂-like responses. The C/R curve for ATP-mediated P2X₂-like responses is shown in Fig. 5. At pH 7.5, the apparent EC₅₀ value (and Hill coefficient) was $7.7 \pm 0.6 \mu\text{M}$ ($n_H = 1.00$) for the P2X₂-like response, which was not significantly different from the determination for homomeric rP2X₂ receptors ($5.6 \pm 0.5 \mu\text{M}$; $n_H = 1.21$) (Fig. 5). At pH 6.5, the EC₅₀ value for the P2X₂-like response was $0.71 \pm 0.06 \mu\text{M}$ ($n_H = 1.95$), similar to the determination for

homomeric rP2X₂ receptors ($1.09 \pm 0.12 \mu\text{M}$; $n_H = 1.81$) (Fig. 5). The ATP C/R curves for P2X₂-like responses and responses by homomeric rP2X₂ receptors appeared to be monophasic at the pH levels tested. Also, the maximum amplitude for P2X₂-like responses, as for homomeric rP2X₂ receptors (King et al., 1996c), was not significantly different at the pH levels tested (Fig. 5B). Furthermore, Ap₆A and α,β -meATP (both 30 μM) were ineffective at eliciting P2X₂-like responses (Fig. 2B) or activating homomeric rP2X₂ receptors (Jacobson et al., 2000).

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).

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 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 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 responses 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 P2X₁-like responses under acidic conditions, this effect was no more than the relaxation of receptor desensitization for a significant proportion of the available homomeric P2X₁ receptor population. However, several lines of evidence disprove this argument. First, the amplitude

of P2X₁-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 P2X₁-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, P2X₁-like responses could be evoked by very low agonist concentrations, not only ATP but also Ap₆A and α,β -meATP, in contrast to parallel experiments where homomeric P2X₁ receptors were studied separately. None of these observations are consistent with the behavior of desensitized homomeric P2X₁ receptors.

Only one in every six P2X₁/P2X₂ cRNA-coinjected oocytes showed pH-potentiated P2X₁-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 P2X₁ and P2X₂ receptors (data not shown). This outcome may explain, in part, why a previous attempt using human embryonic kidney 293 cells to coexpress P2X₁ and P2X₂ 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 P2X₁/P2X₂ cRNA-coinjected cells were studied, agonist activation of the mixed P2X receptor population resulted in complex C/R curves for pH-potentiated P2X₁-like responses. C/R curves extended over a large concentration range and, at pH 7.5, were clearly biphasic for ATP and Ap₆A. It seems likely that biphasic C/R curves resulted from the separate activation of different populations of P2X assemblies capable of generating rapidly inactivating P2X₁-like responses. It is less likely that the second phase of complex C/R curves was caused by a simultaneous activation of homomeric P2X₁ and P2X₂ ion channels and the summation of inward currents, particularly in the case of Ap₆A or α,β -meATP, which are inert at rP2X₂ 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 rP2X₂ receptors (King et al., 1997). Furthermore, the elevation of ATP C/R curves for P2X₁-like responses under acidic conditions (Fig. 4, C and D) was equally incompatible with the involvement of homomeric 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 P2X_{1/5} 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 μ M) concentration range and these values were unrelated to EC₅₀

values for homomeric P2X₁ or P2X₂ receptors (Table 1). Where EC₅₀ 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_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 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_e 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 P2X₁-like responses were mediated by homomeric P2X₂ receptors, for a number of reasons. The P2X₁-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 P2X receptor responses. 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 P2X_{1/2} receptors could involve from one to three P2X₁ 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 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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ABBREVIATIONS

cRNA	capped RNA
C/R	concentration-response
Ap₆A	P ¹ ,P ⁶ -diadenosine hexaphosphate ammonium salt
α,β-meATP	α,β -methylene ATP lithium salt
pH_e	extracellular pH

References

- Bianchi BR, Lynch KJ, Touma E, Niforatos W, Burgard EC, Alexander KM, Park HS, Yu H, Metzger R, Kowaluk E, et al. Pharmacological characterization of recombinant human and rat P2X receptor subtypes. *Eur J Pharmacol.* 1999; 376:127–138. [PubMed: 10440098]
- Brake AJ, Wagenbach MJ, Julius D. New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature (Lond).* 1994; 371:519–523. [PubMed: 7523952]
- Burnstock G, King BF. The numbering of cloned P₂ purinoceptors. *Drug Dev Res.* 1996; 38:67–71.
- Bo X, Zhang Y, Nassar M, Burnstock G, Schoepfer R. A P2X purinoceptor cDNA conferring a novel pharmacological profile. *FEBS Lett.* 1995; 375:129–133. [PubMed: 7498461]
- Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN. A P2X purinoceptor expressed by a subset of sensory neurons. *Nature (Lond).* 1995; 377:428–431. [PubMed: 7566119]
- Collo G, North RA, Kawashima E, Merlo-Pich E, Neidhart S, Surprenant A, Buell G. Cloning of P2X₅ and P2X₆ receptors and the distribution and properties of an extended family of ATP-gated ion channels. *J Neurosci.* 1996; 16:2495–2507. [PubMed: 8786426]
- Ding S, Sachs F. Single channel properties of P2X₂ purinoceptors. *J Gen Physiol.* 1999; 113:695–720. [PubMed: 10228183]
- Greco NJ, Tonon G, Chen W, Luo X, Dalal R, Jamieson GA. Novel structurally altered P2X₁ receptor is preferentially activated by adenosine diphosphate in platelets and megakaryocytic cells. *Blood.* 2001; 98:100–107. [PubMed: 11418468]
- Jacobson KA, King BF, Burnstock G. Pharmacological characterization of P2 (nucleotide) receptors. *Celltransmissions.* 2000; 16:3–16.
- Jiang LH, Rassendren F, Spelta V, Surprenant A, North RA. Amino acid residues involved in gating identified in the first membrane-spanning domain of the rat P2X₂ receptor. *J Biol Chem.* 2001; 276:14902–14908. [PubMed: 11278888]
- Kanjhan R, Housley GD, Thorne PR, Christie DL, Palmer DJ, Luo L, Ryan AF. Localization of ATP-gated ion channels in cerebellum using P2X₂R subunit-specific antisera. *Neuroreport.* 1996; 7:2665–2669. [PubMed: 8981443]
- Khakh BS, Humphrey PP, Surprenant A. Electrophysiological properties of P2X-purinoceptors in rat superior cervical, nodose and guinea-pig coeliac neurones. *J Physiol (Lond).* 1995; 484:385–395. [PubMed: 7602533]

- Kidd EJ, Grahames CB, Simon J, Michel AD, Barnard EA, Humphrey PP. Localization of P2X purinoceptor transcripts in the rat nervous system. *Mol Pharmacol*. 1995; 48:569–573. [PubMed: 7476880]
- Kim M, Yoo OJ, Choe S. Molecular assembly of the extracellular domain of P2X₂, an ATP-gated ion channel. *Biochem Biophys Res Commun*. 1997; 240:618–622. [PubMed: 9398614]
- King BF, Pintor J, Wang S, Ziganshin AU, Ziganshina LE, Burnstock G. A novel P1 purinoceptor activates an outward K⁺ current in follicular oocytes of *Xenopus laevis*. *J Pharmacol Exp Ther*. 1996a; 276:93–100. [PubMed: 8558461]
- King BF, Townsend-Nicholson A, Wildman SS, Thomas T, Spyer KM, Burnstock G. Coexpression of rat P2X₂ and P2X₆ subunits in *Xenopus* oocytes. *J Neurosci*. 2000; 20:4871–4877. [PubMed: 10864944]
- King BF, Wang S, Burnstock G. P2 purinoceptor-activated inward currents in follicular oocytes of *Xenopus laevis*. *J Physiol (Lond)*. 1996b; 494:17–28. [PubMed: 8814603]
- King BF, Wildman SS, Ziganshina LE, Pintor J, Burnstock G. Effects of extracellular pH on agonism and antagonism at a recombinant P2X₂ receptor. *Br J Pharmacol*. 1997; 121:1445–1453. [PubMed: 9257926]
- King BF, Ziganshina LE, Pintor J, Burnstock G. Full sensitivity of P2X₂ purinoceptor to ATP revealed by changing extracellular pH. *Br J Pharmacol*. 1996c; 117:1371–1373. [PubMed: 8730726]
- Lê KT, Babinski K, Séguéla P. Central P2X₄ and P2X₆ channel subunits coassemble into a novel heteromeric ATP receptor. *J Neurosci*. 1998; 18:7152–7159. [PubMed: 9736638]
- Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A. Coexpression of P2X₂ and P2X₃ receptor subunits can account for ATP-gated currents in sensory neurons. *Nature (Lond)*. 1995; 377:432–435. [PubMed: 7566120]
- Liu M, King BF, Dunn PM, Rong W, Townsend-Nicholson A, Burnstock G. Coexpression of P2X₃ and P2X₂ receptor subunits in varying amounts generates heterogeneous populations of P2X receptors that evoke a spectrum of agonist responses comparable to that seen in sensory neurons. *J Pharmacol Exp Ther*. 2001; 296:1043–1050. [PubMed: 11181939]
- Loesch A, Burnstock G. Electron-immunocytochemical localization of P2X₁ receptors in the rat cerebellum. *Cell Tissue Res*. 1998; 294:253–260. [PubMed: 9799441]
- Nakanishi H, Matsuoka I, Ono T, Kimura J. Effect of extracellular pH on contractile responses of the guinea-pig vas deferens. *Clin Exp Pharmacol Physiol*. 1999; 26:35–38. [PubMed: 10027068]
- Nicke A, Baumert HG, Rettinger J, Eichele A, Lambrecht G, Mutschler E, Schmalzing G. P2X₁ and P2X₃ receptors form stable trimers: a novel structural motif of ligand-gated ion channels. *EMBO (Eur Mol Biol Organ) J*. 1998; 17:3016–3028.
- Radford KM, Virginio C, Surprenant A, North RA, Kawashima E. Baculovirus expression provides direct evidence for heteromeric assembly of P2X₂ and P2X₃ receptors. *J Neurosci*. 1997; 17:6529–6533. [PubMed: 9254665]
- Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev*. 1998; 50:413–492. [PubMed: 9755289]
- Stoop R, Quayle JM. Fading and rebound of P2X₂ currents at millimolar ATP concentrations caused by low pH. *Br J Pharmacol*. 1998; 125:235–237. [PubMed: 9786492]
- Stoop R, Surprenant A, North RA. Different sensitivities to pH of ATP-induced currents at four cloned P2X receptors. *J Neurophysiol*. 1997; 78:1837–1840. [PubMed: 9325352]
- Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X₇). *Science (Wash DC)*. 1996; 272:735–738.
- Surprenant A, Schneider DA, Wilson HL, Galligan JJ, North RA. Functional properties of heteromeric P2X_{1/5} receptors expressed in HEK cells and excitatory junction potentials in guinea-pig submucosal arterioles. *J Auton Nerv Syst*. 2000; 81:249–263. [PubMed: 10869729]
- Torres GE, Egan TM, Voigt MM. Hetero-oligomeric assembly of P2X receptor subunits. Specificities exist with regard to possible partners. *J Biol Chem*. 1999; 274:6653–6659. [PubMed: 10037762]
- Torres GE, Haines WR, Egan TM, Voigt MM. Co-expression of P2X₁ and P2X₅ receptor subunits reveals a novel ATP-gated ion channel. *Mol Pharmacol*. 1998; 54:989–993. [PubMed: 9855626]

- Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, Buell G. A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature (Lond)*. 1994; 371:516–519. [PubMed: 7523951]
- Vulchanova L, Arvidsson U, Riedl M, Wang J, Buell G, Surprenant A, North RA, Elde R. Differential distribution of two ATP-gated channels (P2X receptors) determined by immunocytochemistry. *Proc Nat Acad Sci USA*. 1996; 93:8063–8067. [PubMed: 8755603]
- Wildman SS, King BF, Burnstock G. Potentiation of ATP-responses at a recombinant P2X₂ receptor by neurotransmitters and related substances. *Br J Pharmacol*. 1997; 120:221–224. [PubMed: 9117113]
- Wildman SS, King BF, Burnstock G. Modulatory activity of extracellular H⁺ and Zn²⁺ on ATP-responses at rP2X₁ and rP2X₃ receptors. *Br J Pharmacol*. 1999; 128:486–492. [PubMed: 10510462]
- Xiang Z, Bo X, Burnstock G. Localization of ATP-gated P2X receptor immunoreactivity in rat sensory and sympathetic ganglia. *Neurosci Lett*. 1998; 256:105–108. [PubMed: 9853714]
- Xiang Z, Bo X, Burnstock G. P2X receptor immunoreactivity in the rat cochlea, vestibular ganglion and cochlear nucleus. *Heart Res*. 1999; 128:190–196.
- Zhong Y, Dunn PM, Burnstock G. Guinea-pig sympathetic neurons express varying proportions of two distinct P2X receptors. *J Physiol (Lond)*. 2000; 523:391–402. [PubMed: 10699083]

Fig. 1.

Effect of extracellular pH on evoked biphasic responses. In A, example of a subset of rP2X₁/rP2X₂ cRNA-coinjecting oocytes (73 of 87 cells sampled) that responded to extracellular ATP (100 μM) with biphasic inward currents, where the initial P2X₁-like response (open arrows) was inhibited by lowering extracellular pH from 7.5 to 6.5, and the later P2X₂-like response (closed arrows) largely unaffected. Both records in A from the same cell ($V_h = -60$ mV). B, example of another subset of coinjecting oocytes (14 of 87 cells sampled) where the P2X₁-like response was potentiated by lowering extracellular pH from 7.5 to 6.5, and later P2X₂-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 μ M, for 30 s, and 20 min apart) recorded from a single rP2X₁/rP2X₂ cRNA-co-injected oocyte ($V_h = -60$ mV). Monophasic P2X₁-like responses were evoked by low ATP concentrations (< 3 μ M), and biphasic (P2X₁-like and P2X₂-like) responses by higher ATP concentrations (3–300 μ M). B, inward currents evoked by a saturating concentration of ATP (100 μ M) and by known homomeric rP2X₁ receptor agonists Ap₆A (30 μ M) and α,β -meATP (30 μ M) from a single rP2X₁/rP2X₂ cRNA-co-injected oocyte. Records in B from the same cell ($V_h = -60$ mV).

Fig. 3.

Agonist potency at P2X subunit assemblies. A, C/R curves for rapidly inactivating P2X₁-like responses evoked by ATP, Ap₆A and α,β-meATP in oocytes coexpressing rP2X₁ and rP2X₂ subunits. Data given as mean ± S.E.M. (6–9 sets of observations). B, C/R curves for ATP, Ap₆A and α,β-meATP in oocytes expressing homomeric P2X₁ 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).

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 $\pm$ 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; *, $p < 0.05$; **, $p < 0.01$).

Fig. 5.

Effects of extracellular pH of ATP activity at P2X₂-like responses. A, C/R curves for slowly inactivating inward currents evoked by ATP in oocytes either coexpressing P2X₁ and P2X₂ subunits or expressing homomeric rP2X₂ 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 P2X₂-like responses to ATP (100 μ M), at three test pH levels (8.5, 6.5, and 5.5), compared with control data (pH 7.5), in oocytes coexpressing P2X₁ and P2X₂ subunits. Data given as mean $\pm$ S.E.M. (4–9 observations). Data compared by Student's unpaired *t* test (N.S., not significantly different).

Fig. 6.

Reproducibility of P2X₁-like and P2X₂-like responses. ATP (100 μ M, 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 rP2X₁ and rP2X₂ subunits. The reproducibility of P2X₁-like responses at each pHe level supported the conclusion that pH potentiation was not due to a relaxation of rP2X₁ receptor desensitization; the maximum amplitude of P2X₂-like responses was not significantly affected by extracellular pH (Fig. 5B).

TABLE 1

Agonist potency at P2X receptor assemblies

Determinations of EC₅₀ values and Hill coefficients (n_H) for ATP, Ap₆A, and α,β -meATP at homomeric rP2X₁ and rP2X₂ receptors and P2X receptors responsible for the P2X₁-like and P2X₂-like responses. Data for P2X₁-like responses were analyzed for both phases of biphasic C/R curves and reanalyzed for full range of data points.

Receptor	ATP		Ap ₆ A		α,β -meATP	
	EC ₅₀	n_H	EC ₅₀	n_H	EC ₅₀	n_H
Homomeric rP2X ₁	98 ± 11	0.80	2598 ± 721	0.74	3301 ± 492	0.77
P2X ₁ -like	nM		nM		nM	
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	Inactive	
P2X ₂ -like	7726 ± 560	^a	Inactive	Inactive	Inactive	

^aThe 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 μM).

TABLE 2
Effect of extracellular pH at P2X receptor assemblies

Determination of EC₅₀ values and maximum response ($I_{\max}$) for ATP at different extracellular pH levels, at homomeric rP2X₁ receptors, and P2X receptors responsible for P2X₁-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

^aData taken from Wildman et al. (1999).