



Effects of extracellular pH on agonism and antagonism at a recombinant P2X₂ receptor

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- 1 Under voltage-clamp conditions, the activity of agonists and antagonists at a recombinant P2X₂ receptor expressed in *Xenopus* oocytes was examined at different levels of extracellular pH (pH_e).
- 2 In normal Ringer (Mg²⁺ ions absent), the amplitude of submaximal inward currents to ATP was increased by progressively lowering pH_e (8.0–5.5). ATP-responses reached a maximum at pH 6.5 with a 5 fold increase in ATP-affinity; the apparent pK_a was 7.05 ± 0.05.
- 3 Receptor affinity for ATP was lowered when extracellular Ca²⁺ ions were replaced with equimolar Mg²⁺ ions. However, the amplitude of the ATP-responses was still enhanced under acidic conditions, reaching maximal activity at pH 6.5 with a 5 fold increase in ATP-affinity; the apparent pK_a was 7.35 ± 0.05.
- 4 ATP species present in the superfusate (for the above ionic conditions and pH levels) were calculated to determine the forms of ATP which activate P2X₂ receptors: possible candidates include HATP, CaHATP and MgHATP. However, levels of these protonated species increase below pH 6.5, suggesting that receptor protonation rather than agonist protonation is more important.
- 5 The potency order for agonists of P2X₂ receptors was: ATP > 2-MeS-ATP ≥ ATP_γS > ATP_αS > CTP ≥ BzATP, while other nucleotides were inactive. EC₅₀ and n_H values for full agonists were determined at pH 7.4 and re-examined at pH 6.5. Extracellular acidification increased the affinity by approximately 5 fold for full agonists (ATP, 2-MeSATP, ATP_γS and ATP_αS), without altering the potency order.
- 6 The potency order for antagonists at P2X₂ receptors was: Reactive blue-2 > trinitrophenol-ATP ≥ Palatine fast black ≥ Coomassie brilliant blue ≥ PPADS > suramin (at pH 7.4). IC₅₀ values and slopes of the inhibition curves were re-examined at different pH levels. Only blockade by suramin was affected significantly by extracellular acidification (IC₅₀ values: 10.4 ± 2 μM, at pH 7.4; 78 ± 5 nM, at pH 6.5; 30 ± 6 nM, at pH 5.5).
- 7 In summary, a lowered pH_e enhanced the activity of all agonists at P2X₂ receptors but, with the exception of suramin, not antagonists. Since a lowered pH_e is also known to enhance agonist activity at P_{2X} receptors on sensory neurones containing P2X₂ transcripts, the sensitization by metabolic acidosis of native P_{2X} receptors containing P2X₂ subunits may have a significant effect on purinergic cell-to-cell signalling.

Keywords: ATP receptor; P2X receptor; recombinant (P2X₂) receptor; ATP; suramin; pH; acidosis; *Xenopus* oocyte

Introduction

An adenosine 5'-triphosphate (ATP)-gated ion channel (P2X₂ receptor) has been cloned from rat pheochromocytoma PC12 cells (Brake *et al.*, 1994), this receptor representing the second member of a series (P2X_{1–7}) of cloned ionotropic ATP receptors (Burnstock and King, 1996). The recombinant homomeric P2X₂ receptor has an agonist profile (King *et al.*, 1996a) similar to the native P_{2X} receptor on PC12 cells (Rhoads *et al.*, 1993) at which ATP-activated fast excitatory potentials have been recorded (Nakazawa *et al.*, 1991).

By hybridization histochemistry, P2X₂ transcripts have been shown to be widely distributed throughout rat tissues, including brain, spinal cord and peripheral autonomic nerves (where P2X₄ and P2X₆ transcripts also co-localize) and in C-fibre sensory neurones (where P2X₃ transcripts are present) (Collo *et al.*, 1996). From immunostaining with P2X₂-selective antibodies, the distribution and density of P2X₂ receptors in the neuraxis is greater than expected from the distribution of its mRNA (Vulchanova *et al.*, 1996; Kanjhan *et al.*, 1996a,b). Immunopositive nerve fibres were concentrated in the nucleus of the solitary tract (NTS) and Lissauer's tract, indicating the presence of P2X₂ subunits in central terminals of vagal and dorsal root sensory neurones (Vulchanova *et al.*, 1996). P2X₂ appears to be a common

subunit and component of native P_{2X} receptors in CNS neurones and sensory neurones.

We have shown recently (King *et al.*, 1996c) that the sensitivity of the P2X₂ receptor to ATP is affected by extracellular pH (pH_e), with acidification and alkalinization shifting the ATP concentration-response curve to the left and right, respectively, without altering the maximum response. These observations on recombinant P2X₂ receptors have been made more interesting by recent findings of Li and colleagues (1996a,b) who obtained the same effects of pH on ATP-responses at native P_{2X} receptors in sensory neurones of rat nodose ganglion. P2X₂ transcripts are heavily concentrated in sensory neurones in nodose, trigeminal and dorsal root ganglia (Collo *et al.*, 1996) and strong P2X₂-immunoreactivity is also observed at these sites (Vulchanova *et al.*, 1996). The finding that ATP-sensitivity at P_{2X} receptors on sensory neurones is dependent on pH_e raises the possibility that this feature may be common to other native P_{2X} receptors if they comprise P2X₂ subunits.

In the present paper, we have investigated the effects of pH_e on the activity of agonists and antagonists at recombinant homomeric P2X₂ receptors expressed in *Xenopus* oocytes. Our experiments concentrated on the effects of lowering pH_e, since acidosis is more common under physiological circumstances than alkalosis. For example, a localized acidosis often occurs during tissue injury, inflammation and ischaemia, and the effects of acidic shifts on ATP-signalling may contribute to

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sensory nerve sensitization (as discussed by Li *et al.*, 1996a,b). Furthermore, localized acidosis occurs in brain tissue during epileptic seizures (Chesler, 1990) and during injury-related degenerative changes in the CNS (DeSalles *et al.*, 1987; Ransom & Philbin, 1992). Transient acidic shifts also occur during the release of neurotransmitters from storage vesicles (Krishtal *et al.*, 1987; Chesler, 1990; Rose & Deitmer, 1995) which are highly acidic (Johnson & Scarpa, 1976). These transient acidic shifts at the synaptic cleft are known to facilitate excitatory transmission in the CNS (Yanovsky *et al.*, 1995). Thus, we were keen to see whether the activity of agonists and antagonists at the P2X₂ receptor varied when pH_o was lowered. Part of this study has been presented to the Physiological Society (King *et al.*, 1996d).

Methods

Xenopus laevis were anaesthetized with Tricaine (0.1% w/v) and killed by decapitation. The ovarian lobes were removed surgically and stored (at 4°C) in Barth's solution (pH 7.45) containing (mM): NaCl 110, KCl 1, NaHCO₃ 2.4, Tris-HCl 7.5, Ca(NO₃)₂ 0.33, CaCl₂ 0.41, MgSO₄ 0.82 and gentamycin sulphate, 50 µg l⁻¹. Mature oocytes (stages V and VI) were taken from ovarian sacs and defolliculated by a two-step process which involved collagenase treatment (Type 1A, 2 mg ml⁻¹ in a Ca²⁺-free Ringer, for 2–3 h) and mechanical removal of the follicle cell layer with fine forceps. Defolliculation was necessary to remove the native P₁ and P₂ receptors found on the follicle cell monolayer enveloping oocytes (King *et al.*, 1996a,b). The cytosol of defolliculated oocytes was injected with P2X₂ transcripts (40 nl, 0.1–1 µg ml⁻¹); oocytes were incubated at 18°C in Barth's solution for 48 h to allow full receptor expression and stored at 4°C in Barth's solution for up to 12 days until used in electrophysiological experiments.

Membrane currents were measured under voltage-clamp conditions, by a twin-electrode amplifier (Axoclamp 2A). The holding potential (V_h) was set at -30 mV to avoid saturating the amplifier with large currents (>2 µA). The voltage-recording (1–2 MΩ tip resistance) and current-recording (5 MΩ tip resistance) microelectrodes were filled with 0.6 M K₂SO₄ and 3.0 M KCl, respectively. Oocytes were placed in an electrophysiological chamber (0.5 ml vol.) and superfused (at 5 ml min⁻¹) with a Ringer solution (at 18°C) containing (mM): NaCl 110, KCl 2.5, HEPES 5, CaCl₂ 1.8, adjusted to pH 7.45. In some cases, MgCl₂ (1.0 mM) was added to the superfusate to match the experimental conditions of Brake *et al.*, (1994). In other experiments, MgCl₂ (1.8 mM) replaced CaCl₂ (1.8 mM) to give a nominally Ca²⁺-free superfusate; elsewhere, MgCl₂ (1.8 mM) and EGTA (1.0 mM) were added and CaCl₂ (1.8 mM) omitted to give a wholly Ca²⁺-free superfusate. The pH of the bathing Ringer solution (pH_o) was adjusted by the addition of either 1.0 N HCl or 1.0 N NaOH, to achieve the desired acidic or alkaline shift. Electrophysiological data were stored on magnetic tape by a DAT recorder (Sony 1000ES) and displayed with a pen recorder (Gould).

ATP and related nucleotides (at the concentrations shown in the text) were superfused over dual-impaired oocytes by continuous flow which allowed the rapid addition and washout

of drugs. Agonists were applied for 60 s and, thereafter, washed off with Ringer solution for a period of 5–10 min. The P2X₂ receptor is a slowly-desensitizing receptor and recovers rapidly (within 5 min) from agonist activation (Brake *et al.*, 1994; Collo *et al.*, 1996; King *et al.*, 1996c). For concentration-response curves, data were normalized to the maximum current (*I*_{max}) evoked by ATP (100 µM). The agonist concentration that evoked 50% of the maximum response (EC₅₀) was taken from Hill plots of the transform, log (*I*/*I*_{max} - *I*), where *I* is the current evoked by each concentration of agonist. The Hill co-efficient (n_H) was taken from the slope of Hill plots. The activity of P₂ antagonists at P2X₂ was tested by adding each antagonist in cumulative concentrations (as mentioned in the text), each concentration applied 10 min before the addition of submaximal concentrations of ATP (at the EC₅₀ for the pH level tested). The antagonist concentration that reduced ATP-responses by 50% (IC₅₀) was taken from inhibition curves. The reversibility of receptor blockade was tested after prolonged washout (up to 2 h) of antagonists. Data are presented as mean ± s.e.mean of 4 sets of data from different oocyte batches. Significant differences were determined by Student's *t* test (by use of InStat v2.05A, GraphPad). Concentration-response curves and inhibition curves were fitted by non-linear regression analysis by use of commercial software (Prism v. 1.03, GraphPad).

ATP, related nucleotides and other drugs were obtained from Sigma Chemical Co. (U.K.), except 2-methylthio-ATP (2-MeSATP; RBI-SEMAT, U.K.) and adenosine-5'-O-(α -thiotriphosphate) (ATP α S; Boehringer Mannheim, Germany). Suramin (Germanin) was a gift from Bayer plc (U.K.), amiloride and its derivatives from RBI-SEMAT (U.K.) and trinitrophenol-ATP (TNP-ATP) from Molecular Probes Inc. (U.S.A.). All reagents were AnalaR grade from Aldrich Chemicals (U.K.).

Results

Agonist activity at P2X₂ receptors

The selectivity of the recombinant P2X₂ receptor for nucleotidic agonists was tested; the amplitudes of evoked responses were compared against inward currents to 30 µM ATP which, at pH 7.45, is close to the EC₁₀₀. The activity order for agonists was (at 30 µM): ATP ≥ 2-MeSATP ≥ ATP γ S > ATP α S > > cytosine-5'-triphosphate (CTP) ≥ BzATP (see Table 1). ATP and CTP were the only naturally-occurring nucleoside triphosphates to activate the P2X₂ receptor; uridine 5'-triphosphate (UTP), inosine 5'-triphosphate (ITP) and guanosine 5'-triphosphate (GTP) were inert. The nucleoside monophosphate (AMP) and nucleoside diphosphates (ADP, 2-MeSADP and ADP β S) also were inactive. Both α , β -methylene ATP (α , β -meATP) and β , γ -meATP were inactive, as were adenosine and two deoxy-ATP analogues (2'-dATP and 3'-dATP). Thus, the P2X₂ receptor is primarily a purine receptor which does not tolerate changes to the ribose moiety of agonists (as in: 2'-dATP and 3'-dATP). The P2X₂ receptor also required a chain of three phosphates on agonists for activation and does not tolerate the insertion of a methylene bridge in this phosphate

Table 1 Activity of ATP and related substances

Agonist	Activity	Agonist	Activity	Agonist	Activity
ATP	1 (12)	ADP	0 (3)	UTP	0 (3)
2-MeSATP	0.95 ± 0.09 (3)	2-MeSADP	0 (3)	ITP	0 (3)
ATP γ S	0.85 ± 0.14 (3)	ADP β S	0 (3)	GTP	0 (3)
ATP α S	0.80 ± 0.09 (3)	AMP	0 (3)	2'-dATP	0 (3)
CTP	0.20 ± 0.03 (3)	α , β -meATP	0 (3)	3'-dATP	0 (3)
BzATP	0.17 ± 0.06 (3)	β , γ -meATP	0 (3)	Adenosine	0 (3)

The amplitude of inward currents (V_h = -30 mV) evoked by a broad range of nucleotides (tested at 30 µM) was compared with the activity of ATP (30 µM) which was taken as 1. Data are mean ± s.e.mean, for the number of observations (*n*) given in parentheses.

chain. These data agreed with, and extended, the initial characterization of P2X₂ receptors (Brake *et al.*, 1994). Concentration-response curves were constructed for ATP, 2-MeSATP, ATP γ S and ATP α S which are full agonists of P2X₂ receptors (Figure 1a). Affinity constants (as EC₅₀ values) were (at pH 7.45): ATP, $4.6 \pm 1.1 \mu\text{M}$ ($n=4$); 2-MeSATP, $7.1 \pm 1.0 \mu\text{M}$ ($n=4$); ATP γ S, $7.4 \pm 0.5 \mu\text{M}$ ($n=4$); ATP α S, $13.2 \pm 6.8 \mu\text{M}$ ($n=4$). The Hill co-efficient (n_H) for each full agonist was: ATP, 2.1 ± 0.2 ($n=4$); 2-MeSATP, 2.1 ± 0.3 ($n=4$); ATP γ S, 2.3 ± 0.2 ($n=4$); ATP α S, 2.2 ± 0.1 ($n=4$).

Both extracellular Mg²⁺ and Ca²⁺ have been shown to inhibit the opening of ATP-activated P2X₂ receptors on PC12 cells (Nakazawa & Hess, 1993) from which the P2X₂ subunit was cloned. Thus, we retested ATP-activity at different concentrations of Mg²⁺ and Ca²⁺ in the superfusate. With Mg²⁺ (1.0 mM) added to the superfusate (as in Brake *et al.*, 1994), the EC₅₀ value for ATP was $25.8 \pm 4.9 \mu\text{M}$ ($n=4$) and the ATP concentration-response curve was displaced to the right of the control curve without a change in the maximum activity or slope of the curve ($n_H=2.1 \pm 0.1$) (Figure 1b). With Mg²⁺ (1.8 mM) replacing Ca²⁺ (1.8 mM) to give a nominally Ca²⁺-free superfusate, the EC₅₀ value for ATP was similar ($26.1 \pm 2.1 \mu\text{M}$, $n=3$) and the ATP concentration-response curve displaced to the right of the control curve, while the slope remained 2.1 ± 0.1 (Figure 1b). With Mg²⁺ (1.8 mM) and EGTA (1.0 mM) present and Ca²⁺ (1.8 mM) omitted to give a wholly Ca²⁺-free superfusate, the EC₅₀ value for ATP was $29.3 \pm 1.8 \mu\text{M}$ ($n=3$) and the slope of the curve was 2.1 ± 0.1 (Figure 1b).

Antagonist activity at P2X₂ receptors

A series of known and novel P2 receptor antagonists were tested against ATP-activated inward currents in oocytes expressing P2X₂ receptors. Known antagonists included: suramin (see Dunn & Blakeley, 1988); Reactive blue 2 (RB-2; see Manzini *et al.*, 1986); Coomassie brilliant blue-G (CBB-G; see Soltoff *et al.*, 1989) pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS; see Lambrecht *et al.*, 1992); trinitrophenol-ATP (TNP-ATP; see Mockett *et al.*, 1994); 2,2'-pyridylisatogen tosylate (PIT; see Spedding *et al.*, 1975); apamin (see Brown & Burnstock, 1981). Also, a novel P₂ receptor antagonist Palatine fast black (PFB) was identified and tested.

After ATP-responses of consistent amplitude (with $5 \mu\text{M}$, a value close to the EC₅₀ at pH 7.45) had been obtained, each antagonist was added to the superfusate in cumulative concentrations (0.01–100 μM ; apamin was tested at 0.3 μM only) and the degree of inhibition of ATP-responses monitored (Figure 2). RB-2 and suramin were the most and least effective blocking agents, respectively, for the P2X₂ receptor. TNP-ATP, PFB, CBB-G and PPADS were equally effective, some 4 fold less potent than RB-2 and 8 fold more potent than suramin. Their activity indices (IC₅₀ value) were (in descending order): RB-2, $0.36 \pm 0.08 \mu\text{M}$ ($n=3$); TNP-ATP, $1.13 \pm 0.19 \mu\text{M}$ ($n=3$); PFB, $1.29 \pm 0.33 \mu\text{M}$ ($n=4$); CBB-G, $1.4 \pm 0.3 \mu\text{M}$ ($n=3$); PPADS, $1.6 \pm 0.08 \mu\text{M}$ ($n=3$); suramin, $10.4 \pm 1.2 \mu\text{M}$ ($n=4$); PIT ($n=4$) and apamin ($n=3$) were inactive. The slope of the inhibition curves was 2 for RB-2, TNP-ATP, PFB and PPADS, suggesting that two molecules of each antagonist are required to block the P2X₂ receptor. However, the slope of the inhibition curves was 1 for suramin and CBB-G, indicating only one molecule of each antagonist was necessary (see Discussion). The blocking actions of suramin, TNP-ATP and PPADS were reversible with washout (1 h); blockade by RB-2 and CBB-G was partially reversible after washout (2 h) while PFB was irreversible. The blocking actions of suramin and RB-2 (at 100 μM) at P2X₂ receptors expressed in oocytes has been demonstrated previously (Brake *et al.*, 1994). The blocking actions of suramin and PPADS (0.1–100 μM) have been studied on P2X₂ receptors expressed in HEK-293 cells (Evans *et al.*, 1995), with similar activity and IC₅₀ values as found here.

The P2X₂ receptor was shown to possess structural features and overall topology similar to amiloride-sensitive Na⁺-

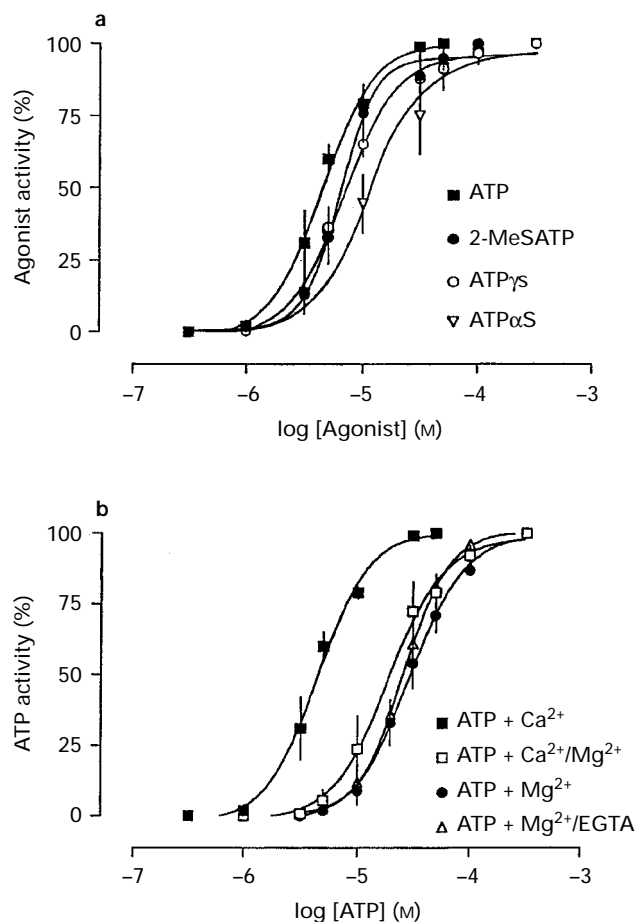


Figure 1 Activity of ATP and analogues at P2X₂ receptors. In (a) concentration-response curves for evoked inward currents to ATP 2-MeSATP, ATP γ S and ATP α S (each at 0.5–300 μM) at pH 7.45. Data were normalized to the maximum response to ATP. In (b), concentration-response curves for ATP (0.5–300 μM) in a normal Ringer solution (NRS, pH 7.45) which contained either calcium or magnesium ions or both of these divalent cation species. These solutions included: NRS and calcium (1.8 mM) ions (ATP + Ca²⁺); NRS and calcium (1.8 mM) and magnesium (1.0 mM) ions (ATP + Ca²⁺/Mg²⁺); NRS and magnesium (1.8 mM) ions (ATP + Mg²⁺); NRS and magnesium ions (1.8 mM) in the presence of EGTA (1.0 mM) (ATP + Mg²⁺/EGTA). Data are mean of 4 sets of observations for each curve; vertical lines show s.e. mean. Curves fitted by a logistic equation, by use of commercial software (Prism V1.03, GraphPad).

channels (Brake *et al.*, 1994). Therefore, we tested the blocking activity of amiloride and three related analogues on the recombinant receptor. However, neither amiloride (0.01–100 μM) ($n=3$), 5-(N-methyl-N-isobutyl)-amiloride (0.01–100 μM) ($n=3$), 5-(N,N-dimethyl)-amiloride (0.01–100 μM) ($n=3$) nor 5-(N,N-hexamethylene)-amiloride (0.01–100 μM) ($n=3$) were effective antagonists of ATP-responses (data not shown).

Effect of pH on agonist activity

The amplitude of ATP-responses at P2X₂ receptors has been shown to depend on extracellular pH (pH_e) (King *et al.*, 1996c), with acidic shifts enhancing and alkaline shifts inhibiting the amplitude of inward currents to submaximal concentrations of ATP. The effects of pH_e (8.0–5.5) on the amplitude of ATP-responses are summarized in Figure 3a, and reveal a non-linear (sigmoid) relationship between pH_e and ATP activity. With experiments carried out with either Ca²⁺ (1.8 mM) or Mg²⁺ (1.8 mM) and EGTA (1 mM) in the superfusate, maximal ATP activity occurred at pH 6.5 and lowering

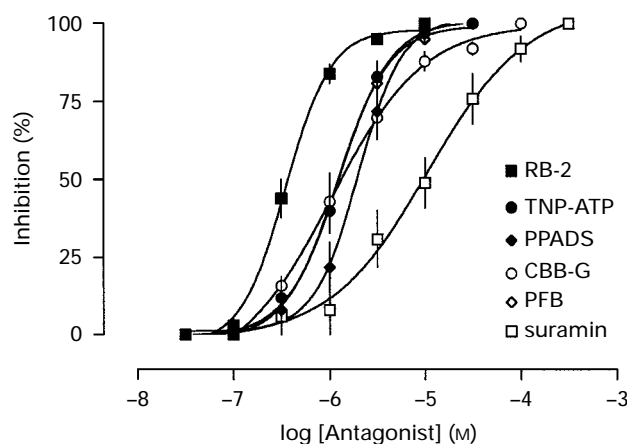


Figure 2 Activity of antagonists at P2X₂ receptors. Inhibition curves (at pH 7.45) for reactive blue 2 (RB-2), trinitrophenyl-ATP (TNP-ATP), pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), Coomassie brilliant blue G (CBB-G), Palatine fast black (PFB) and suramin as antagonists of submaximal ATP-responses (at 5 μ M, which is close to the EC₅₀ value). Data are mean of 4 sets of observations for each inhibition curve; vertical lines show s.e.mean. Curves fitted by a logistic equation, by use of commercial software (Prism V1.03, GraphPad).

pH_e to pH 5.5 caused no further increase in ATP-responses. ATP-responses were reduced or abolished at levels greater than pH 8.0. The apparent pK_a was 7.05 ± 0.05 ($n=3$) with Ca²⁺ (1.8 mM) present in the Ringer solution, and 7.35 ± 0.05 ($n=3$) when extracellular Ca²⁺ was replaced with Mg²⁺ (1.8 mM). These values lie close to the pK_a (7.2 ± 0.1) at which ATP-responses in rat sensory neurones reached half-maximal potentiation (Li *et al.*, 1996a,b).

Earlier analyses of the ATP concentration-response relationship for P2X₂ receptors (King *et al.*, 1996c) showed that a raised pH_e displaced the ATP-curve to the right while a lowered pH_e displaced the ATP-curve to the left; neither procedure altered the maximum response nor significantly altered the slope (n_H) of ATP-curves. With Ca²⁺ (1.8 mM) included in the superfusate, EC₅₀ values ($n=4$) for ATP were: pH 8.0, 28 ± 2 μ M; pH 7.4, 4.6 ± 1 μ M; pH 6.5, 1.2 ± 0.1 μ M; pH 5.5, 1.1 ± 0.1 μ M. With Mg²⁺ (1.8 mM) and EGTA (1 mM) replacing Ca²⁺ in the superfusate, EC₅₀ values ($n=3$) for ATP were: pH 7.4, 29.3 ± 1.8 μ M; pH 6.5, 5.5 ± 0.5 μ M (Figure 3b). On comparison of EC₅₀ values, ATP showed a 5 fold increase in affinity for P2X₂ receptors at pH 6.5, with either Ca²⁺ or Mg²⁺ present in the superfusate (Figure 3b).

We have shown that the potency order of agonists remained unchanged at different pH_e levels although P2X₂ receptor affinity for ATP was altered (King *et al.*, 1996c). Since acidic shifts from pH 7.45 to pH 6.5 gave the greatest increase in ATP-affinity, the activity of other agonists of P2X₂ receptors was tested at pH 7.4 and retested at pH 6.5 to see if their affinity for P2X₂ receptors was similarly altered. Concentration-response curves for 2-MeSATP, ATP γ S and ATP α S were displaced to the left after lowering pH_e, without altering the maximum response or significantly altering the slope (n_H) of each curve (Figure 4). EC₅₀ values ($n=4$) at pH 7.4 and pH 6.5 for each full agonist are shown in Table 2, revealing a 4–7 fold change in affinity for P2X₂ receptors after a lowering of pH_e.

ATP species activating P2X₂ receptors

The ATP species which activates P2X₂ receptors was investigated by use of an analytical software programme (Brooks & Storey, 1992) to compute the fractions of each form of ATP, taking into account the ionic composition and pH_e of the bathing medium. With these data, we compared the ratios of

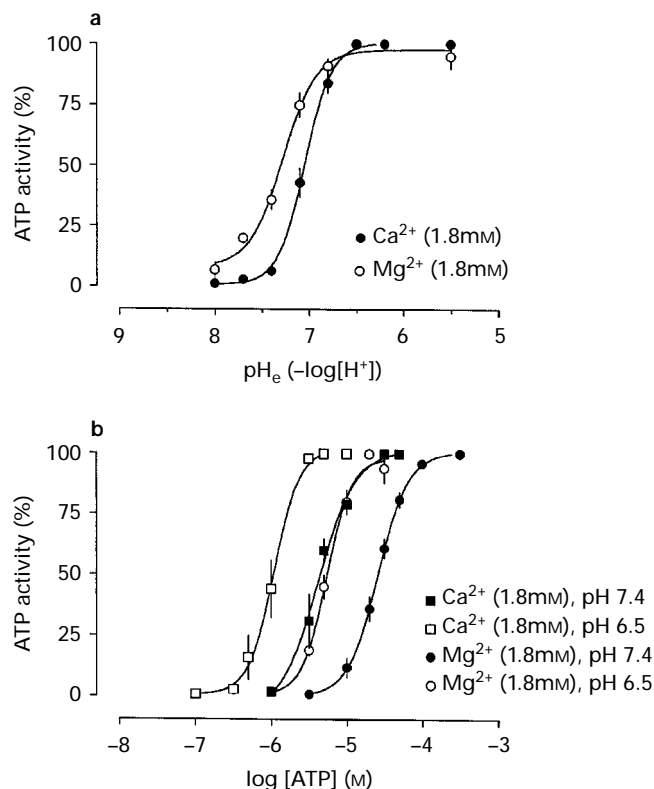


Figure 3 pH-dependence of ATP-activity at P2X₂ receptors. In (a), the effect of extracellular pH (8.0–5.5) on the activity of submaximal concentrations of ATP (3 μ M and 20 μ M) in a normal Ringer solution containing either calcium (1.8 mM) or magnesium (1.8 mM) ions as the divalent cation species. Data were normalized to the maximum response to ATP (100 μ M, at pH 7.45) and each point represents the mean of 3 sets of observations; vertical lines show s.e.mean. In (b), the concentration-response relationship for ATP, with either calcium (1.8 mM) or magnesium (1.8 mM) ions present in the Ringer solution, at pH 7.4 and pH 6.5. Data were normalized to the maximum response to ATP and are the mean of 3–4 observations; vertical lines show s.e.mean. In (a) and (b), curves were fitted by use of commercial software (Prism v1.03, GraphPad).

EC₅₀ values against fractional ratios for each form of ATP to see if the fractional amount of any ATP species increased proportionally to explain the observed potentiation of ATP-responses with acidic shifts (see Table 3).

It seemed unlikely that free ATP (ATP⁴⁻) activated the P2X₂ receptor since the fractional amount of free ATP fell as pH_e was changed from 8.0 to 5.5 (Table 3). Similarly, the fractional amount of NaATP, KATP, CaATP, Ca₂ATP MgATP and Mg₂ATP fell with acidification. Also, H₂ATP could be discounted since it is not formed at pH 7.4 and pH 8.0. Instead, the fractional amounts of CaHATP, MgHATP and HATP were seen to increase with acidification and these greater amounts might explain an apparent increase in P2X₂ receptor affinity for ATP. However, this argument does not fully explain our results, since the amounts of CaHATP and HATP increased further when changing pH_e from 6.5 to 5.5, although ATP-affinity (as judged by the EC₅₀ value) remained constant at these two pH levels. Therefore, the effects of progressive acidification do not appear to rest solely with increasing the amount of a particular form of ATP but also appear to involve additional factors (*e.g.*, the protonation of residues on the P2X₂ receptor itself: (see Discussion)).

Effect of pH on antagonist activity

The activity of antagonists was investigated at different pH levels to see if their blocking actions were enhanced at lowered pH_e levels. RB-2 and suramin were tested since they

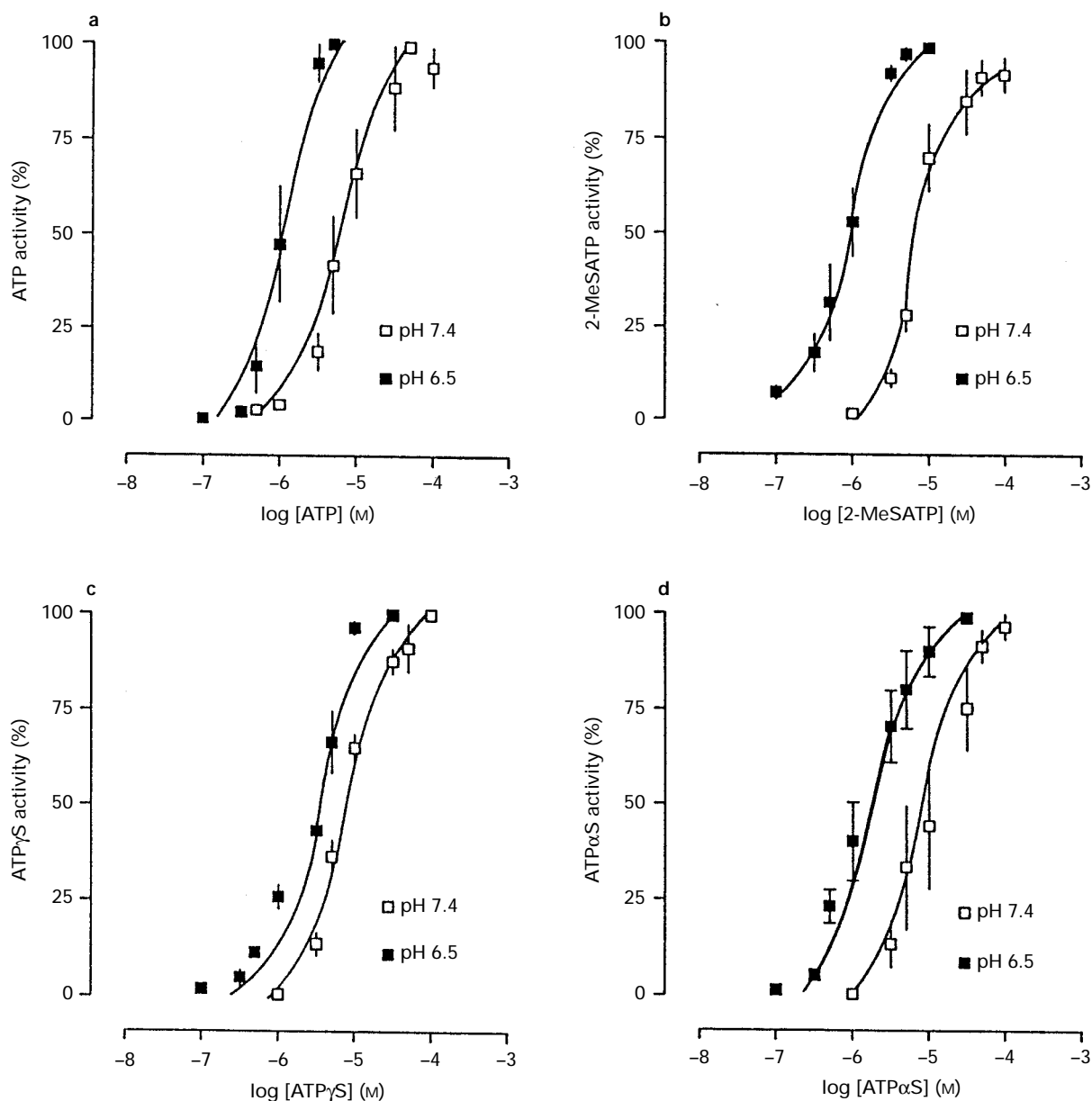


Figure 4 pH-dependency of agonist activity at P2X₂ receptors. Concentration-response curves for ATP (a), 2-MeSATP (b), ATPγS (c) and ATPαS (d), at pH 7.4 and pH 6.5. Data were normalized to the maximum response to ATP (100 μ M, at pH 7.4) and are the mean of 3–4 observations; vertical lines show s.e.mean. Curves were fitted by use of commercial software (Prism v1.03, GraphPad).

Table 2 Comparison of agonist activity indices at different pH levels

Agonist	EC_{50} value (pH 7.4)	EC_{50} value (pH 6.5)	Dose-ratio
ATP	$4.6 \pm 1 \mu$ M	$1.2 \pm 0.1 \mu$ M	3.85 ± 0.27
2-MeSATP	$7.1 \pm 1 \mu$ M	$1.1 \pm 0.2 \mu$ M	6.60 ± 0.47
ATPγS	$7.4 \pm 1 \mu$ M	$2.1 \pm 0.5 \mu$ M	3.67 ± 0.31
ATPαS	$13.2 \pm 6 \mu$ M	$2.0 \pm 1.0 \mu$ M	7.00 ± 1.87

EC_{50} values ($n=3-4$) for full agonists of the P2X₂ receptor at pH 7.4 and pH 6.5, and dose-ratios of these activity indices. Data from concentration-response relationships shown in Figure 4.

were the most and least effective antagonists of P2X₂ receptor. PPADS also was tested since it is a relatively selective P_{2X} antagonist (Lambrecht *et al.*, 1992; but see, Brown *et al.*,

1995) and TNP-ATP was chosen because it is chemically related to ATP and, like ATP, might be similarly affected by acidification. Inhibition curves for these four antagonists are shown in Figure 5.

The activity of RB-2 was not changed significantly by lowering pH_e from pH 7.4 to pH 6.5 (IC_{50} values: pH 7.4, $0.36 \pm 0.08 \mu$ M ($n=3$); pH 6.5, $0.17 \pm 0.05 \mu$ M ($n=4$); $P < 0.1$ by paired t test) and the activity of PPADS also remained unchanged (IC_{50} values: pH 7.4, $1.6 \pm 0.8 \mu$ M ($n=3$); pH 6.5, $1.2 \pm 0.2 \mu$ M ($n=4$); NS). The activity of TNP-ATP was reduced (IC_{50} values: pH 7.4, $1.13 \pm 0.19 \mu$ M ($n=3$); pH 6.5, $9.8 \pm 4.9 \mu$ M ($n=4$); $P < 0.05$). The slopes of inhibition curves for these three antagonists were more shallow under acidic conditions, n_H changing from 2 at pH 7.4 to 1 at pH 6.5. Since the Hill co-efficient for agonists is 2 (indicating a requirement of two molecules for receptor activation), it is possible that acidic shifts affected the affinity of these two putative ATP-binding sites to different degrees. Thus, the above antagonists tested might compete for two binding sites

at pH 7.4 but only for one binding site at pH 6.5. The most significant change in antagonist efficacy was seen with suramin which was enhanced 130 fold at pH 6.5 and 350 fold at pH 5.5 (IC₅₀ values: pH 7.4, 10.4 ± 1.2 μM (*n* = 4); pH 6.5, 78 ± 5 nM (*n* = 4); pH 5.5, 30 ± 6 nM (*n* = 4); *P* < 0.05, by paired *t* test). The slope of inhibition curves remained unchanged at 1 during acidic shifts.

Discussion

Agonist activity at the recombinant P2X₂ receptor

We have extended the pharmacological characterization of agonist activity at the recombinant P2X₂ receptor cloned from rat PC12 cells. The potency order of agonists observed in the

Table 3 Fractional ratios of ATP species

ATP species	Ca-Ringer						Mg-Ringer					
	pH 8.0		pH 7.4		pH 6.5		pH 5.5		pH 7.4		pH 6.5	
	Amount	Ratio	Amount	Ratio	Amount	Ratio	Amount	Ratio	Amount	Ratio	Amount	Ratio
Total ATP (as EC ₅₀ values)	28 μM	0.26	4.6 μM	1	12 μM	3.8	1.1 μM	4.2	29.3 μM	1	5.5 μM	5.3
Free ATP	15.5%	1.04	14.9%	1	13.4%	0.9	6.4%	0.43	14.5%	1	12.7%	0.88
NaATP	17.9%	1.02	17.5%	1	15.7%	0.89	7.4%	0.42	17.2%	1	14.9%	0.87
KATP	0.3%	1	0.3%	1	0.3%	1	0.13%	0.43	0.31%	1	0.26%	0.87
CaATP	57.3%	1.01	56.9%	1	50.8%	0.89	24%	0.42	—	—	—	—
Ca ₂ ATP	8.6%	0.97	8.8%	1	7.7%	0.88	3.6%	0.41	—	—	—	—
CaHATP	0.03%	0.3	0.1%	1	0.8%	8	3.9%	39	—	—	—	—
MgATP	—	—	—	—	—	—	—	—	64.9%	1	59.4%	0.92
Mg ₂ ATP	—	—	—	—	—	—	—	—	1.7%	1	1.6%	0.94
MgHATP	—	—	—	—	—	—	—	—	0.1%	1	0.7%	7
HATP	0.4%	0.26	1.5%	1	11.1%	7.4	52.6%	35.1	1.5%	1	10.3%	6.9
H ₂ ATP	0%	—	0%	—	0.04%	—	1.9%	—	0	—	0.04%	—

The amounts and fractional ratios (at 18°C) of ATP species in two solutions, a Ca²⁺-containing Ringer (Ca-Ringer) and a Ca²⁺-free Ringer (Mg-Ringer) (for compositions, see Methods). Total ATP was taken as the EC₅₀ values at each pH level and the amounts of each ATP species were calculated by BAD v.435 (Brooks & Storey, 1992). Concentration-ratios and fractional-ratios were calculated after taking values at pH 7.4 to be 1.

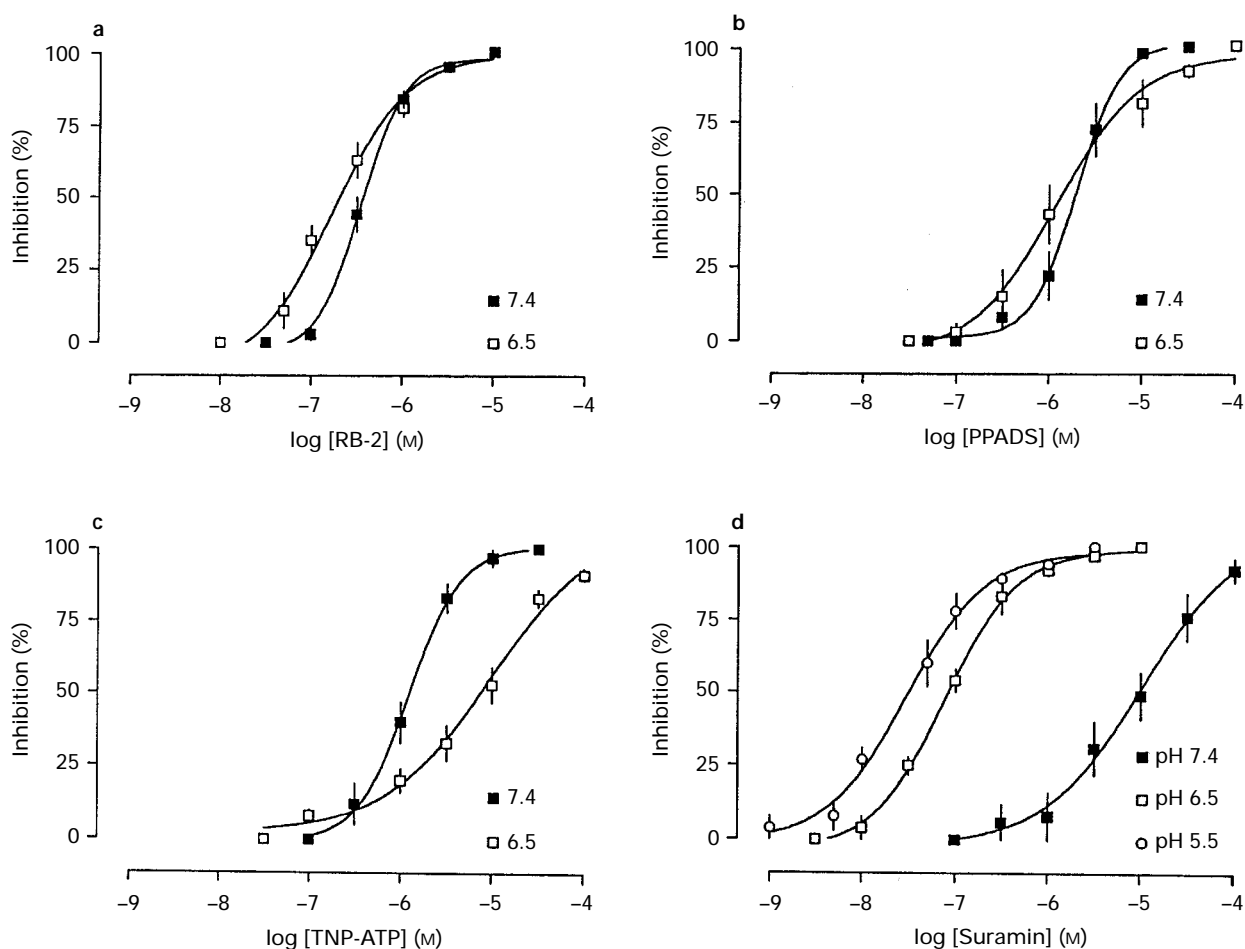


Figure 5 pH-dependency of antagonist activity at P2X₂ receptors. Inhibition curves for RB-2 (a), PPADS (b), TNP-ATP (c) and suramin (d) as antagonists of submaximal ATP-responses (at 5 μM) at pH 7.4, pH 6.5 and pH 5.5. Data are mean of 4 sets of observations for each inhibition curve; vertical lines show s.e.mean. Curves were fitted by use of commercial software (Prism V1.03, GraphPad).

present study (ATP > 2-MeSATP ≥ ATP_γS > ATP_αS > CTP ≥ BzATP) was similar to the potency order (ATP = ATP_γS = 2-MeSATP) first determined for recombinant P2X₂ receptors expressed in *Xenopus* oocytes (Brake *et al.*, 1994) and similar to the potency order (2-MeSATP = 2-Cl-ATP > ATP > ATP_γS > BzATP > ADP) shown for recombinant P2X₂ receptors expressed in HEK-293 cells (Evans *et al.*, 1995). The EC₅₀ value for ATP (4.6 ± 1.1 μM) determined in our study is similar to the value (7.7 ± 1 μM) obtained by Evans *et al.* (1995) but different from the value (59.9 ± 5.3 μM) found by Brake *et al.* (1994). When Mg²⁺ and Ca²⁺ ions were present in the superfusate (as in both of the above-cited studies), the EC₅₀ value for ATP in our experiments (25.8 ± 4.9 μM) lay between the above values. These differences in measurements of P2X₂-affinity for ATP may reflect subtle differences in the extracellular pH (pH_e) and differences in the concentration/activity of divalent cations used in respective studies.

Three studies (Brake *et al.*, 1994; Evans *et al.*, 1995; the present paper) have shown that α,β-meATP and β,γ-meATP do not activate (or desensitize: B. King, unpublished results) the recombinant P2X₂ receptor. A picture is emerging that recombinant P_{2X} receptor subtypes which desensitize rapidly (*i.e.*, P2X₁ and P2X₃) are activated by methylene-phosphonate analogues of ATP (Valera *et al.*, 1994; 1995; Chen *et al.*, 1995; Lewis *et al.*, 1995) while subtypes which desensitize slowly (*i.e.*, P2X₂, P2X₄₋₇) are not activated by these analogues (Brake *et al.*, 1994; Bo *et al.*, 1995; Evans *et al.*, 1995; Buell *et al.*, 1996; Collo *et al.*, 1996; Garcia-Guzman *et al.*, 1996; Séguéla *et al.*, 1996; Soto *et al.*, 1996a,b; Surprenant *et al.*, 1996; Wang *et al.*, 1996). We found that the recombinant P2X₂ receptor is activated by CTP, as did Brake *et al.* (1994), and this pharmacological feature is shared by P2X₃ (Chen *et al.*, 1995) and P2X₄ (Soto *et al.*, 1996a) subtypes. Thus, like many metabotropic ATP receptor subtypes, some ionotropic ATP receptors can be activated by some pyrimidine nucleotides, although they are not as potent as purine nucleotides at P2X₂₋₄ subtypes. It was notable that the P2X₂ receptor was not activated by ADP, 2-MeSADP and ADPβS. Brake *et al.* (1994) also found that ADP did not activate the P2X₂ subtype, while Evans *et al.* (1995) showed the converse, although ADP-activity was only seen over a concentration range 100 fold higher than required for ATP activation. A 1% contamination by ATP of ADP stocks or the presence of a nucleotide diphosphokinase (converting diphosphates to triphosphates) in HEK-293 cells might account for the apparent ADP activation of P2X₂ receptors.

ATP-affinity at P2X₂ was strongly affected by extracellular Mg²⁺ ions present in the superfusate, decreasing the EC₅₀ for ATP by 6 fold. Trezise and colleagues (1994) have commented on a similar sensitivity to Mg²⁺ cations by P_{2X} receptors on the rat vagus nerve where P2X₂ transcripts (Collo *et al.*, 1996) and P2X₂-immunoreactivity (Vulchanova *et al.*, 1996) have been localized. The observed enhancement of ATP-activity at the rat isolated vagus nerve upon removal of extracellular Mg²⁺ and Ca²⁺ has been explained in terms of a reduction in Mg²⁺/Ca²⁺-dependent ecto-nucleotidase activity (Trezise *et al.*, 1994; Kennedy & Leff, 1995). However, this does not explain our results with P2X₂ since defolliculated oocytes are largely devoid of ecto-ATPases (Ziganshin *et al.*, 1995). Nakazawa and Hess (1993) found that Na⁺ permeation of native P_{2X} receptors on rat PC12 cells was reduced by extracellular divalent cations including Mg²⁺, with a potency order Cd²⁺ > Mn²⁺ > Mg²⁺ = Ca²⁺ > Ba²⁺. The P2X₂ receptor was cloned from rat PC12 cells by Brake *et al.* (1994) who suggested that the H5 region on the extracellular loop of the receptor forms an ion-binding site which guards the ion pore formed by TM2 transmembrane spanning domains. Thus, it is possible that this ion-binding site for divalent cations influences the ease with which ATP opens the ion pore at the recombinant P2X₂ receptor.

Antagonist activity at the recombinant P2X₂ receptor

So far, there has been limited data on the activity of known P₂ receptor antagonists at the P2X₂ subtype. Brake *et al.* (1994)

showed that suramin (100 μM) and Reactive blue 2 (RB-2) (100 μM) blocked the P2X₂ subtype but a detailed analysis of these drugs was not carried out. Evans *et al.* (1995) showed that PPADS, suramin and pyridoxal-5-phosphate block P2X₂ receptors (IC₅₀ values: 1 μM, 5 μM and 5 μM, respectively). These activity indices for PPADS and suramin agree reasonably well with IC₅₀ values (1.6 μM and 10.4 μM, respectively) determined by us. However, our data show that RB-2 is more potent than either PPADS or suramin, and as active at P2X₂ receptors (IC₅₀ = 0.36 ± 0.08 μM) as at P2Y₁ receptors (IC₅₀ = 0.58 ± 0.13 μM) (Simon *et al.*, 1995). This similar efficacy in blocking recombinant P_{2X} and P_{2Y} receptors now casts doubt on earlier claims, based on data from studies of mesenteric arteries (Burnstock & Warland, 1987), that RB-2 can be used as a selective antagonist over a narrow concentration range for native P_{2Y} receptors in mammalian tissues. In agreement with Mockett and colleagues (1994), we found that the fluoroprobe TNP-ATP was an effective antagonist of P2X₂ receptors although our results represent the first occasion where its blocking activity has been fully assessed. Along with oxidized ATP (see Wiley *et al.*, 1994) and adenosine-3'-phosphate-5'-phosphosulphate (Boyer *et al.*, 1996), TNP-ATP represents yet another ATP analogue which can function as a P₂ receptor antagonist. A new P₂ antagonist, Palatine fast black (PFB), was identified but its usefulness was limited because it caused an irreversible blockade of P₂ receptors; at > 100 μM, PFB was found to be toxic to oocytes.

The slope of inhibition curves for RB-2, PPADS, PFB and TNP-ATP was 2 (at pH 7.4), while the slope for inhibition curves for suramin and Coomassie brilliant blue G (CBB-G) was 1 (at pH 6.5). The results suggested that the first group of antagonists interacted with two binding sites and the second group with one binding site. The chemical structures of suramin and CBB-G reveal that each molecule possesses two identical side chains. It is tempting to suggest that these two side chains interact with two binding sites as do RB-2, PPADS, PFB and TNP-ATP. However, the slopes of inhibition curves for RB-2, PPADS and TNP-ATP changed to 1 at pH 6.5. It may be true that the two agonist binding sites on P2X₂ have unequal affinity for antagonists and that suramin and CBB-G cannot compete for one of these binding sites and, at pH 6.5, other antagonists can only compete for this single binding site.

Agonist and antagonist activity at lowered pH_e levels

Acidic pH_e levels had different effects on agonist and antagonist activity at P2X₂ receptors. Receptor affinity for four full agonists (ATP, 2-MeSATP, ATP_γS and ATP_αS) was enhanced approximately 5 fold at pH 6.5, although no further enhancement was observed at lower pH levels. Of the antagonists tested, only suramin was affected by acidic conditions and acid-induced potentiation of its blocking action was greater at pH 5.5 than at pH 6.5. The reasons why suramin alone was affected by lowering pH_e remain to be determined.

Receptor affinity for agonists and antagonists is also enhanced at low pH_e at the γ-aminobutyric acid_A (GABA_A) receptor (Robello *et al.*, 1994; Krishek *et al.*, 1996), GABA ρ1 subunits (Wang *et al.*, 1995), and combinations of GABA α1 and β1 subunits (Krishek *et al.*, 1996), as well as adenosine A₁ (Hoppe & Reddington, 1991; Van der Wenden *et al.*, 1991; Allende *et al.*, 1993) and A_{2A} receptors (Askalan & Richardson, 1994; Kirk & Richardson, 1995). For A₁ and A_{2A} subtypes, low pH_e had a greater effect on agonist activity than antagonist activity (Allende *et al.*, 1991; Kirk & Richardson, 1995). A role for one or more histidine residues has been established for this pH-dependent enhancement of agonist activity at the above adenosine and GABA receptors. Where strategic histidine residues have been modified chemically (Allende *et al.*, 1993) or substituted (Van der Wenden *et al.*, 1991; Wang *et al.*, 1995), the potentiating effect of extracellular pH has been diminished or nullified. It is noteworthy that the putative extracellular loop of the P2X₂ receptor contains 9 histidine residues interspersed between 10 cysteine residues, the

latter being conserved throughout the P2X₁₋₇ series. Allende *et al.* (1993) showed that cysteine and histidine residues are both important for agonist and antagonist binding at the pH-sensitive A₁ receptor. It is tempting to speculate that these two types of amino acid residues also play a similar role in ATP binding at the pH-sensitive P2X₂ receptor. It is interesting to note that agonist binding/activity at pH-sensitive GABA receptors is modulated by Zn²⁺ ions acting on specific histidine residues (Wang *et al.*, 1995). In the initial characterization of P2X₂ (Brake *et al.*, 1994) receptor affinity for ATP was increased 4 fold by adding Zn²⁺ to the bathing medium. The locus for pH- and Zn²⁺-potentiation of agonist activity may rest with amino acid residues on the extracellular loop of P2X₂ receptors.

ATP-species activating P2X₂ receptors

Brooks and Storey (1992) have designed an iterative process to calculate the species of ATP present in a solution of known ionic strength, pH and temperature. This process is contained in a software programme (BAD) which is freely available and, according to the compilers, is as accurate as earlier programmes of a similar purpose. We have used this programme to determine the ATP species present in the superfusate at different pH levels and ionic strength. Calculations were based on the EC₅₀ values for ATP under different experimental conditions, to yield fractional ratios of the ATP-species for the amount of ATP described by the EC₅₀. A preliminary account of these data has been presented (King *et al.*, 1996d).

A comparison of EC₅₀ ratios and fractional ratios revealed that the amounts of HATP, CaHATP and MgHATP in solution increased proportionately with a progressive acidification of the bathing medium, over the range of pH 8.0–6.5. Li *et al.* (1996b) have carried out a similar analysis and also find that the amounts of HATP, CaHATP and MgHATP increase as pH_e is lowered. Although our findings are corroborated by Li *et al.* (1996b), we believe it is unlikely that these three species identified by BAD represent the only forms of ATP that activate P2X₂ receptors. It seems more likely that all forms of ATP will activate the P2X₂ receptor but there will be significant differences in receptor affinity for each species. This belief is supported by data from *I/V* relationships for ATP currents where, in ion substitution experiments with only one cationic

species present (*e.g.*, only Na⁺ or K⁺), ATP can still evoke responses of similar amplitude (King: unpublished observations).

With regard to the involvement of free ATP (ATP⁴⁻) in the activation of P2X₂ receptors, its pK for protonation is 6.51 indicating that the amount of ATP⁴⁻ falls and HATP³⁻ increases with acidification (as shown in Table 3). Thus, it is difficult to explain how ATP⁴⁻ can activate the P2X₂ receptor with a greater affinity at pH 6.5. In fact, Sela *et al.* (1991) predicted that ATP⁴⁻ did not activate the native P_{2X} receptor on PC12 cells from which P2X₂ was cloned. Other workers have suggested the contrary opinion (Rhoads *et al.*, 1993), since they found that the addition of Mg²⁺ to the extracellular solution reduces the secretory activity of ATP at PC12 cells. We observed that the addition of extracellular Mg²⁺ (1 mM) to the superfusate decreased ATP-affinity at P2X₂ (see Figure 1b) and calculations with BAD showed that the amount of free ATP⁴⁻ decreased by 27% when Mg²⁺ (1 mM) was present. Notwithstanding the value of calculating the amounts of ATP species present, increases in ATP-affinity can not be easily explained by an increased availability of one or more ATP species at lower pH_e levels, since there is an upper limit to the pH effect on receptor sensitivity but not to protonation of ATP-species. Krishek and colleagues (1996), in testing a similar pH-sensitivity at recombinant GABA receptors, concluded that receptor protonation rather than agonist protonation explained their results since some combinations of GABA subunits (*e.g.*, $\alpha 1, \beta 1 \gamma 2S$) are not affected by a change in pH. In our case, we too believe that receptor protonation is more likely, since lower pH levels reduce, rather than enhance, ATP activity at recombinant homomeric P2X₃ and P2X₄ receptors (King: unpublished results).

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