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Effects of extracellular pH on agonism and antagonism at a recombinant $P2X_2$ receptor

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1 Under voltage-clamp conditions, the activity of agonists and antagonists at a recombinant $P2X_2$ receptor expressed in *Xenopus* oocytes was examined at different levels of extracellular pH (pH_e).

2 In normal Ringer (Mg^{2+} 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 Ca^{2+} ions were replaced with equimolar Mg^{2+} 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 $P2X_2$ 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 $P2X_2$ receptors was: ATP > 2-MeS-ATP $\geqslant$ ATPγS > ATPαS > > CTP $\geqslant$ BzATP, while other nucleotides were inactive. EC_{50} 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, ATPγS and ATPαS), without altering the potency order.

6 The potency order for antagonists at $P2X_2$ receptors was: Reactive blue-2 > trinitrophenol-ATP $\geqslant$ Palatine fast black $\geqslant$ Coomassie brilliant blue $\geqslant$ PPADS > suramin (at pH 7.4). IC_{50} values and slopes of the inhibition curves were re-examined at different pH levels. Only blockade by suramin was affected significantly by extracellular acidification (IC_{50} 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 $P2X_2$ 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 $P2X_2$ transcripts, the sensitization by metabolic acidosis of native P_{2X} receptors containing $P2X_2$ subunits may have a significant effect on purinergic cell-to-cell signalling.

Keywords: ATP receptor; P2X receptor; recombinant ($P2X_2$) receptor; ATP; suramin; pH; acidosis; *Xenopus* oocyte

Introduction

An adenosine 5′-triphosphate (ATP)-gated ion channel ($P2X_2$ receptor) has been cloned from rat phaeochromocytoma 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 $P2X_2$ 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, $P2X_2$ transcripts have been shown to be widely distributed throughout rat tissues, including brain, spinal cord and peripheral autonomic nerves (where $P2X_4$ and $P2X_6$ transcripts also co-localize) and in C-fibre sensory neurones (where $P2X_3$ transcripts are present) (Collo *et al*., 1996). From immunostaining with $P2X_2$-selective antibodies, the distribution and density of $P2X_2$ 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 $P2X_2$ subunits in central terminals of vagal and dorsal root sensory neurones (Vulchanova *et al*., 1996). $P2X_2$ 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 $P2X_2$ 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 $P2X_2$ 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. $P2X_2$ transcripts are heavily concentrated in sensory neurones in nodose, trigeminal and dorsal root ganglia (Collo *et al*., 1996) and strong $P2X_2$-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 $P2X_2$ subunits.

In the present paper, we have investigated the effects of pH_e on the activity of agonists and antagonists at recombinant homomeric $P2X_2$ 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 $P2X_2$ receptor varied when pH_e 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, $NaHCO_3$ 2.4, Tris-HCl 7.5, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82 and gentamycin sulphate, 50 $\mu g\ l^{-1}$. 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 Ca^{2+}-free Ringer, for 2–3 h) and mechanical removal of the follicle cell layer with fine forceps. Defolliculation was necessary to remove the native P_1 and P_2 receptors found on the follicle cell monolayer enveloping oocytes (King *et al*., 1996a,b). The cytosol of defolliculated oocytes was injected with $P2X_2$ transcripts (40 nl, 0.1–1 $\mu g\ ml^{-1}$); 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 K_2SO_4 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, $CaCl_2$ 1.8, adjusted to pH 7.45. In some cases, $MgCl_2$ (1.0 mM) was added to the superfusate to match the experimental conditions of Brake *et al*., (1994). In other experiments, $MgCl_2$ (1.8 mM) replaced $CaCl_2$ (1.8 mM) to give a nominally Ca^{2+}-free superfusate; elsewhere, $MgCl_2$ (1.8 mM) and EGTA (1.0 mM) were added and $CaCl_2$ (1.8 mM) omitted to give a wholly Ca^{2+}-free superfusate. The pH of the bathing Ringer solution (pH_e) 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-impaled 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 $P2X_2$ 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 (EC_{50}) 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 P_2 antagonists at $P2X_2$ 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 EC_{50} for the pH level tested). The antagonist concentration that reduced ATP-responses by 50% (IC_{50}) 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) (ATPαS; 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 $P2X_2$ receptors

The selectivity of the recombinant $P2X_2$ 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 EC_{100}. The activity order for agonists was (at 30 μM): ATP ⩾ 2-MeSATP ⩾ ATPγS > ATPαS >> cytosine-5′-triphosphate(CTP) ⩾ BzATP (see Table 1). ATP and CTP were the only naturally-occurring nucleoside triphosphates to activate the $P2X_2$ 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 ADPβS) 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 $P2X_2$ 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 $P2X_2$ 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)
ATPγS	0.85 ± 0.14 (3)	ADPβS	0 (3)	GTP	0 (3)
ATPαS	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 $P2X_2$ receptors (Brake *et al*., 1994). Concentration-response curves were constructed for ATP, 2-MeSATP, ATPγS and ATPαS which are full agonists of $P2X_2$ receptors (Figure 1a). Affinity constants (as EC_{50} values) were (at pH 7.45): ATP, 4.6 ± 1.1 μM ($n=4$); 2-MeSATP, 7.1 ± 1.0 μM ($n=4$); ATPγS, 7.4 ± 0.5 μM ($n=4$); ATPαS, 13.2 ± 6.8 μM ($n=4$). The Hill co-efficient (n_H) for each full agonist was: ATP, 2.1 ± 0.2 ($n=4$); 2-MeSATP, 2.1 ± 0.3 ($n=4$); ATPγS, 2.3 ± 0.2 ($n=4$); ATPαS, 2.2 ± 0.1 ($n=4$).

Both extracellular Mg^{2+} and Ca^{2+} have been shown to inhibit the opening of ATP-activated $P2X_2$ receptors on PC12 cells (Nakazawa & Hess, 1993) from which the $P2X_2$ subunit was cloned. Thus, we retested ATP-activity at different concentrations of Mg^{2+} and Ca^{2+} in the superfusate. With Mg^{2+} (1.0 mM) added to the superfusate (as in Brake *et al*., 1994), the EC_{50} value for ATP was 25.8 ± 4.9 μM ($n=4$) 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 Mg^{2+} (1.8 mM) replacing Ca^{2+} (1.8 mM) to give a nominally Ca^{2+}-free superfusate, the EC_{50} value for ATP was similar (26.1 ± 2.1 μM, $n=3$) 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 Mg^{2+} (1.8 mM) and EGTA (1.0 mM) present and Ca^{2+} (1.8 mM) omitted to give a wholly Ca^{2+}-free superfusate, the EC_{50} value for ATP was 29.3 ± 1.8 μM ($n=3$) and the slope of the curve was 2.1 ± 0.1 (Figure 1b).

Antagonist activity at $P2X_2$ receptors

A series of known and novel P2 receptor antagonists were tested against ATP-activated inward currents in oocytes expressing $P2X_2$ 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 P_2 receptor antagonist Palatine fast black (PFB) was identified and tested.

After ATP-responses of consistent amplitude (with 5 μM, a value close to the EC_{50} 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 $P2X_2$ 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 (IC_{50} value) were (in descending order): RB-2, 0.36 ± 0.08 μM ($n=3$); TNP-ATP, 1.13 ± 0.19 μM ($n=3$); PFB, 1.29 ± 0.33 μM ($n=4$); CBB-G, 1.4 ± 0.3 μM ($n=3$); PPADS, 1.6 ± 0.08 μM ($n=3$); suramin, 10.4 ± 1.2 μM ($n=4$); 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 $P2X_2$ 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 $P2X_2$ 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 $P2X_2$ receptors expressed in HEK-293 cells (Evans *et al*., 1995), with similar activity and IC_{50} values as found here.

The $P2X_2$ receptor was shown to possess structural features and overall topology similar to amiloride-sensitive Na^+-

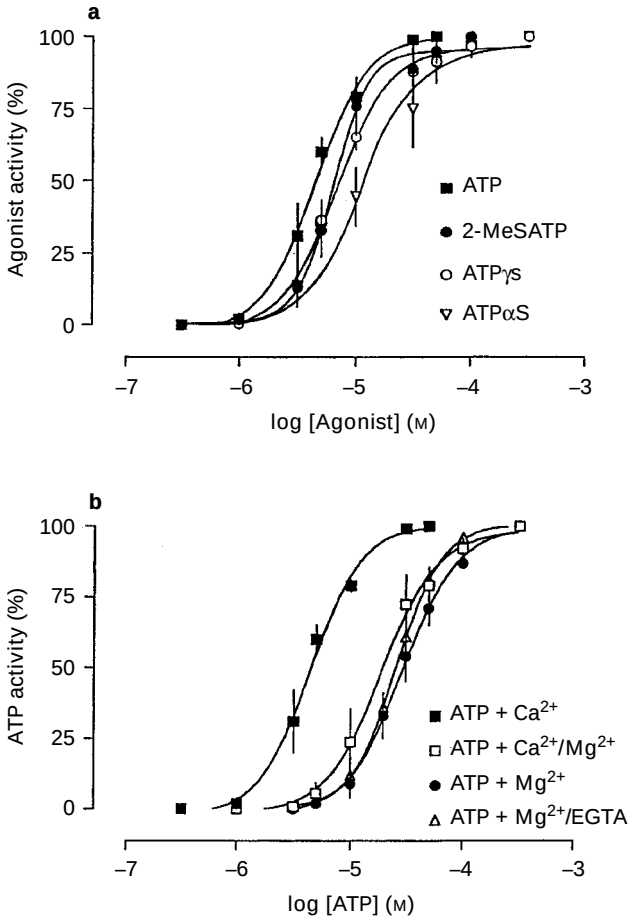

Figure 1 Activity of ATP and analogues at $P2X_2$ receptors. In (a) concentration-response curves for evoked inward currents to ATP 2-MeSATP, ATPαS and ATPγS (each at 0.5 – 300 μM) at pH 7.45. Data were normalized to the maximum response to ATP. In (b), concentration-response curves for ATP (0.5 – 300 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 + Ca^{2+}); NRS and calcium (1.8 mM) and magnesium (1.0 mM) ions (ATP + Ca^{2+}/Mg^{2+}); NRS and magnesium (1.8 mM) ions (ATP + Mg^{2+}); NRS and magnesium ions (1.8 mM) in the presence of EGTA (1.0 mM) (ATP + Mg^{2+}/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 $P2X_2$ 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 Ca^{2+} (1.8 mM) or Mg^{2+} (1.8 mM) and EGTA (1 mM) in the superfusate, maximal ATP activity occurred at pH 6.5 and lowering

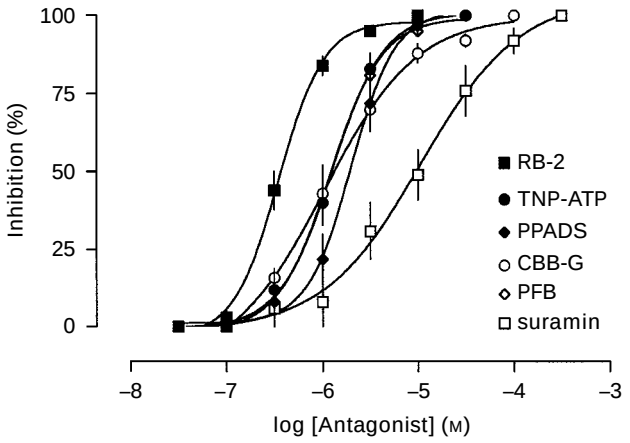

Figure 2 Activity of antagonists at P2X$_2$ receptors. Inhibition curves (at pH 7.45) for reactive blue 2 (RB-2), trinitrophenyl-ATP (TNP-ATP), pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), Coomassie brilliant blue G (CBB-G), Palatine fast black (PFB) and suramin as antagonists of submaximal ATP-responses (at 5 μM, which is close to the EC$_{50}$ 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).

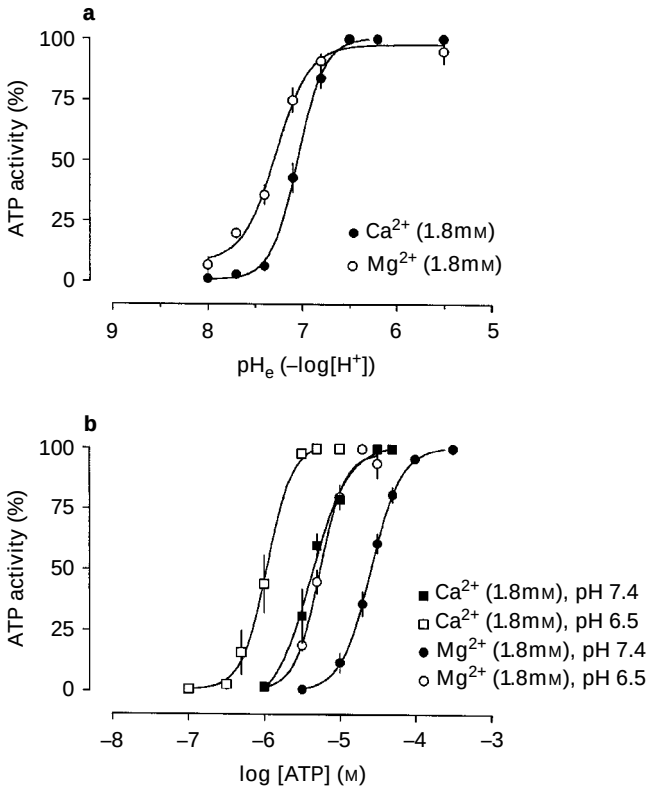

Figure 3 pH-dependence of ATP-activity at P2X$_2$ receptors. In (a), the effect of extracellular pH (8.0–5.5) on the activity of submaximal concentrations of ATP (3 μM and 20 μM) in a normal Ringer solution containing either calcium (1.8 mM) or magnesium (1.8 mM) ions as the divalent cation species. Data were normalized to the maximum response to ATP (100 μM, 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).

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 Ca^{2+} (1.8 mM) present in the Ringer solution, and 7.35±0.05 (n=3) when extracellular Ca^{2+} was replaced with Mg^{2+} (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 P2X$_2$ 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 Ca^{2+} (1.8 mM) included in the superfusate, EC$_{50}$ values (n=4) for ATP were: pH 8.0, 28±2 μM; pH 7.4, 4.6±1 μM; pH 6.5, 1.2±0.1 μM; pH 5.5, 1.1±0.1 μM. With Mg^{2+} (1.8 mM) and EGTA (1 mM) replacing Ca^{2+} in the superfusate, EC$_{50}$ values (n=3) for ATP were: pH 7.4, 29.3±1.8 μM; pH 6.5, 5.5±0.5 μM (Figure 3b). On comparison of EC$_{50}$ values, ATP showed a 5 fold increase in affinity for P2X$_2$ receptors at pH 6.5, with either Ca^{2+} or Mg^{2+} present in the superfusate (Figure 3b).

We have shown that the potency order of agonists remained unchanged at different pH$_e$ levels although P2X$_2$ 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 P2X$_2$ receptors was tested at pH 7.4 and retested at pH 6.5 to see if their affinity for P2X$_2$ receptors was similarly altered. Concentration-response curves for 2-MeSATP, ATPγS and ATPαS 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). EC$_{50}$ 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 P2X$_2$ receptors after a lowering of pH$_e$.

ATP species activating P2X$_2$ receptors

The ATP species which activates P2X$_2$ 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 EC$_{50}$ 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 (ATP^{4-}) activated the P2X$_2$ 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, Ca_2ATP MgATP and Mg_2ATP fell with acidification. Also, H_2ATP 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 P2X$_2$ 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 EC$_{50}$ 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 P2X$_2$ 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

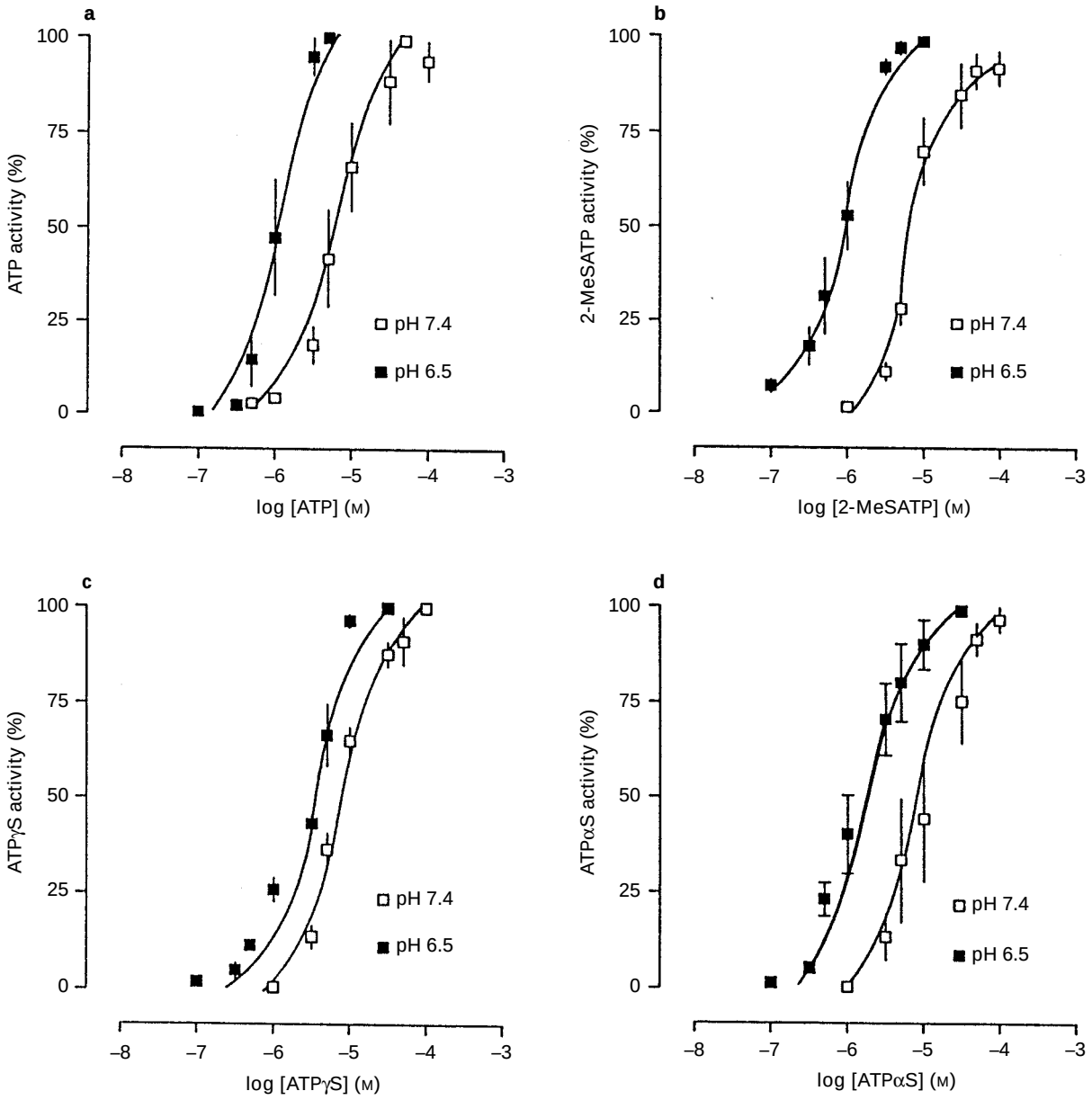

Figure 4 pH-dependency of agonist activity at P2X$_2$ receptors. Concentration-response curves for ATP (a), 2-MeSATP (b), ATPγS (c) and ATPαS (d), at pH 7.4 and pH 6.5. Data were normalized to the maximum response to ATP (100 μM, at pH 7.4) and are the mean of 3–4 observations; vertical lines show s.e.mean. 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±1 μM	1.2±0.1 μM	3.85±0.27
2-MeSATP	7.1±1 μM	1.1±0.2 μM	6.60±0.47
ATPγS	7.4±1 μM	2.1±0.5 μM	3.67±0.31
ATPαS	13.2±6 μM	2.0±1.0 μM	7.00±1.87

EC_{50} values (n=3–4) for full agonists of the P2X$_2$ 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 P2X$_2$ 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±0.08 μM (n=3); pH 6.5, 0.17±0.05 μM (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±0.8 μM (n=3); pH 6.5, 1.2±0.2 μM (n=4); NS). The activity of TNP-ATP was reduced (IC_{50} values: pH 7.4, 1.13±0.19 μM (n=3); pH 6.5, 9.8±4.9 μM (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 (IC_{50} 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 $P2X_2$ receptor

We have extended the pharmacological characterization of agonist activity at the recombinant $P2X_2$ receptor cloned from rat PC12 cells. The potency order of agonists observed in the

Table 3 Fractional rations of ATP species

	Ca-Ringer								*Mg-Ringer*			
ATP	*pH 8.0*		*pH 7.4*		*pH 6.5*		*pH 5.5*		*pH 7.4*		*pH 6.5*	
species	*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_2ATP	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_2ATP	–	–	–	–	–	–	–	–	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_2ATP	0%	–	0%	–	0.04%	–	1.9%	–	0	–	0.04%	–

The amounts and fractional ratios (at 18°C) of ATP species in two solutions, a Ca^{2+}-containing Ringer (Ca-Ringer) and a Ca^{2+}-free Ringer (Mg-Ringer) (for compositions, see Methods). Total ATP was taken as the EC_{50} 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.

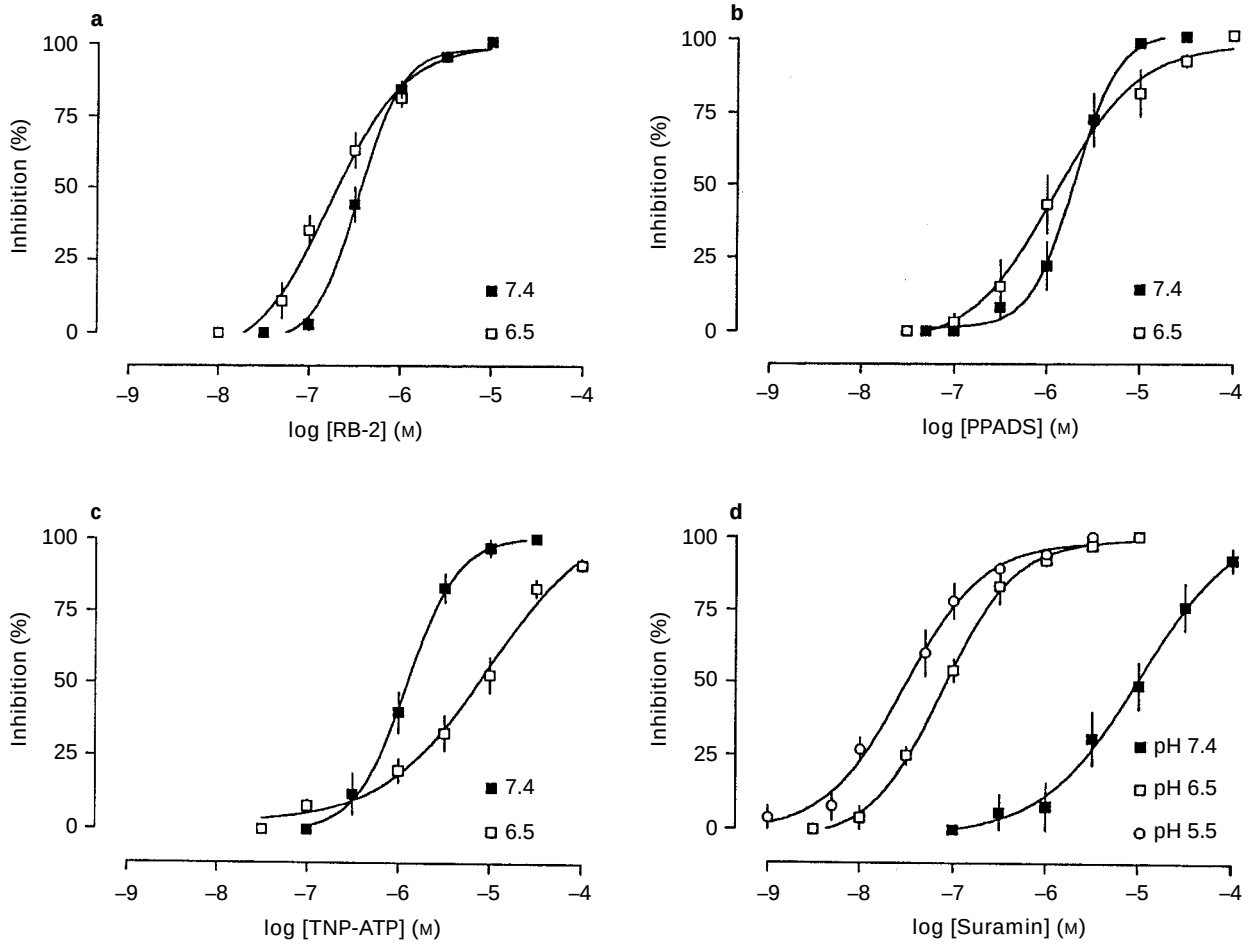

Figure 5 pH-dependency of antagonist activity at $P2X_2$ receptors. Inhibition curves for RB-2 (a), PPADS (b), TNP-ATP (c) and suramin (d) as antagonists of submaximal ATP-responses (at 5 μM) at pH 7.4, pH 6.5 and pH 5.5. Data are mean of 4 sets of observations for each inhibition curve; vertical lines show s.e.mean. Curves were fitted by use of commercial software (Prism V1.03, GraphPad).

present study (ATP > 2-MeSATP ≥ ATPγS > ATPαS > > CTP ≥ BzATP) was similar to the potency order (ATP = ATPγS = 2-MeSATP) first determined for recombinant $P2X_2$ receptors expressed in *Xenopus* oocytes (Brake *et al*., 1994) and similar to the potency order (2-MeSATP = 2-Cl-ATP > ATP > ATPγS > BzATP > ADP) shown for recombinant $P2X_2$ receptors expressed in HEK-293 cells (Evans *et al*., 1995). The EC_{50} 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 Mg^{2+} and Ca^{2+} ions were present in the superfusate (as in both of the above-cited studies), the EC_{50} value for ATP in our experiments (25.8 ± 4.9 μM) lay between the above values. These differences in measurements of $P2X_2$-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 $P2X_2$ receptor. A picture is emerging that recombinant P_{2X} receptor subtypes which desensitize rapidly (*i.e*., $P2X_1$ and $P2X_3$) 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*., $P2X_2$, P_{2X4-7}) 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 $P2X_2$ receptor is activated by CTP, as did Brake *et al*. (1994), and this pharmacological feature is shared by $P2X_3$ (Chen *et al*., 1995) and $P2X_4$ (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 $P2X_{2-4}$ subtypes. It was notable that the $P2X_2$ receptor was not activated by ADP, 2-MeSADP and ADPβS. Brake *et al*. (1994) also found that ADP did not activate the $P2X_2$ 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 $P2X_2$ receptors.

ATP-affinity at $P2X_2$ was strongly affected by extracellular Mg^{2+} ions present in the superfusate, decreasing the EC_{50} for ATP by 6 fold. Trezise and colleagues (1994) have commented on a similar sensitivity to Mg^{2+} cations by P_{2X} receptors on the rat vagus nerve where $P2X_2$ transcripts (Collo *et al*., 1996) and $P2X_2$-immunoreactivity (Vulchanova *et al*., 1996) have been localized. The observed enhancement of ATP-activity at the rat isolated vagus nerve upon removal of extracellular Mg^{2+} and Ca^{2+} has been explained in terms of a reduction in Mg^{2+}/Ca^{2+}-dependent ecto-nucleotidase activity (Trezise *et al*., 1994; Kennedy & Leff, 1995). However, this does not explain our results with $P2X_2$ 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 Mg^{2+}, with a potency order $Cd^{2+} > Mn^{2+} > Mg^{2+} = Ca^{2+} > Ba^{2+}$. The $P2X_2$ 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 $P2X_2$ receptor.

Antagonist activity at the recombinant $P2X_2$ receptor

So far, there has been limited data on the activity of known P_2 receptor antagonists at the $P2X_2$ subtype. Brake *et al*. (1994) showed that suramin (100 μM) and Reactive blue 2 (RB-2) (100 μM) blocked the $P2X_2$ 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 $P2X_2$ receptors (IC_{50} values: 1 μM, 5 μM and 5 μM, respectively). These activity indices for PPADS and suramin agree reasonably well with IC_{50} 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 $P2X_2$ receptors ($IC_{50} = 0.36 \pm 0.08$ μM) as at $P2Y_1$ receptors ($IC_{50} = 0.58 \pm 0.13$ 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 $P2X_2$ 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 P_2 receptor antagonist. A new P_2 antagonist, Palatine fast black (PFB), was identified but its usefulness was limited because it caused an irreversible blockade of P_2 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 $P2X_2$ 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 $P2X_2$ receptors. Receptor affinity for four full agonists (ATP, 2-MeSATP, ATPγS and ATPαS) 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 A_1 (Hoppe & Reddington, 1991; Van der Wenden *et al*., 1991; Allende *et al*., 1993) and A_{2A} receptors (Askalan & Richardson, 1994; Kirk & Richardson, 1995). For A_1 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 $P2X_2$ receptor contains 9 histidine residues interspersed between 10 cysteine residues, the

latter being conserved throughout the $P2X_{1-7}$ series. Allende *et al*. (1993) showed that cysteine and histidine residues are both important for agonist and antagonist binding at the pH-sensitive A_1 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 $P2X_2$ receptor. It is interesting to note that agonist binding/activity at pH-sensitive GABA receptors is modulated by Zn^{2+} ions acting on specific histidine residues (Wang *et al*., 1995). In the initial characterization of $P2X_2$ (Brake *et al*., 1994) receptor affinity for ATP was increased 4 fold by adding Zn^{2+} to the bathing medium. The locus for pH- and Zn^{2+}-potentiation of agonist activity may rest with amino acid residues on the extracellular loop of $P2X_2$ receptors.

ATP-species activating $P2X_2$ 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 EC_{50} values for ATP under different experimental conditions, to yield fractional ratios of the ATP-species for the amount of ATP described by the EC_{50}. A preliminary account of these data has been presented (King *et al*., 1996d).

A comparison of EC_{50} 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 $P2X_2$ receptors. It seems more likely that all forms of ATP will activate the $P2X_2$ 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 (ATP^{4-}) in the activation of $P2X_2$ receptors, its p*K* for protonation is 6.51 indicating that the amount of ATP^{4-} falls and $HATP^{3-}$ increases with acidification (as shown in Table 3). Thus, it is difficult to explain how ATP^{4-} can activate the $P2X_2$ receptor with a greater affinity at pH 6.5. In fact, Sela *et al*. (1991) predicted that ATP^{4-} did not activate the native P_{2X} receptor on PC12 cells from which $P2X_2$ was cloned. Other workers have suggested the contrary opinion (Rhoads *et al*., 1993), since they found that the addition of Mg^{2+} to the extracellular solution reduces the secretory activity of ATP at PC12 cells. We observed that the addition of extracellular Mg^{2+} (1 mM) to the superfusate decreased ATP-affinity at $P2X_2$ (see Figure 1b) and calculations with BAD showed that the amount of free ATP^{4-} decreased by 27% when Mg^{2+} (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*., $\alpha1,\beta1\gamma2S$) 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 $P2X_3$ and $P2X_4$ receptors (King: unpublished results).

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