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Extended pharmacological profiles of rat $P2Y_2$ and rat $P2Y_4$ receptors and their sensitivity to extracellular H^+ and Zn^{2+} ions

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1 Two molecularly distinct rat P2Y receptors activated equally by adenosine-5′-triphosphate (ATP) and uridine-5′-triphosphate (UTP) ($rP2Y_2$ and $rP2Y_4$ receptors) were expressed in *Xenopus* oocytes and studied extensively to find ways to pharmacologically distinguish one from the other.

2 Both P2Y subtypes were activated fully by a number of nucleotides. Tested nucleotides were equipotent at $rP2Y_4$ (ATP = UTP = CTP = GTP = ITP), but not at $rP2Y_2$ (ATP = UTP > CTP > GTP > ITP). For dinucleotides (Ap_nA, $n = 2–6$), $rP2Y_4$ was only fully activated by Ap_4A, which was as potent as ATP. All tested dinucleotides, except for Ap_2A, fully activated $rP2Y_2$, but none were as potent as ATP. ATPγS and BzATP fully activated $rP2Y_2$, whereas ATPγS was a weak agonist and BzATP was inactive (as an agonist) at $rP2Y_4$ receptors.

3 Each P2Y subtype showed different sensitivities to known P2 receptor antagonists. For $rP2Y_2$, the potency order was suramin > > PPADS = RB-2 > TNP-ATP and suramin was a competitive antagonist (pA_2, 5.40). For $rP2Y_4$, the order was RB-2 > > suramin > PPADS > TNP-ATP and RB-2 was a competitive antagonist (pA_2, 6.43). Also, BzATP was an antagonist at $rP2Y_4$ receptors.

4 Extracellular acidification (from pH 8.0 to pH 5.5) enhanced the potency of ATP and UTP by 8–10-fold at $rP2Y_4$ but did not affect agonist responses at $rP2Y_2$ receptors.

5 Extracellular Zn^{2+} ions (0.1–300 μM) coapplied with ATP inhibited agonist responses at $rP2Y_4$ but not at $rP2Y_2$ receptors.

6 These two P2Y receptors differ significantly in terms of agonist and antagonist profiles, and the modulatory activities of extracellular H^+ and Zn^{2+} ions. These pharmacological differences will help to distinguish between $rP2Y_2$ and $rP2Y_4$ receptors, *in vivo*.
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Keywords: GPCR; purinoceptor; P2Y receptor; $P2Y_2$ receptor; $P2Y_4$ receptor; P2U receptor; extracellular pH; Zinc; oocyte

Abbreviations: ATP, adenosine-5′-triphosphate; ATPγS, adenosine-5′-*O*-(thiotriphosphate); 2-MeSATP, 2-methylthioATP; $\alpha\beta$-meATP, α,β-methyleneATP; $\beta\gamma$-meATP, β,γ-methyleneATP; BPAE, bovine pulmonary-artery endothelium; BzATP, 2′,3′-*O*-(4-benzoylbenzoyl)ATP; CTP, cytosine-5′-triphosphate; GTP, guanosine-5′-triphosphate; ITP, inosine-5′-triphosphate; UTP, uridine-5′-triphosphate; Ap_nA, P^1-$P^{(n)}$-di(adenosine-5′)phosphate; GPCR, G protein-coupled receptor; LGIC, ligand-gated ion-channel; PPADS, pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid; RB-2, Reactive blue 2; SAR, structure-activity relationship; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)ATP; TPEN, *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenedi-amine; suramin, suramin hexasodium salt; Up_nU, P^1-$P^{(n)}$-di(uridine-5′)phosphate

Introduction

Prior to expression cloning, the P2U receptor was defined as a G-protein-coupled receptor activated equally by the purine and pyrimidine nucleotides adenosine-5′-triphosphate (ATP) and uridine-5′-triphosphate (UTP) (Dubyak, 1991; O'Connor *et al*., 1991; Dubyak & El-Moatassim, 1993). Cell-surface receptors sensitive to these nucleotides (and, in some cases, other naturally occurring nucleoside triphosphates) have a widespread distribution in tissues and cell types of various species – including astrocytes, leukocytes, epithelium, endothelium, endocrine and exocrine glands, hepatocytes, vascular and visceral smooth muscle and others (Ralevic & Burnstock, 1998). A subdivision of ATP/UTP sensitive receptors was proposed on the basis that suramin hexasodium salt (suramin) blocked some, but not all, P2U receptors in mammalian tissues (Dainty *et al*., 1994; Ralevic & Burnstock, 1998).

With the introduction of expression cloning, an ATP/UTP receptor was isolated from mouse neuroblastoma (NG108-15) cells and human airway epithelial (CF/T43) cells (Lustig *et al*., 1993; Parr *et al*., 1994). Heterologous expression of these P2 receptors, in either *Xenopus laevis* oocytes or human 1321N1 astrocytoma cells, resulted in ATP- and UTP-mediated responses that signalled through the $G_q/PLC_\beta/IP_3/Ca^{2+}$ pathway (Lustig *et al*., 1993; Parr *et al*., 1994). The encoding cDNAs for these P2 receptors were defined as genes for the mouse and human $P2Y_2$ receptor, whereas cDNAs for another two P2 purinoceptors isolated from chick brain were classed as $P2Y_1$ and $P2Y_3$ (Barnard *et al*., 1994; Von Kügelgen *et al*., 1987). This nomenclature system still remains in place and has been expanded to include cDNAs for $P2Y_4$ to $P2Y_{14}$, most of

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which encode functional nucleotide receptors (Abbracchio *et al*., 2003).

Shortly after the molecular identification of the $P2Y_2$ receptor, the human $P2Y_4$ receptor was cloned and, when expressed in 1321N1 cells, this metabotropic receptor was shown to be sensitive to UTP and insensitive to ATP (Communi *et al*., 1995; Nguyen *et al*., 1995). This human $P2Y_4$ receptor was viewed as a pyrimidinoceptor that was both structurally and phenotypically distinct from the ATP/UTP-sensitive $P2Y_2$ receptors. However, the subsequent cloning of rat and mouse $P2Y_4$ receptors – each greater than 80% identical to the human isoform of $P2Y_4$ – resulted in a metabotropic receptor that was activated equally by ATP and UTP in all expression systems employed (*Xenopus* oocytes, Jurkat cells and 1321N1 cells) (Bogdanov *et al*., 1998; Webb *et al*., 1998; Kennedy *et al*., 2000; Lazarowski *et al*., 2001; Suarez-Heurta *et al*., 2001). Therefore, the rodent $P2Y_4$ was found to be phenotypically similar to the rat and human $P2Y_2$ receptor.

From the functional evidence available at the time, it was proposed that several pharmacological features might help to distinguish $P2Y_2$ isoforms from the rat $P2Y_4$ receptor (King *et al*., 1998). These features included: (i) the potency of UTP and ATP seemed higher at $P2Y_2$ receptors; (ii) adenosine-5′-*O*-(thiotriphosphate) (ATPγS) was a full agonist at $P2Y_2$, but weakly stimulated $P2Y_4$; (iii) in contrast, inosine-5′-triphosphate (ITP) was a full agonist at $P2Y_4$, but weakly stimulated $P2Y_2$; (iv) suramin blocked $P2Y_2$, but not $P2Y_4$ receptors. However, many of these distinguishing pharmacological features were gleaned from published data taken from unrelated studies and have not been tested directly.

In the present study, the issue of differentiating rat $P2Y_2$ from rat $P2Y_4$ receptors has been revisited. For the first time, we demonstrate the pharmacological means to distinguish between these two ATP/UTP-sensitive P2Y receptor subtypes. A part of this study has been presented elsewhere (Wildman *et al*., 2002).

Methods

Oocyte preparation

Defolliculated *Xenopus* oocytes were selected to express recombinant P2Y receptors because they do not possess endogenous P2 receptors, lack functional P1 receptors and, furthermore, surface ATPase activity is exceedingly low with no effective breakdown of superfused UTP, ATP and adenine-based dinucleotides (P^1-$P^{(n)}$-di(adenosine-5′)phosphate (Ap_nA) family) for the superfusion periods used in this study (King *et al*., 1996a; Pintor *et al*., 1996). To prepare these cells, *Xenopus laevis* frogs were anaesthetized with Tricaine (0.4% w v^{-1} in tap water), killed by decapitation, and the ovarian lobes surgically removed. Oocytes (stages V and VI) were defolliculated by a two-step process involving collagenase treatment (Type IA, 1 mg ml^{-1} in a Ca^{2+}-free Ringer solution, for 2 h) followed by stripping away the follicular layer with fine forceps (in double-strength Ca^{2+}-free Ringer solution to help shrink oocytes).

Prepared oocytes were stored in a Barth's solution (pH 7.50) containing 110 mM NaCl, 1 mM KCl, 7.5 mM Tris-HCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, supplemented with 50 μg l^{-1} gentamycin sulphate. Defolliculated oocytes were injected cytosolically (40 nl) with either $rP2Y_4$ or $P2Y_2$ cRNA (1 μg μl^{-1}), then incubated for 48 h at 18°C in Barth's solution and, thereafter, kept at 4°C in Barth's solution for up to 7 days and until used in electrophysiological experiments.

Electrophysiological recordings

Agonist-activated membrane currents were recorded under voltage-clamp conditions ($V_h = -60$ mV) from cRNA-injected oocytes using twin-electrode amplifiers (Axoclamp 2A and 2B; Axon Instruments, Union City, CA, U.S.A.). The voltage-recording and current-recording microelectrodes (1–5 MΩ tip resistance) were filled with 3.0 M KCl. The evoked membrane currents were calcium-activated chloride currents ($I_{Cl,Ca}$), which resulted from P2Y receptor stimulation of the G_q/PLC_β/IP_3/Ca^{2+} pathway in oocytes (Lustig *et al*., 1993). Oocytes were superfused with a Ca^{2+}-Ringer solution (12 ml min^{-1}, pH 7.50, at 18°C) containing 110 mM NaCl, 2.5 mM KCl, 5 mM HEPES and 1.8 mM $CaCl_2$. Where stated, the pH of the bathing solution was adjusted using either 1.0 N HCl or 1.0 N NaOH to achieve the desired level. Electrophysiological data were stored on a computer using an MP100 WSW interface (Biopac Systems, Goleta, CA, U.S.A.) and analysed using the software package *Acqknowledge III* (Biopac Systems).

Analysis of drug actions

All drugs were superfused by a gravity-fed continuous flow system, which allowed for rapid addition and washout. Agonists were added for 90 s or until the evoked current reached a peak, then washed out with Ringer solution for a period of 1 h. For all agonist concentration–response (C/R) curves, data were normalised to the maximum current (I_{max}) evoked by ATP (30 μM or 100 μM for $rP2Y_4$ and $rP2Y_2$, respectively; at pH 7.5) in the oocyte under study. 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 coefficient (n_H) was taken from the slope of the Hill plots.

The inhibitory activity of P2 antagonists was tested by adding each antagonist in increasing concentrations, each applied 20 min prior to and during the addition of ATP ($\sim EC_{70}$ concentration, 3 or 10 μM ATP for $rP2Y_4$ and $rP2Y_2$, respectively). Where the activity of antagonists was tested at a lower pH level (pH 5.5), the EC_{70} concentrations of ATP were 300 nM and 10 μM for $rP2Y_4$ and $rP2Y_2$, respectively. The antagonist concentration that reduced ATP responses by 50% (IC_{50}) was taken from the inhibition curves. The reversibility of receptor blockade was tested after prolonged (>2 h) washout of antagonists. pA_2 values were determined by Schild analysis.

C/R curves for the modulatory activity of Zn^{2+} ions were constructed against submaximal concentrations of ATP (3 or 10 μM ATP for $rP2Y_4$ and $rP2Y_2$, respectively) and thereafter data were normalized to the amplitude of control ATP responses.

Data are presented as mean ± s.e.m. of three or more sets of data obtained from different oocyte batches. Significant differences were determined by Student's t-test (using Instat

v2.05A, GraphPad Software, San Diego, CA, U.S.A.). Concentration–response curves and inhibition curves were fitted by nonlinear regression analysis using commercial software (Prism v3.0, GraphPad).

Drugs

ATP, other nucleotides and drugs were purchased from Sigma-Aldrich (Poole, Dorset, U.K.). Suramin was a gift from Bayer plc (Newbury, Berkshire, U.K.), while 2′,3′-*O*-(2,4,6-trinitrophenyl)ATP (TNP-ATP) was obtained from Molecular Probes (Eugene, OR, U.S.A.). All reagents, including $ZnCl_2$, were AnalaR grade from Sigma-Aldrich. Drugs were prepared in a Ca^{2+}-Ringer solution and the extracellular pH was adjusted to the desired level.

DNA constructs

The rat $P2Y_2$ receptor cDNA (U56839) was kindly provided by Dr Claude Desgranges and Dr Cheikh Seye (INSERM U441, Pessac, FRANCE) (as described in Seye *et al*., 1997). We have previously isolated the rat $P2Y_4$ receptor cDNA (Y14705) (as described in Bogdanov *et al*., 1998).

Results

Activation by nucleoside triphosphates

Previously, rat $P2Y_4$ (rPY_4) receptors expressed in human 1321N1 astrocytoma cells were shown to be activated by a wide range of nucleoside triphosphates (Kennedy *et al*., 2000). Here, we have confirmed that UTP, ATP, cytosine-5′-triphosphate (CTP), guanosine-5′-triphosphate (GTP) and ITP were active at $rP2Y_4$ expressed in *Xenopus* oocytes (Figure 1a,c), also established that the same range of nucleotides were active at rat $P2Y_2$ ($rP2Y_2$) receptors expressed in oocytes (Figure 1d) and, in both cases, all tested nucleotides were found to be full agonists. These five nucleotides were equipotent at $rP2Y_4$ (Table 1), whereas at $rP2Y_2$ only UTP and ATP were equipotent and CTP, GTP and ITP – in this order – were progressively less potent (Table 1). It has already been shown that BzATP (2′,3′-*O*-(4-benzoylbenzoyl)ATP) is an agonist at human $P2Y_2$ expressed in 1321N1 cells (Erb *et al*., 1993) and, therefore, we confirmed that BzATP also activated rat $P2Y_2$ in oocytes (Figure 1b,d). Here BzATP was as potent as UTP and ATP (Table 1), but it proved to be virtually inactive at $rP2Y_4$ receptors (at 1 mM, $6.4 \pm 0.9\%$ of the maximum ATP response; $n = 3$) (Figure 1b,c).

Activation by adenine dinucleotides

Diadenosine polyphosphates (Ap_nAs, $n = 2–6$) are active at a number of native and recombinant P2 receptors (Hoyle *et al*., 2001) and, so, the Ap_nA family was tested at rat P2Y receptor subtypes. At $rP2Y_4$, the tetraphosphate molecule Ap_4A (EC_{50}, $2.6 \pm 1.6\,\mu M$, $n = 4$) was as potent as ATP, whereas the remainder of the tested dinucleotides were either partial agonists (<25% of the maximum ATP response) or inactive (Figure 2a; Table 1). The $rP2Y_2$ receptor was found to be less selective and was fully activated by all of the tested adenine dinucleotides, except for the diphosphate molecule Ap_2A, which was a partial agonist (at 10 mM, $21.2 \pm 3.9\%$ of the maximum ATP response) (Figure 2b; Table 1). At $rP2Y_2$, the most potent dinucleotide was also Ap_4A (EC_{50}, $6.5 \pm 0.7\,\mu M$, $n = 4$).

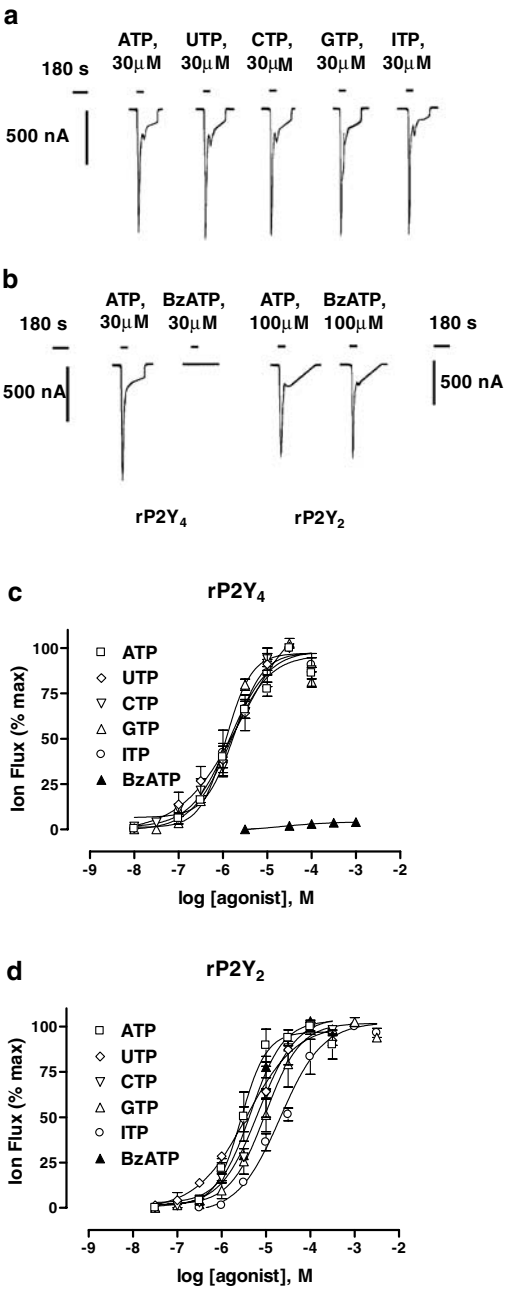

Figure 1 Activation by nucleoside triphosphates. (a) Naturally occurring nucleoside triphosphates (ATP, UTP, CTP, GTP and ITP; at 30 μM, the EC_{100}) activated $rP2Y_4$ receptors and evoked calcium-dependent chloride currents ($I_{Cl,Ca}$) in *Xenopus* oocytes. Water-injected control oocytes failed to respond to any of these nucleotides. However, the same range of agonists also activated $rP2Y_2$ receptors (records not shown). (b) The synthetic nucleotide BzATP (30 μM) was inactive at $rP2Y_4$ receptors, but the same compound (100 μM, the EC_{100}) was fully active at $rP2Y_2$ receptors. (c, d) The concentration/response (C/R) curves for agonists at each of the two P2Y receptor subtypes. Data are expressed as mean ± s.e.m. ($n = 3–6$). The EC_{50} values of agonists and Hill coefficients (n_H) for C/R curves are given in Table 1. In (a), all records were evoked from the same oocyte whereas in (b), paired records were from separate oocytes ($V_h = -60$ mV).

Table 1 Agonist and antagonist potency at recombinant P2Y receptors

	rP2Y$_4$		*rP2Y*$_2$	
	EC_{50} (μM)	n_H	EC_{50} (μM)	n_H
UTP	1.8±0.9	1.3±0.3	3.6±0.3	0.9±0.3
ATP	1.5±0.8	1.0±0.2	2.7±0.9	1.1±0.3
CTP	1.2±0.1	1.1±0.2	6.8±0.5	1.0±0.2
GTP	1.4±0.1	1.0±0.3	9.7±0.8	1.1±0.2
ITP	1.6±0.3	1.2±0.2	20.9±0.9	0.9±0.3
Ap_2A	Inactive		1,029±21[a]	1.1±0.2
Ap_3A	5.7±1.9[a]	1.4±0.7	23.8±1.2	1.0±0.2
Ap_4A	2.6±1.6	0.7±0.2	6.5±0.7	1.1±0.3
Ap_5A	3.4±1.3[a]	1.4±0.5	20.3±0.6	1.3±0.1
Ap_6A	Inactive		26.5±0.8	1.0±0.2
BzATP	nd[a]	nd	4.7±0.2	1.1±0.2
ATPγS	5.4±0.3[a]	1.9±0.4	10.5±0.7	1.1±0.3
2-MeSATP	1.4±0.2[a]	1.5±0.3	9.4±0.5[a]	1.4±0.3
	IC_{50} (μM)	n_H	IC_{50} (μM)	n_H
BzATP	159±19	1.2±0.4	Nd	nd
PPADS	>1,000	nd	>10,000	nd
RB-2	18.5±1.4	0.8±0.2	>10,000	nd
Suramin	1027±32	0.9±0.2	8.9±3.3	1.3±0.5
TNP-ATP	>1000	nd	>10,000	nd

[a]Partial agonist; nd, not determined.
Potency indices (EC_{50}, IC_{50}) and Hill coefficients (n_H) for a range of agonists and antagonists at rP2Y$_4$ receptors (left) and rP2Y$_2$ receptors (right) expressed in *Xenopus* oocytes. Data are expressed as mean±s.e.m. (n=3–6).

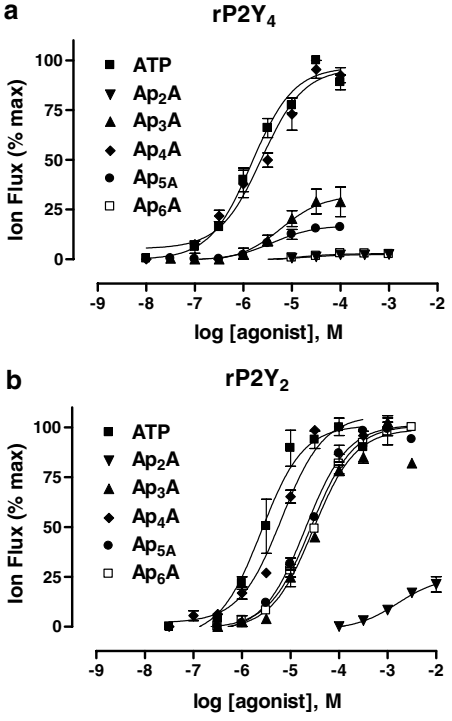

Figure 2 Activation by adenine dinucleotides. Concentration/response (C/R) relationships (a, b) for the adenine dinucleotide series (Ap_nA, n=2–6) as agonists of rP2Y$_4$ receptors (top) and rP2Y$_2$ receptors (bottom) expressed in *Xenopus* oocytes. Ap_nA responses were normalised to the maximum ATP response evoked at each P2Y subtype. Data are expressed as mean±s.e.m. (n=3–7). Further details are given in the text and Table 1.

Activation by synthetic nucleotides

To conclude the study of agonists, some well-known synthetic nucleotides were tested at both P2Y receptor subtypes (Table 1). At rP2Y$_2$, ATPγS was a full agonist but only a partial agonist at rP2Y$_4$ (24.2±5.1% of maximum ATP response; n=4). 2-methylthioATP (2-MeSATP) was a partial agonist at rP2Y$_2$ (33.8±4.9% of maximum ATP response; n=4) and rP2Y$_4$ (23.5±3.9% of maximum ATP response; n=4). Both α,β-methyleneATP ($\alpha\beta$meATP) and β,γ-methyleneATP ($\beta\gamma$meATP) (up to 100 μM) were inactive at each P2Y receptor subtype.

Blockade of P2Y receptors

Comparatively little is known about the potency of classical P2 receptor antagonists at native P2U receptors in mammalian tissues – other than that some are suramin sensitive and some are not (Dainty *et al*., 1994; Ralevic & Burnstock, 1998). Here, a short series of known P2 antagonists – Reactive blue 2 (RB-2), suramin, PPADS (pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid) and TNP-ATP – was tested at rP2Y$_4$ and rP2Y$_2$ receptors (Figure 3a,b).

For rP2Y$_4$, RB-2 was the most potent blocking agent (IC_{50}, 18.5±1.4 μM, n=4) and, in further experiments, it behaved as a competitive antagonist (pA_2, 6.43)–displacing the, C/R curve for ATP to the right without reducing its maximum response (Figure 3c). Suramin and PPADS were relatively weak antagonists, requiring very high concentrations to block rP2Y$_4$ (Figure 3a), and each antagonist yielded predictive IC_{50} values in the region of 1–10 mM (Table 1). TNP-ATP was inactive at 1 mM, although it did reduce ATP responses at higher concentrations (Figure 3a). Since BzATP was an agonist of very low efficacy, it was tested for antagonist activity and, at high concentrations, it blocked rP2Y$_4$ receptors (IC_{50}, 159±19 μM, n=4) (Figure 3a).

For rP2Y$_2$, suramin was the most potent blocking agent (IC_{50}, 8.9±3.3 μM), and, in further experiments, it behaved as a competitive antagonist (pA_2, 5.40) – displacing the C/R curve for ATP to the right without reducing its maximum response (Figure 3d). PPADS, RB-2 and TNP-ATP were very weak antagonists (between 20 and 50% inhibition at 10 mM).

The blocking actions of suramin at both P2Y receptor subtypes, as well as that of BzATP and TNP-ATP, were reversible following a 1 h washout period. However, blockade by either RB-2 or PPADS required in excess of 2 h washout.

Modulatory actions of pH

It has been previously reported that changing extracellular pH altered the potency of agonists at native P1 (adenosine) receptors (Hiley *et al*., 1995) and recombinant P2X receptors (King *et al*., 1996b). Thus the effects of changing extracellular pH – over the range of pH 8.0–5.5 – were investigated for rP2Y$_4$ and rP2Y$_2$ receptors (Figure 4a,b).

With rP2Y$_2$, there were no significant differences in agonist potency at the four pH levels investigated (Figure 4b). However, a different picture emerged for rP2Y$_4$, where agonist potency was significantly altered under acidic conditions (Figure 4a, Table 2). ATP potency was increased three-fold at pH 6.5 and 10-fold at pH 5.5 (compared to agonist activity

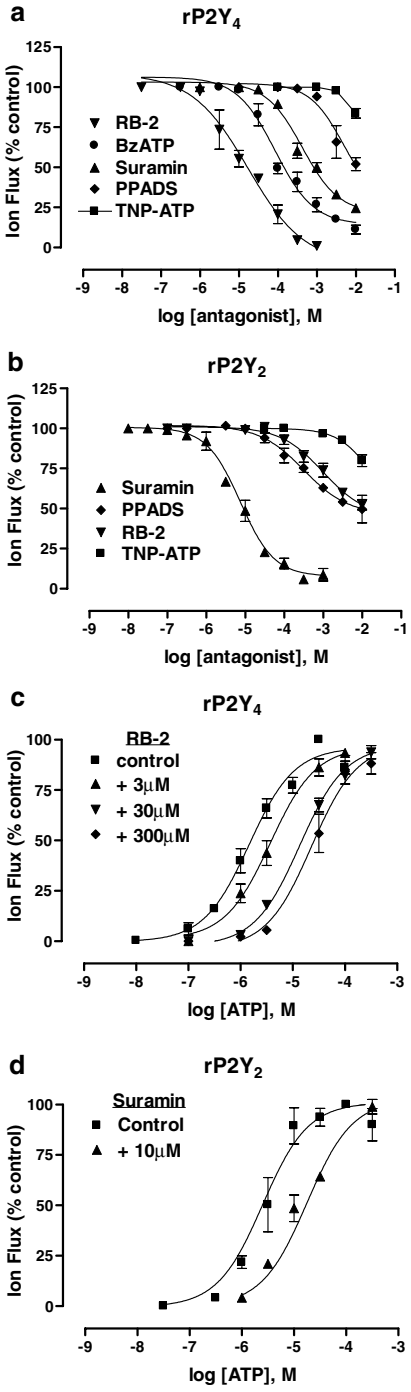

Figure 3 Blockade of P2Y receptors. (a, b) Inhibition curves for a range of P2 receptor antagonists superfused (for 20 min) prior to and during (90 s) the superfusion of ATP (EC_{70} value, 3 or 10 μM for $rP2Y_4$ and $rP2Y_2$, respectively) at $rP2Y_4$ receptors (a) and $rP2Y_2$ receptors (b) expressed in *Xenopus* oocytes. Data are expressed as mean$\pm$s.e.m. ($n=3-4$). IC_{50} values and, where possible, Hill coefficients (n_H) are given in Table 1. (c, d) Concentration/responses (C/R) curves for ATP activation of $rP2Y_4$ receptors (c) and $rP2Y_2$ receptors (d) in the presence of either Reactive blue 2 (RB-2, in (c) or suramin (in D) at the given concentrations. In the presence these antagonists, the C/R curves were shifted to the right, without reducing the maximum agonist response, indicating competitive antagonism. From these rightward shifts, pA_2 values (RB-2, 6.43; suramin, 5.40) were determined by Schild analysis (not shown).

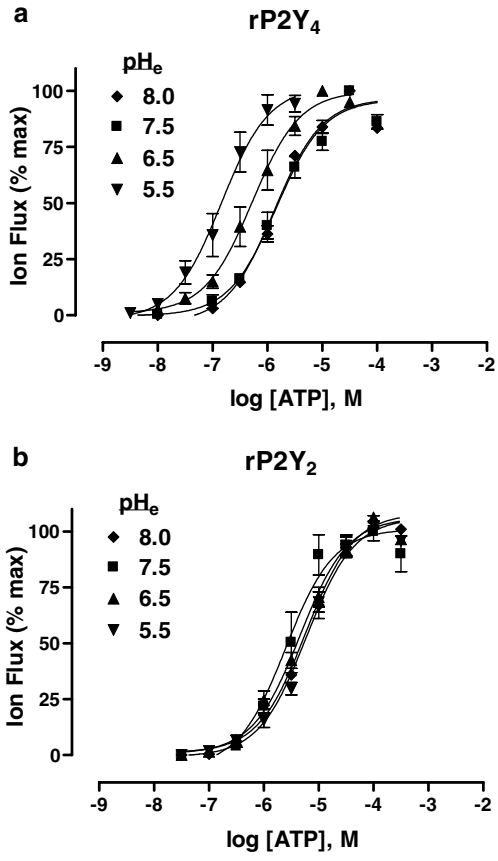

Figure 4 Modulatory actions of extracellular pH. (a, b) Concentration/response (C/R) curves for ATP activation of $rP2Y_4$ receptors (a) and $rP2Y_2$ receptors (b) expressed in *Xenopus* oocytes and examined at the levels of extracellular pH indicated. Data are expressed as mean$\pm$s.e.m. ($n=3-7$). The EC_{50} values and Hill coefficients (n_H) are given in Table 2. Further details on the forms of ATP-ion species activating these two P2Y receptors are given in the text and Table 3.

Table 2 H^+ modulation of agonist potency at recombinant P2Y receptors

	$rP2Y_4$		$rP2Y_2$	
	EC_{50} (μM)	n_H	EC_{50} (μM)	n_H
ATP				
pH 8.0	1.33$\pm$0.47	1.2$\pm$0.2	5.80$\pm$0.63	1.2$\pm$0.2
pH 7.5	1.46$\pm$0.82	1.0$\pm$0.2	2.51$\pm$0.93	1.1$\pm$0.3
pH 6.5	0.53$\pm$0.09	1.0$\pm$0.2	4.57$\pm$0.60	1.0$\pm$0.3
pH 5.5	0.15$\pm$0.13	1.3$\pm$0.3	5.96$\pm$0.73	1.1$\pm$0.3
UTP				
pH 7.5	1.79$\pm$0.92	1.3$\pm$0.3	3.63$\pm$0.28	0.9$\pm$0.3
pH 5.5	0.23$\pm$0.08	0.9$\pm$0.1	3.04$\pm$0.49	1.1$\pm$0.3

Potency indices (EC_{50}) and Hill coefficients (n_H) for ATP/UTP activation of $rP2Y_4$ receptors (left) and $rP2Y_2$ receptors (right) expressed in *Xenopus* oocytes and examined under different levels of extracellular pH (pH 8.0–5.5). Data are expressed as mean$\pm$s.e.m. ($n=3-6$).

at pH 7.5), with similar changes in potency when using UTP (Table 2), and also for Ap_4A (data not shown). Use of the program "*Bound and Determined*" (v.4.35) (Brooks & Storey, 1992) showed that the proportional changes in agonist potency at $rP2Y_4$ were unrelated to the levels of free ATP (i.e. ATP^{4-})

Table 3 Analysis of amounts of ATP-ion species in EC_{50} concentrations

	rP2Y$_4$				*RP2Y$_2$*			
	pH 8.0	*pH 7.5*	*pH 6.5*	*pH 5.5*	*pH 8.0*	*pH 7.5*	*pH 6.5*	*pH 5.5*
Mean EC_{50} (nM)	1332	1458	529	145	5801	2511	4566	5955
	ATP species (nM)				*ATP species (nM)*			
ATP^{4-}	106	115	38	6	460	197	329	230
$HATP^{3-}$	4	13	43	63	16	22	373	2594
H_2ATP^{2-}	0	0	0	3	0	0	2	130
$NaATP^{3-}$	182	198	66	10	794	340	568	395
$KATP^{3-}$	3	4	1	0	15	6	10	7
$CaATP^{2-}$	849	922	307	45	3698	1590	2648	1842
Ca_2ATP	187	203	68	10	817	352	585	407
$CaHATP^{-}$	1	2	6	9	2	3	50	351
Total	1332	1457	529	146	5802	2510	4565	5956

Computational determinations of the amounts (in nM) of each ATP-ion species present in the oocyte bathing solution (Ringer's solution; composition in Methods, temperature 18°C) for the mean concentration of ATP (in nM) evoking the 50% maximum response (EC_{50}) at $rP2Y_4$ receptors (left) and $rP2Y_2$ receptors (right) examined at four extracellular pH levels (pH 8.0–5.5). The amounts of each ATP-ion species were determined by the programme "*Bound and Determined*" (Brooks & Storey, 1992).

(Table 3). The calculated levels for ATP^{4-} ion species progressively decreased with acidification, although agonist potency had increased. Also, changes in the calculated levels of other major ATP-ion species did not match the proportional increase in EC_{50} values upon changing to lower extracellular pH levels. These computational results suggested that the effect of pH was on the receptor and not on the ligand. Changing extracellular pH had no significant effect ($P>0.1$) on the potency of either RB-2 or suramin at $rP2Y_4$ and $P2Y_2$ receptors.

Modulatory actions of divalent cations

Where extracellular pH has a strong modulatory effect, it is not uncommon for metal cations (especially Zn^{2+}) to modulate the same receptor subtype – whether they are nonpurinergic G protein-coupled receptors (LGICs) and G protein-coupled receptors (GPCRs) (Smart *et al*., 1994) or purinergic LGICs (Wildman *et al*., 1998) and GPCRs (Hiley *et al*., 1995; Mundell & Kelly, 1998). Here, a modulatory effect by extracellular Zn^{2+} ions was revealed at both P2Y subtypes (Figure 5a,b).

For $rP2Y_4$, Zn^{2+} ions (0.1–1000 μM) coapplied with the ATP inhibited agonist-evoked responses in a concentration-dependent manner (IC_{50}, $29.8\pm3.1\,\mu$M; slope, -1.2 ± 0.1; $n=4$) (Figure 5a). ATP responses were further inhibited by Zn^{2+} ions, if this cation was applied either for 15 min (IC_{50}, $4.0\pm0.4\,\mu$M; slope, -1.0 ± 0.1; $n=4$) or for 30 min (IC_{50}, $2.5\pm0.6\,\mu$M; slope, -1.0 ± 0.1; $n=4$) prior to addition of the agonist. The inhibitory effect of Zn^{2+} ions (10 μM) was abolished by the Zn^{2+} chelator, *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenedi-amine (TPEN) (30 μM, 15 min pre-incubation), and restored upon washout of TPEN (data not shown). The modulatory effects of Zn^{2+} were reversed after washout.

For $rP2Y_2$, the modulatory effect of extracellular Zn^{2+} ions was much less pronounced (Figure 5b). Without preincubation, Zn^{2+} ions (1 mM) barely inhibited ATP responses, whereas a more potent inhibitory effect was seen following preincubation periods of either 15 min (IC_{50}, $186\pm24\,\mu$M; slope, -1.2 ± 0.2; $n=4$) or 30 min (IC_{50} $198\pm19\,\mu$M; slope, -1.3 ± 0.1; $n=4$). This inhibitory effect was reversed after washout.

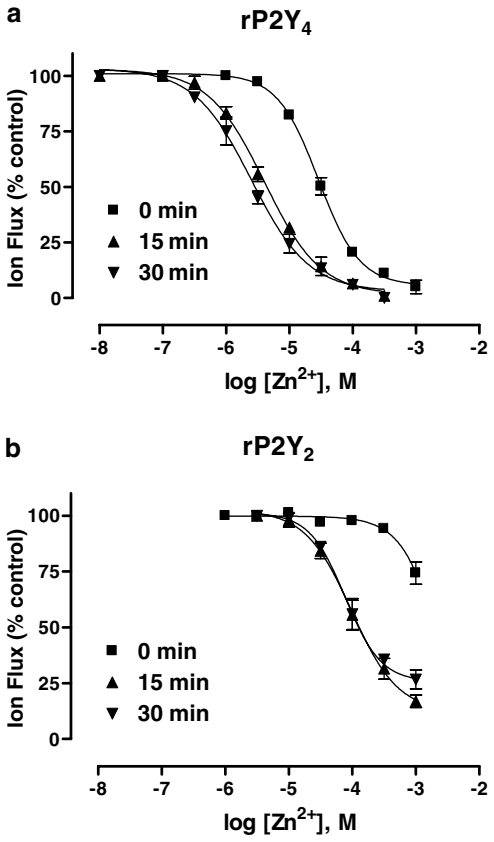

Figure 5 Modulatory actions of Zn^{2+} ions. (a, b) Inhibition curves for Zn^{2+} ions (0.01–1000 μM) applied in the superfusate, either prior to (15, 30 min) or simultaneously (0 min), with ATP (EC_{70} value, 3 or 10 μM for $rP2Y_4$ and $rP2Y_2$, respectively) at $rP2Y_4$ receptors (a) and $rP2Y_2$ receptors (b) expressed in *Xenopus* oocytes. Data are expressed as mean±s.e.m. ($n=4$). IC_{50} values and further details are given in the text.

Discussion

The present investigations provided for the first time a direct comparison of two structurally distinct rat P2Y receptor subtypes that are known to be activated equally by ATP and UTP yet, at a superficial level, appear to share the same phenotype. In the past, some pharmacological procedures have been proposed to separate these two P2Y receptors (King *et al*., 1998; also see a list of procedures in the Introduction), but few of these suggestions have been tested directly, or validated, until now.

The rat $P2Y_4$ receptor did not select for UTP, ATP, CTP, GTP or ITP when expressed in oocytes. All five of these nucleotides were equipotent at $rP2Y_4$ in oocytes, but not at rat $P2Y_4$ nor murine $P2Y_4$ expressed in 1321N1 cells, where only ATP and UTP are equipotent and CTP, GTP and ITP are significantly less potent (Kennedy *et al*., 2000; Lazarowski *et al*., 2001). In contrast, the human $P2Y_4$ receptor expressed in 1321N1 cells is only stimulated by three of these five nucleotides (UTP > ITP = GTP), while ATP is an antagonist (Kennedy *et al*., 2000). Our EC_{50} values for ATP and UTP at $rP2Y_4$ were approximately two-fold lower than equivalent data for other $P2Y_4$ isoforms expressed in 1321N1 cells, suggesting that we had not overexpressed $rP2Y_4$ and unduly altered the potency of ATP, UTP and other nucleotides by affecting receptor reserve (Kenakin, 2002). Others have shown that UTP potency can be higher than other nucleotides at $rP2Y_4$ receptors, depending on the type of G protein to which it is coupled (Filippov *et al*., 2003). Thus, if the present findings are to be used as discriminatory tools of P2Y subtypes *in vivo,* it is important to apply our findings to P2Y receptors in rat tissues and coupling through the $G_q/PLC_\beta/IP_3/Ca^{2+}$ pathway.

In the present study, rat $P2Y_2$ was fully activated by the above five nucleotides, but only ATP and UTP were equipotent and the remainder were significantly less potent (i.e. ATP = UTP > CTP > GTP > ITP). UTP and ATP also are equipotent at mouse $P2Y_2$ (Lustig *et al*., 1993) and human $P2Y_2$ (Parr *et al*., 1994), although only CTP and GTP have been tested at murine $P2Y_2$ where they were found to be inactive (Erb *et al*., 1993). The agonist activity of some or all of the above five nucleotides at species orthologues of $P2Y_2$ and $P2Y_4$ receptors suggests that the negatively charged triphosphate-ribose moiety common to each nucleotide is critically important for receptor activation, while the nature of the purine or pyrimidine moiety is much less important. In accordance with this suggestion, neutralization of positively charged amino-acid residues in TM6 and TM7 to limit triphosphate binding greatly reduced the potency of ATP and UTP at the mutated murine $P2Y_2$ receptor (Erb *et al*., 1995).

In our experiments, we tried to match the levels of receptor expression for both P2Y subtypes by adjusting the amounts of injected cRNA so that the maximum ATP response by oocytes was of similar amplitude (about 1 μA). While this is a relatively crude and imperfect procedure to control the P2Y receptor number, it helped us nonetheless to avoid the worst effects of differences in the receptor reserve on agonist potency (Kenakin, 2002) and allowed us to broadly compare the relative potencies of nucleotides at these two P2Y subtypes. Our findings indicate that $rP2Y_4$ was slightly more sensitive to ATP and UTP than at $rP2Y_2$ when receptor reserve appears to be similar, a conclusion which ran counter to earlier suggestions that $P2Y_2$ is more sensitive. This earlier misconception probably resulted from one of us (King *et al*., 1998) comparing potency data from different cell types expressing significantly different P2Y receptor numbers. Our findings also showed that the two P2Y receptor subtypes can be distinguished by differences in the relative potency of CTP (five-fold), GTP (seven-fold) and, notably, ITP (13-fold). In particular, the lower potency of ITP at $rP2Y_2$ is in agreement with earlier proposed procedures to separate these two P2Y receptor subtypes.

The pharmacological activity of BzATP was more clear-cut in our experiments: it activated $rP2Y_2$ and inhibited $rP2Y_4$. BzATP has been shown to activate murine $P2Y_2$ receptors (Erb *et al*., 1993), but it has not been tested beforehand on other isoforms of $P2Y_4$ receptors. This synthetic compound also activates human $P2Y_{11}$ (Communi *et al*., 1999) and inhibits rat and human $P2Y_1$ (Vigne *et al*., 1999), so the power of BzATP to discriminate between $P2Y_2$ and $P2Y_4$ receptors in native tissues is not wholly reliable, although for that purpose BzATP could still be combined with other pharmacological tools. For example, human $P2Y_{11}$ is activated by α,β-meATP (van der Weyden *et al*., 2000) and rat and human $P2Y_1$ receptors are fully activated by either 2-MeSADP or 2-MeSATP and blocked by PPADS. In the present study, α,β-meATP was inactive at both $rP2Y_2$ and $rP2Y_4$ and 2-MeSATP was a partial agonist, whereas neither receptor was potently inhibited by PPADS. We also found that ATPγS was a full agonist at $rP2Y_2$ receptors and a partial agonist at $rP2Y_4$. The mouse $P2Y_2$ receptor is also fully activated by ATPγS (Erb *et al*., 1993). Taken together, ATPγS may also help to distinguish $P2Y_2$ and $P2Y_4$ subtypes *in vivo*, but with the caveat that ATPγS can also activate other P2Y ($P2Y_{1,11}$) and P2X ($P2X_{1-3,5}$) receptor subtypes. Thus, ATPγS (like BzATP) could be used as a discriminatory tool for $P2Y_2$ and $P2Y_4$ receptors *in vivo*, but only in conjunction with other pharmacological agents to exclude the presence of other P2 receptor subtypes.

The observed actions of the adenine dinucleotides (Ap_nA family) revealed some similarities, and differences, between the two P2Y receptor subtypes. First, in the oocyte system, Ap_4A fully activated both P2Y receptor subtypes. Ap_4A also fully activated rat and human $P2Y_4$ receptors expressed in 1321N1 cells (Kennedy *et al*., 2000). Ap_4A has never before been tested at rat and mouse $P2Y_2$ isoforms, but is known to be equipotent with ATP and UTP at human $P2Y_2$ (Hoyle *et al*., 2001). In the present study, rat $P2Y_2$ was also activated by several other dinucleotides (Ap_3A, Ap_5A and Ap_6A). We have already shown in an earlier study that *Xenopus* oocytes do not rapidly break down Ap_nA molecules (Pintor *et al*., 1996), and so the activity of many of the Ap_nA family at rat $P2Y_2$ cannot be explained by their breakdown to ATP. Therefore, the activity profile of the Ap_nA series might also be considered when trying to distinguish between $P2Y_2$ and $P2Y_4$ subtypes in rat tissues. Our data for the Ap_nA series mirrored the structure–activity relationship (SAR) for the P^1-$P^{(n)}$-di(uridine-5′)phosphate (Up_nU) series ($n = 2–6$) at the equivalent human P2Y receptors (Pendergast *et al*., 2001). Thus human and rat $P2Y_4$ isoforms are only activated fully by the tetraphosphates of the Up_nU and Ap_nA series, respectively, and these tetraphosphates are as potent as either UTP or ATP. On the other hand, human and rat $P2Y_2$ isoforms are activated by the same range of Up_nU and Ap_nA compounds – with the

tetraphosphates most active and almost as potent as either UTP and ATP, other dinucleotides marginally less potent and the diphosphates being either weakly active or inactive.

Prior to this study, it had been shown that suramin shifted the C/R curve to the right for UTP acting on rat aortic strips but had no effect on the C/R for UTP acting on canine tracheal epithelium, indicating that there may be two subtypes of P2U receptors *in vivo* (Dainty *et al*., 1994). Examples of P2U/$P2Y_2$-like receptors in other tissues have also been reported to show different sensitivities to suramin (Ralevic & Burnstock, 1998). Rat $P2Y_2$ was competitively antagonised by suramin (pA_2, 5.40) (this study) and also at human $P2Y_2$ (pA_2, 4.26) (Charlton *et al*., 1996). Suramin has not yet been tested on canine $P2Y_2$ (Zambon *et al*., 2000). In contrast, suramin was effective only at high concentrations at rat $P2Y_4$ receptors (mean IC_{50}, 1 mM). It is possible that blockade by high concentrations of suramin (100 μM and greater) was due to the inhibition of G-protein signalling rather than, or as well as, P2Y receptor blockade (Freissmuth *et al*., 1999). The observed differences in suramin sensitivity at $rP2Y_2$ and $rP2Y_4$ might account for the possibility of two P2U receptors, *in vivo*.

Of the other antagonists tested, rat $P2Y_4$ was competitively antagonised by RB-2 (pA_2, 6.43), which contrasted with negative data for $rP2Y_2$ and provided another way of differentiating between these two P2Y subtypes. The P2X receptor antagonist TNP-ATP also was inactive at both $rP2Y_2$ and $rP2Y_4$ receptors – raising the possibility that TNP-ATP might be one of the few P2 antagonists selective only for ATP-gated ion channels. We found that rat $P2Y_4$ was incompletely blocked by PPADS (by 47% at 10 mM), as also seen with PPADS at human $P2Y_4$ receptors expressed in 1321N1 cells (30% at 100 μM) (Charlton *et al*., 1996). In contrast, it has been reported that PPADS blocked both the rat $P2Y_4$ (mean IC_{50}, 25 M) and mouse $P2Y_4$ receptors (mean IC_{50}, 45 μM) expressed in 1321N1cells (Suarez-Heurta *et al*., 2001). We did protect PPADS for white light, which promotes the breakdown of the azo-bridge in PPADS, and its limited activity in our hands cannot be due to degradation. Also, differences in PPADS activity at $rP2Y_4$ cannot be due to the oocyte expression system since the antagonist is not active at $hP2Y_4$ in 1321N1 cells. Also, we did not find any significant differences in antagonist activity when the extracellular pH was changed. Therefore, at this time, we cannot explain the differences in the activity of PPADS at $rP2Y_4$ seen in separate studies and, therefore, caution against the use of PPADS to identify the $P2Y_4$ subtype.

Another new aspect of the present study involved the modulation of agonist activity at P2Y receptors. This is the first time that extracellular pH has been shown to affect recombinant P2Y receptors, although H^+ ions are known to enhance agonist potency at native $P1(A_{2A})$ receptors (Hiley *et al*., 1995) and recombinant P2X subtypes – especially rat $P2X_2$ receptors (King *et al*., 1996b). This potentiating effect was peculiar to the $rP2Y_4$ subtype, whereas agonist potency at the $rP2Y_2$ subtype was unaffected. This latter negative finding agreed with an earlier study of native P2U receptors in adenosine-5′-triphosphate (BPAE) cells, which were believed to be activated by free ATP (ATP^{4-}) and unaffected by changing pH (Lustig *et al*., 1992).

We carried out a computational analysis of the available ATP-ion species, using "*Bound and Determined*" (Brooks & Storey, 1992), and, at the four pH levels tested, the amounts of ATP^{4-} present (197–460 nM) were proportionally similar to the EC_{50} ratios determined for $rP2Y_2$ receptors. However, in our experiments with $rP2Y_4$, the available ATP^{4-} levels progressively decreased (106–6 nM) with extracellular acidification – even though agonist potency was progressively enhanced. Thus, free ATP (ATP^{4-}) may be able to activate $rP2Y_2$ receptors, as suggested for P2U receptors in BPAE cells (Lustig *et al*., 1992), but this did not seem to be the case for $rP2Y_4$ receptors since our EC_{50} values were not in proportion with the available amounts of free ATP.

It was not possible to identify the ATP-ion species that activated $rP2Y_4$ receptors and, instead, the observed pH-induced changes in agonist potency may involve an action on the receptor itself. The pH modulation of agonist potency at the $P2X_2$ receptor is thought to be due to the acidification of histidine residue(s) close to the ATP-binding site (Clyne *et al*., 2002a). However, the amino-acid sequences of $rP2Y_4$ and $rP2Y_2$ show eight conserved histidine residues and only 1 unique histidine in the intracellular C-terminus of $rP2Y_4$ – a site unlikely to be instantly accessible to extracellular pH. The substitution of cysteine with alanine residues can also blunt the pH modulation of agonist potency at $P2X_2$ receptors (Clyne *et al*., 2002b). Of the 13 cysteine residues found in $rP2Y_4$, 10 are shared with $rP2Y_2$ receptors. The three unique cysteine residues are located on IL2, TM4 and C-terminus of $rP2Y_4$. The precise contribution of these cysteine residues – particularly in TM4 – awaits mutational analysis of the $P2Y_4$ receptor.

The inhibitory action of Zn^{2+} ions on agonist activity represented another novel finding for P2Y receptors. This divalent cation caused an inhibition of $rP2Y_4$ receptors when coapplied with ATP, and the immediacy of its effect suggested an extracellular site-of-action – perhaps one of the histidine and/or cysteine residues on $rP2Y_4$, since these residues have also been causally linked with the modulatory actions of Zn^{2+} ions at $P2X_2$ receptors (Clyne *et al*., 2002a, b). However, the actions of Zn^{2+} ions were not duplicated by H^+ ions at $rP2Y_4$ receptors and it seems unlikely that the same histidine and cysteine residues can be involved in the modulatory effects of both ion species yet still have divergent effects. Zn^{2+} effects were time dependent and enhanced by preincubation, which might indicate additional sites-of-action – either deeper in the agonist-binding pocket or at an intracellular site. Intracellular Zn^{2+} ions can potentiate agonist responses at A_{2A} and A_{2B} receptors in NG108-15 cells, by slowing down the rate of GRK2 kinase-mediated receptor desensitisation, and can also affect the rate of desensitisation at β_2-adrenoceptors and 5-HT_4 receptors by a similar mechanism (Mundell & Kelly, 1998, and references therein). However, we observed an inhibitory effect – not a potentiation – with Zn^{2+} ions at P2Y receptors, suggesting that these cations might not easily gain access to the inside of oocytes.

In summary, the pharmacological profiles of rat $P2Y_2$ and rat $P2Y_4$ were more divergent than previously supposed. There were differences in agonist potency for naturally occurring nucleotides, greater differences in the selectivity of dinucleotides and significant differences in the pharmacological actions of some synthetic nucleotides. The actions of extracellular pH on ATP/UTP activity were significantly different at these two P2Y receptors, as was the blocking activity of a range of P2 receptor antagonists on ATP/UTP

responses. Finally, agonist activity was modulated by Zn^{2+} ions at the rP2Y$_4$ receptor, which was much more sensitive than rP2Y$_2$. Collectively, these pharmacological differences should help to determine the presence of either of the P2Y receptor subtype in rat cell lines and tissues.

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