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Selectivity and activity of adenine dinucleotides at recombinant $P2X_2$ and $P2Y_1$ purinoceptors

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1 Adenine dinucleotides (Ap_xA, x=2-6) are naturally-occurring polyphosphated nucleotidic substances which are found in the CNS and are known to be released in a calcium-dependent manner from storage vesicles in brain synaptosomes. The selectivity and activity of adenine dinucleotides for neuronally-derived recombinant P_2 purinoceptors were studied using $P2X_2$ and $P2Y_1$ subtypes expressed in *Xenopus* oocytes.

2 For the $P2Y_1$ subtype derived from chick brain, Ap_3A was equipotent and as active as ATP (EC_{50} values: 375±86 nM and 334±25 nM, respectively). Ap4A was a weak partial agonist and other dinucleotides were inactive as agonists. None of the inactive dinucleotides were antagonists nor modulated the acitivity of Ap_3A and ATP.

3 For the $P2X_2$ subtype derived from rat PC12 cells, Ap_4A was as active as ATP but less potent (EC_{50} values: 15.2±1 μM and 3.7±0.7 μM, respectively). Other adenosine dinucleotides were inactive as either agonists or antagonists.

4 Ap_5A (1-100 nM) potentiated ATP-responses at the $P2X_2$ subtype, showing an EC_{50} of 2.95±0.7 nM for this modulatory effect. Ap_5A (10 nM) shifted the concentration-response curves for ATP to the left by one-half $\log_{10}$ unit but did not alter the Hill co-efficient for ATP (n_H=2.1±0.1). Ap_5A (10 nM) failed to potentiate Ap_4A-responses but did enhance the efficacy of the P_2 purinoceptor antagonist, suramin, by 12 fold at the $P2X_2$ subtype.

5 In conclusion, the results show that ionotropic ($P2X_2$) and metabotropic ($P2Y_1$) ATP receptors which occur in the CNS are activated selectively by naturally-occurring adenine dinucleotides which are known to be released with nucleotides from storage vesicles. The observed potentiation of $P2X_2$-responses by Ap_5A, where co-released with ATP by brain synaptosomes, may have a functional bearing in purinergic signalling in the CNS.

Keywords: Adenine dinucleotide; diadenosine polyphosphate; ATP; purine; P_2 purinoceptors; recombinant receptor; *Xenopus* oocytes

Introduction

Adenine dinucleotides, also termed diadenosine polyphosphates or ApxA (where x indicates the number of phosphates), are a family of naturally-occurring nucleotides where two adenosine molecules are linked at the 5′ position on their ribose moiety by a chain of phosphates varying from 2 to 6 in length (for review, see McLennan & Zamecnik, 1992). Like mononucleotides, this family of dinucleotides displays activity inside and outside cells. Intracellularly, adenine dinucleotides can exert a variety of actions on cell metabolism such as triggering DNA synthesis (Grummt, 1978) and they can also act as second messengers during cell stress (Denisenko, 1984). Extracellularly, adenine dinucleotides can induce platelet aggregation (Luthje & Ogilvie, 1983; Schlüter *et al.*, 1994), act as modulatory substances in neural systems (Pintor & Miras-Portugal, 1993) and affect calcium mobilization in vascular tissues (Tepel *et al.*, 1996).

Dinucleotides possess several features attributable to neurotransmitters or neuromodulators. For example, diadenosine tetraphosphate (Ap_4A), diadenosine pentaphosphate (Ap_5A) and diadenosine hexaphosphate (Ap_6A) are concentrated in *Torpedo* cholinergic vesicles (Pintor *et al.*, 1992b), in synaptic vesicles of chromaffin cells (Rodriguez del Castillo *et al.*, 1988; Pintor *et al.*, 1992c) and in rat brain synaptic terminals (Pintor *et al.*, 1992a). They are released exocytotically in a Ca^{2+}-dependent manner from synaptosomal preparations by either secretagogue induction or by depolarizing agents (Pintor *et al.*, 1992a,b,c). In the extracellular space, these substances bind to and activate most of the naturally-occurring P_2 purinoceptors described to date (Hoyle, 1990; Pintor *et al.*, 1993; Williams, 1994; Abbracchio & Burnstock, 1995; Pintor & Miras-Portugal, 1995a,b).

Recent advances in molecular biology have helped to reclassify P_2 purinoceptor subtypes into two broad families of ionotropic ($P2X_{1-n}$) and metabotropic ($P2Y_{1-n}$) ATP receptors (Abbracchio & Burnstock, 1995). So far, cloned ionotropic ATP receptors ($P2X_{1-7}$ subtypes) show considerable structural diversity (Brake *et al.*, 1994; Valera *et al.*, 1994; Chen *et al.*, 1995; Bo *et al.*, 1995; Collo *et al.*, 1996; Surprenant *et al.*, 1996) and possess pharmacological profiles that are often distinct from agonist potencies observed for native P_{2X} purinoceptors in many tissues. This discrepancy has prompted the suggestion that some native P_{2X} purinoceptors are assembled as heteromultimers of cloned P2X subtypes (Lewis *et al.*, 1995). Structurally-distinct subtypes of metabotropic ATP receptors ($P2Y_{1-7}$ subtypes) have also been isolated (Lustig *et al.*, 1993; Webb *et al.*, 1993; Chang *et al.*, 1995; Communi *et al.*, 1995; Webb *et al.*, 1996a,b; Akbar *et al.*, 1996), and possess distinct pharmacological profiles, including a marked preference for either ATP ($P2Y_1$) or UTP ($P2Y_4$) or, to varying extents, both of these naturally-occurring nucleotides ($P2Y_{2,3,5,6,7}$).

Ionotropic and metabotropic ATP receptors have been cloned from neural tissues, including a $P2X_2$ purinoceptor from rat phaeochromatocytoma PC12 cells (Brake *et al.*, 1994) and a $P2Y_1$ purinoceptor from embryonic chick brain (Webb *et al.*, 1993). These particular recombinant P_2 purinoceptors are distinguished by possessing pharmacological profiles similar to neuronal P_{2X} and P_{2Y} purinoceptors native to their source tissues (Simon *et al.*, 1995; King *et al.*, 1996c). Since adenine

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dinucleotides have been reported to activate native P_{2X} and P_{2Y} purinoceptors in neural tissues (Stone & Perkins, 1981; Castro *et al.*, 1990; Klishin *et al.*, 1994), we decided to investigate the actions of the full family of adenine dinucleotides (Ap_xA, $x=2-6$) on these two recombinant P_2 purinoceptor subtypes. Recently, it was reported that Ap_4A is a potent agonist of the recombinant human $P2Y_2$ purinoceptor from non-neural (epithelial) tissue (Lazarowski *et al.*, 1995) and, therefore, we were also interested to compare this earlier study with agonist actions of adenine dinucleotides at neurally-derived $P2X_2$ and $P2Y_1$ subtypes of recombinant P_2 purinoceptors.

Methods

Oocyte preparation

Xenopus laevis frogs were anaesthetized with tricaine (0.1% w/v), killed by decapitation and the ovarian lobes were removed. Oocytes (stages V and VI) were plucked off the inner ovarian epithelial layer with fine forceps and stored (at 4°C) in Barths' solution (pH 7.4) containing (in 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, gentamycin 50 $\mu g\ l^{-1}$. *Xenopus* oocytes were stripped of their follicle cell layer (defolliculated) by a three-step method, as described previously (Ziganshin *et al.*, 1995). Briefly, oocytes were treated for 3 h with collagenase (Type 1A, 2 mg ml^{-1}) in a Ca^{2+}-free Ringer solution containing (in mM): NaCl 110, KCl 2.5, HEPES 5, pH 7.4. Then, the follicle cell monolayer was removed with fine forceps and denuded oocytes were rolled in poly-L-lysine coated dishes to remove adhering debris and small plaques of follicle cells.

Oocyte injection, receptor expression and electrophysiological measurements

Defolliculated *Xenopus* oocytes were injected with mRNA (40 nl, 2.0 $\mu g\ \mu l^{-1}$) for chick $P2Y_1$ purinoceptor (Webb *et al.*, 1993) and mRNA (40 nl, 0.1 $\mu g\ \mu l^{-1}$) for rat $P2X_2$ purinoceptor (Brake *et al.*, 1994) using a Drummond microinjector. Injected oocytes were stored at 18°C for two days in Barth's solution to allow full expression of recombinant P_2 purinoceptors, then kept at 4°C for up to 12 days until used in electrophysiological studies (as in Webb *et al.*, 1993; Simon *et al.*, 1995; King *et al.*, 1996c).

Ionic currents evoked by nucleotides and dinucleotides were measured under voltage-clamp conditions with a twin-electrode amplifier (Axoclamp 2A). The voltage-recording and current-recording microelectrodes (1–2 MΩ tip resistance) were filled with 0.6 M K_2SO_4 and 3 M KCl, respectively. Oocytes were placed in a 0.5 ml bath and superfused at a flow rate of 5 ml min^{-1} with an amphibian Ringer containing (mM): NaCl 110, KCl 2.5, HEPES 5, $CaCl_2$ 1.8, pH 7.4. Dual-impaled oocytes were studied if their resting membrane potential was more negative than −40 mV and their input resistance greater than 0.5 MΩ. Data were stored on magnetic tape using a DAT recorder (Sony 1000ES) and displayed using a pen recorder (Gould).

Adenine dinucleotides and ATP (at the concentrations shown in the text) were superfused over dual-impaled oocytes by continuous flow, which allowed the rapid addition and washout of test substances. Agonists were applied in Ringer for 1 min and, thereafter, agonists washed out with Ringer for a period of 20 min to allow receptors to recover from any desensitization. For concentration-response curves, data were normalized with respect to the maximum current (I_{max}) evoked by ATP in each experiment. The concentration that evoked 50% of the maximum response (EC_{50}) was taken from Hill plots of the transform log $I/I\text{-}I_{max}$ (I being the current evoked at each concentration of agonist). The Hill co-efficient (n_H) was taken from the slope of Hill plots. The efficacy of the P_2 antagonist suramin on $P2X_2$ was tested by adding the antagonist in cumulative concentrations (0.1–100 μM), applied for 10 min prior to the addition of a submaximal concentration of ATP (3 μM, which is close to EC_{50}). The antagonist concentration that reduced ATP-responses by 50% (IC_{50}) was taken from inhibition curves. Data are presented as mean ± s.e.mean of at least four determinations using different oocyte batches. Significant differences were determined by Student's two-tailed t test. Single experimental traces shown in the text are representative of four determinations showing identical results. Concentration-response curves and inhibition curves were fitted by non-linear regression analysis using commercially-available software packages ('FigP' Software, v.6.0c and Prism v.1.03, Graphpad).

Nucleotide stability

The stability and purity of adenine dinucleotides and ATP were determined by high performance liquid chromatography (h.p.l.c.), as described previously (King *et al.*, 1996a). The chromatographic system comprised a Beckman (114M) solvent delivery module coupled to a UV/VIS absorbance detector (Savern Analytical, SA6500). Chromatographic runs were carried out using a Spherisorb ODS2 column (25 cm length, 0.46 cm i.d.; Hichrom). The column was equilibrated overnight with a mobile phase containing 0.2 mM KH_2PO_4 and 3% methanol, pH 6.0, at a flow rate of 0.2 ml min^{-1}. This mobile phase was filtered through HAFT filters (Millipore) and degassed under vacuum before use. The analysis of samples (10 μl volume) was carried out at a flow rate of 1.5 ml min^{-1}. The separation of dinucleotides and nucleotides was carried out by isocratic reverse-phase chromatography with spectrophotometric detection at a wavelength of 260 nm.

Drugs

ATP and adenine dinucleotides were obtained from Sigma Chemical Co. (UK). Other reagents were analytical grade and obtained from Merck (Darmstadt, Germany). Suramin (Germanin) was kindly provided by Bayer plc (U.K.).

Results

Dinucleotide stability

Adenine dinucleotides were tested by h.p.l.c. to verify their purity and stability. Chromatographs (Figure 1) showed one major peak for each dinucleotide assayed, each peak showing a significantly different mobility to the peak for ATP. The results confirmed adenine dinucleotides were stable, that each sample contained only one species of dinucleotide and none was contaminated with ATP. Defolliculated *Xenopus* oocytes were incubated (at 18°C) with dinucleotides and ATP in Ringer solution for 1 min (a period of time where dinucleotide-evoked currents reached maximum amplitude in electrophysiological experiments), then samples (100 μl) were withdrawn and assayed by h.p.l.c. Analysis of chromatographs showed no significant difference in the height of peaks in chromatographs (see Figure 1), indicating that dinucleotides and ATP were not degraded significantly by surface enzymes (ecto-ATPase) on oocytes nor released or taken up by oocytes. In another series of experiments where *Xenopus* oocytes were incubated (at 18°C) with dinucleotides (100 μM) for 3 h, the observed rate of breakdown was (in terms of pmol h^{-1} per oocyte): Ap_2A, 239 ± 25; Ap_3A, 310 ± 41; Ap_4A, 300 ± 65; Ap_5A, 108 ± 10; Ap_6A, 169 ± 19 ($n=8$). This low rate of breakdown confirmed the stability of dinucleotides in the presence of oocytes.

Effect of diadenosine polyphosphates on $P2Y_1$ purinoceptor

Superfusions of either ATP, Ap_3A or Ap_4A (0.03–100 μM) evoked inward currents ($I_{Cl,Ca}$) in oocytes injected with mRNA for chick $P2Y_1$ purinoceptor; Ap_2A, Ap_5A and Ap_6A (0.03–

100 μM) were inactive as agonists (Figure 2). Ap_3A was equipotent with and as active as ATP, while Ap_4A was a weak partial agonist (Figure 2). Analysis of concentration-response curves for Ap_3A and ATP revealed EC_{50} values of 375±86 nM and 334±25 nM, respectively. The Hill co-efficients (n_H) for Ap_3A and ATP were 1.1±1 and 1.2±0.1 respectively, which indicated only one agonist molecule was necessary to activate the $P2Y_1$ purinoceptor.

When co-superfused with ATP (3 μM), non-agonist adenine dinucleotides (0.1–100 μM) neither inhibited nor potentiated ATP-responses. Uninjected defolliculated oocytes were not activated by any of the adenine dinucleotides (100 μM) nor by ATP (100 μM). These results indicated that defolliculated oocytes do not possess endogenous P_{2X} and P_{2Y} purinoceptors, so confirming earlier observations (King *et al.*, 1995; 1996b).

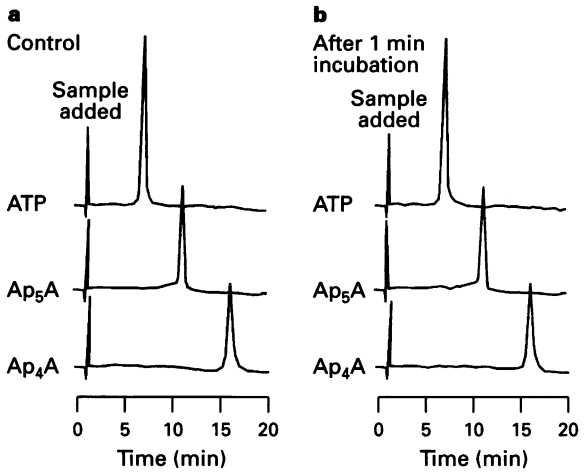

Figure 1 Stability of ATP, Ap_4A and Ap_5A in solution: h.p.l.c. elution profiles (a) of adenosine 5′-triphosphate (ATP), diadenosine tetraphosphate (Ap_4A) and diadenosine pentaphosphate (Ap_5A) in Ringer solution (control, 10 μM). Each chromatograph showed one major peak which followed the sharp biphasic injection artefact (at $t=1$ min), indicating the presence of one substance in each run. These three nucleotides (10 μM) also were incubated with mRNA-injected oocytes for 1 min to test whether dinucleotides and ATP were rapidly broken down. The h.p.l.c. elution profiles (b) confirmed there was little breakdown of these substances, as judged by no significant difference in the height of peaks and absence of secondary peaks.

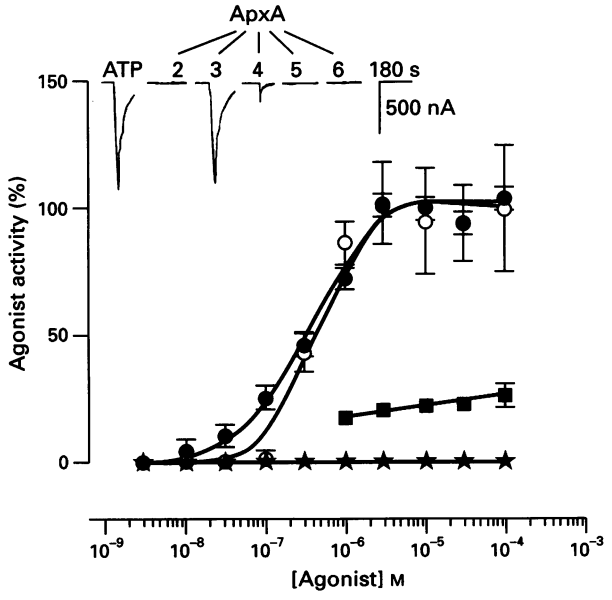

Figure 2 Adenine dinucleotide and ATP activity at $P2Y_1$ purinoceptor: concentration-response curves ($n=4$) for adenine dinucleotides and ATP, where only Ap_3A (○), Ap_4A (■) and ATP (●) stimulated the recombinant $P2Y_1$ purinoceptor. Other dinucleotides (Ap_2A, Ap_5A and Ap_6A; see ★) were ineffective as agonists. The amplitude of currents evoked by dinucleotides was normalized to the maximum response to ATP. Analyses of concentration-response curves are given in the text. Inset: matched responses of dinucleotides and ATP (at 10 μM) on the same mRNA-injected oocyte ($V_h=-40$ mV).

Effect of diadenosine polyphosphates on $P2X_2$ purinoceptor

Superfusions of ATP and Ap_4A (0.1–500 μM) evoked inward currents ($I_{Na/K}$) in oocytes injected mRNA for the rat $P2X_2$ purinoceptor, while Ap_2A, Ap_3A, Ap_5A and Ap_6A (0.1–500 μM) were inactive (Figure 3). Ap_4A was less potent than ATP, but showed the same maximal activity (Figure 3). EC_{50} values for Ap_4A and ATP were 15.2±1 μM and 3.7±0.7 μM respectively, diadenosine tetraphosphate being 4 fold less potent than ATP. The Hill co-efficient (n_H) for Ap_4A and ATP were 1.8±0.1 and 2.0±0.1, respectively. Values for n_H indicated a requirement for two molecules of each agonist to activate this receptor, as observed in other studies of agonists at $P2X_2$ (Brake *et al.*, 1994; King *et al.*, 1996c).

Effect of diadenosine pentaphosphate on ATP-responses at $P2X_2$ purinoceptor

In light of reports that some adenine dinucleotides inhibited synaptic transmission in the nervous system (Stone & Perkins, 1981; Klishin *et al.*, 1994; Schubert *et al.*, 1994), the possibility was investigated that adenine dinucleotides might affect ATP-responses at the $P2X_2$ purinoceptor. Ap_2A, Ap_3A, Ap_5A and Ap_6A (0.1–100 μM) did not antagonize responses to ATP (3 μM); however, diadenosine pentaphosphate (Ap_5A, 1–100 nM) markedly potentiated ATP-responses (Figure 4a). Low concentrations (1 nM) of Ap_5A were effective and maximal potentiation occurred using 10 mmol Ap_5A, whereas higher concentrations (>10 nM) of Ap_5A did not induce a

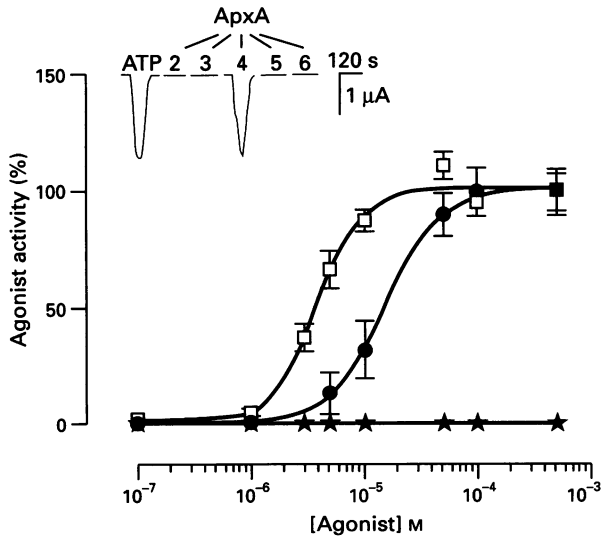

Figure 3 Adenine dinucleotide and ATP activity at $P2X_2$ purinoceptor: concentration-response curves ($n=4$) for adenine dinucleotides and ATP, where only Ap_4A (□) and ATP (●) stimulated the recombinant $P2X_2$ purinoceptor. Other dinucleotides (Ap_2A, Ap_3A, Ap_5A and Ap_6A; see ★) were ineffective as agonists. The amplitude of currents evoked by dinucleotides was normalized to the maximum response to ATP. Analyses of concentration-response curves are given in the text. Inset: matched responses of dinucleotides and ATP (at 100 μM) on the same mRNA-injected oocyte ($V_h=-30$ mV).

greater potentiation (Figure 4b). An EC_{50} value of 2.95 ± 0.7 nM was calculated for this Ap_5A-potentiating effect; n_H was 1.88 ± 0.27 which indicated that two molecules of Ap_5A bind to the $P2X_2$ purinoceptor. Since n_H for ATP-activation is 2.0 ± 0.1, it is quite likely that one molecule of Ap_5A binds to (or near to) each of the two ATP binding sites. The potentiating effect of Ap_5A (> 10 nM) was maintained as long as the dinucleotide was present in the superfusate, but was fully reversible after 20 min washout when ATP-responses returned to control amplitude (Figure 4a). Ap_5A (10 nM) did not alter the slope nor reversal potential of I/V relationship of I_{ATP} activated by the $P2X_2$ purinoceptor (data not shown), indicating the potentiating effect was due to a change in $P2X_2$ sensitivity to ATP rather than an effect on $P2X_2$ channel conductance. However, Ap_5A (10 nM) did not alter the pH of the superfusate (7.3 ± 0.1 before and during dinucleotide superfusion). Thus, the observed potentiation was unrelated to the effects of acidification on ATP-sensitivity at $P2X_2$ (King *et al.*, 1996c).

Maximal Ap_5A-potentiation amounted to $51 \pm 14\%$ above the amplitude of control responses to ATP (3 μM, approximately EC_{50}). Therefore, the effect of Ap_5A (10 nM) was tested on a wide range of ATP concentrations to see if the ATP concentration-response curve was displaced leftwards. Ap_5A only enhanced submaximal ATP-responses (Figure 5a) but failed to increase the maximum activity of ATP; thus, its effect was due to an increase in $P2X_2$ sensitivity to ATP (Figure 5b). EC_{50} values for ATP were 4.0 ± 1 μM and 1.4 ± 0.1 μM before and during Ap_5A (Figure 5b). In these experiments, n_H for ATP alone was 2.1 ± 0.1 which was not significantly different (2.2 ± 0.1) in the presence of Ap_5A.

Ap_5A (10 nM) also was tested on Ap_4A-evoked currents at the $P2X_2$ purinoceptor and caused a decrease ($12 \pm 4\%$) in submaximal responses to Ap_4A (10 μM); a higher concentration of Ap_5A (100 nM) failed to further inhibit Ap_4A-evoked currents. None of the other adenine dinucleotides (Ap_2A, Ap_3A and Ap_6A) were tested on Ap_4A-responses.

Effect of Ap_5A on suramin blockade of $P2X_2$ purinoceptor

By itself, suramin (0.1 – 100 μM) reversibly antagonized ATP-responses in a concentration-dependent manner with an IC_{50} of 10.4 ± 1.2 μM ($n = 4$) (Figure 6b). The efficacy of suramin was increased in the presence of Ap_5A (10 nM) and this potentiation was fully reversible with washout. Thus, suramin (1 μM) weakly inhibited ATP-responses by $10 \pm 6\%$ ($n = 4$) in the absence of Ap_5A, but strongly inhibited ATP responses by $77 \pm 4\%$ ($n = 4$) when the dinucleotide was added to the superfusate (Figure 6a). The inhibition curve for suramin was determined under control conditions and, subsequently, shown to be displaced leftwards when Ap_5A (10 nM) was present. With dinucleotide present, suramin showed a 12 fold increase in efficacy with an IC_{50} of 0.86 ± 0.2 μM ($n = 4$) (Figure 6b). The slope of the inhibition curve was 0.9 ± 0.1 under control conditions and 0.8 ± 0.2 when Ap_5A (10 nM) was present; these similar values for the slope revealed a parallel shift of the inhibition curve while the leftwards shift indicated an increased affinity for suramin. A slope of ~1 also indicated that only one molecule of suramin was necessary to block $P2X_2$, yet it required 2 molecules of ATP for receptor activation (since $n_H = 2.1 \pm 0.1$ for ATP).

Discussion

Dinucleotide selectivity and activity at $P2Y_1$ purinoceptor

ATP and adenine dinucleotide activation of the recombinant chick $P2Y_1$ purinoceptor resulted in large (~1 μA) inward currents in defolliculated *Xenopus* oocytes. Agonist activation of $P2Y_1$ is known to stimulate the phospholipase $C/IP_3/Ca^{2+}$ pathway (Simon *et al.*, 1995) and, hence, open calcium-activated chloride channels on the oocyte membrane (Barish, 1983). This resultant inward current ($I_{Ca,Cl}$) is now widely

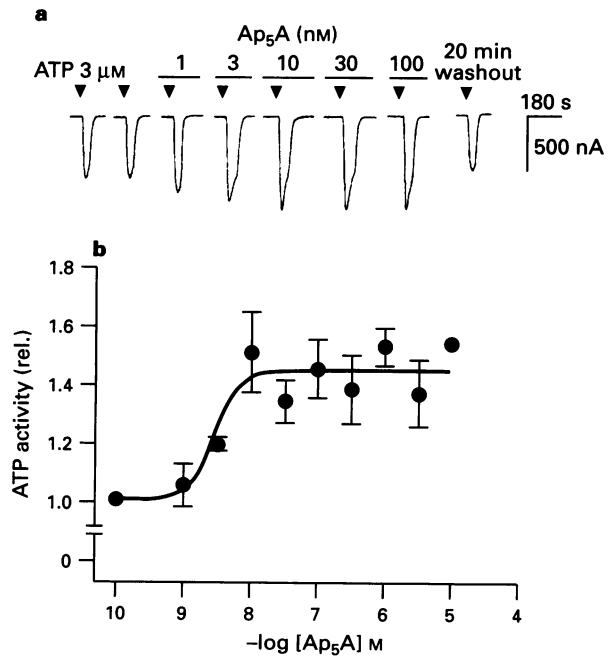

Figure 4 Potentiation of ATP-responses at $P2X_2$ purinoceptor by Ap_5A: in (a) is shown the potentiating effect of Ap_5A (1 – 100 nM) on inward currents evoked by ATP (3 μM). This effect of Ap_5A fully reversed after 20 min washout. ($V_h = -30$ mV.) In (b) concentration-response curve ($n = 4$) for the Ap_5A-induced potentiation of the inward current evoked by fixed concentration of ATP (3 μM). The amplitude of inward currents was normalized to control ATP-responses (taken as 1.0) prior to the addition of the dinucleotide.

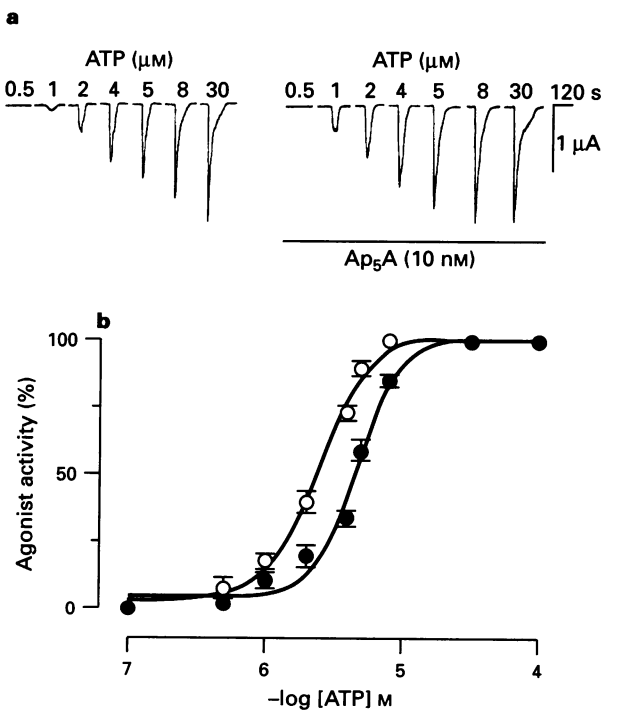

Figure 5 Potentiation of ATP-responses at $P2X_2$ purinoceptor by Ap_5A: in (a) inward currents to ATP (0.5 – 30 μM) before (left records) and during (right records) the presence of Ap_5A (10 nM). ($V_h = -30$ mV). In (b) concentration-response curves ($n = 4$) for ATP in the absence (●) and presence ○) of Ap_5A (10 nM). Analyses of concentration-response curves are given in the text.

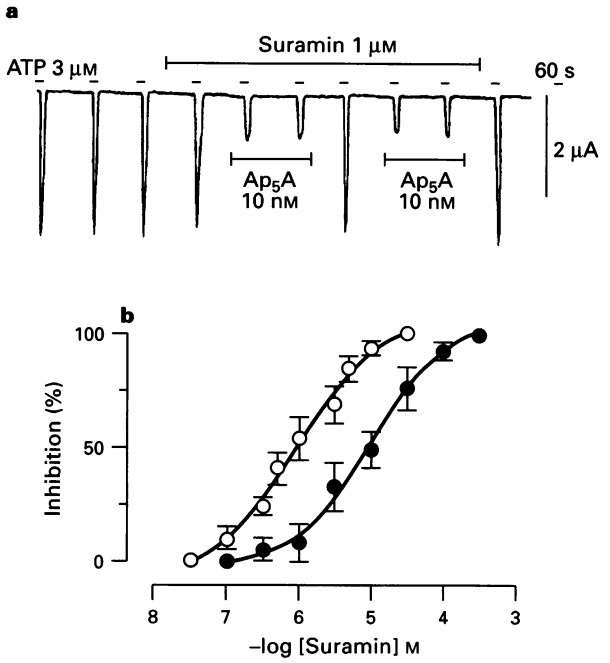

Figure 6 Potentiation of suramin blockade at $P2X_2$ purinoceptor by Ap_5A: in (a), suramin (1 μM) blockade of inward current to ATP (3 μM) was enhanced in the presence of Ap_5A (10 nM). This potentiating effect was temporarily reversed by removing Ap_5A from the superfusate and restored by its re-addition. ($V_h = -30$ mV.) In (b) inhibition-curves ($n = 4$) showing suramin antagonism of ATP-responses (at 3 μM) in the absence (●) and presence (○) of Ap_5A (10 nM). Analyses of the inhibition curves for suramin are given in the text.

considered to be an effective reporter of G-protein-coupled receptors stimulating the PLCβ/IP_3/Ca^{2+} intracellular system in the oocyte (Landau & Blitzer, 1994). Diadenosine triphosphate (Ap_3A) and, to a lesser extent, diadenosine tetraphosphate (Ap_4A) activated the recombinant $P2Y_1$ purinoceptor. Compared to ATP, Ap_3A was a full and equipotent agonist while Ap_4A was a weak partial agonist. A different order of activity for dinucleotides has been reported for the recombinant $P2Y_2$ purinoceptor of human epithelia (Lazarowski *et al.*, 1995), where Ap_4A is as active as ATP but Ap_3A much less active and other dinucleotides (Ap_2A, Ap_5A and Ap_6A) inactive. Thus one important difference between recombinant $P2Y_1$ and $P2Y_2$ purinoceptors is their selectivity for Ap_3A and Ap_4A, respectively.

Apart from recombinant $P2Y_1$ and $P2Y_2$ purinoceptors, adenine dinucleotides also activate P2Y purinoceptors native to a number of mammalian tissues (Castro *et al.*, 1990; Hoyle, 1990; Pintor & Miras-Portugal, 1995b; Ralevic *et al.*, 1995). The recombinant form of chick $P2Y_1$ purinoceptor appears to be similar to a P_{2Y} purinoceptor found in rat mesenteric arterial bed (Ralevic *et al.*, 1995), inasmuch as both are activated fully by Ap_3A while other adenine dinucleotides are inactive. However, the recombinant $P2Y_1$ purinoceptor appears to be pharmacologically unlike the native P_{2Y} purinoceptor on chromaffin cells in bovine adrenal medulla which is activated slightly by Ap_3A but fully by Ap_5A and Ap_6A (Castro *et al.*, 1990; Pintor *et al.*, 1991). The P_{2Y} purinoceptor in circular muscle of human colon (Hoyle & Burnstock, 1992) is activated by Ap_5A but not Ap_3A and, to this extent, is unlike the recombinant form of chick $P2Y_1$ purinoceptor. Ap_3A is known to activate a P_2 purinoceptor present in rat hepatocytes, but this P_2 purinoceptor has been characterized pharmacologically as an ADP receptor (Green *et al.*, 1995) whereas ATP > ADP at chick $P2Y_1$.

It was once thought that all adenine dinucleotides acted broadly on all P_{2Y} purinoceptors. However, it has become clear from the results of this study (and the comparison of results discussed above) that dinucleotides act selectively on P_{2Y} purinoceptors in different tissues. This selectivity may prove useful in the future characterization of members of the P_{2Y} purinoceptor family, whether studied *in situ* or as recombinant receptors.

Dinucleotide selectivity and activity at $P2X_2$ purinoceptor

ATP and adenine dinucleotide activation of the recombinant $P2X_2$ purinoceptor from rat PC12 cells resulted in large ($\leqslant 5$ μA) inward currents in defolliculated *Xenopus* oocytes. The $P2X_2$ purinoceptor forms an ATP-gated ion channel which shows inward rectification and a reversal potential for inward currents in the region of -5 mV (Brake *et al.*, 1994). The ion channel is equally permeable to Na^+ and K^+, while replacement of extracellular Ca^{2+} by Ba^{2+} fails to decrease evoked currents and indicates little or no contribution by Ca^{2+}-activated chloride channels in ATP-evoked inward currents (Brake *et al.*, 1994).

In the present study, the EC_{50} for ATP (3.7 μM) was smaller than an EC_{50} value of 15 μM reported originally for the recombinant $P2X_2$ (Brake *et al.*, 1994). We have found that ATP-potency at $P2X_2$ is very sensitive to extracellular pH and is increased by acidification (up to pH 6.5) but decreased by alkalinization (up to pH 8.0) of the bathing medium (King *et al.*, 1996c). Thus, the observed difference in EC_{50} values for ATP may be have been due to slight differences in extracellular pH. The recombinant $P2X_2$ purinoceptor was selective for diadenosine tetraphosphate (Ap_4A) but the dinucleotide was 5 fold less potent than ATP. This potency order (ATP > Ap_4A) was different from the potency order for recombinant chick $P2Y_1$ (ATP = Ap_3A > > Ap_4A) seen in the present study and human $P2Y_2$ (ATP = Ap_4A > Ap_3A) reported earlier (Lazarowski *et al.*, 1995). Thus, $P2X_2$ can be clearly distinguished from $P2Y_1$ and $P2Y_2$ purinoceptors on the basis of selectivity, potency and activity of adenine dinucleotides.

Adenine dinucleotides also have been tested on native P_{2X} purinoceptors in various mammalian tissues (Krishtal *et al.*, 1988; MacKenzie *et al.*, 1988; Marchenko *et al.*, 1988; Hoyle, 1990; Ralevic *et al.*, 1995). Ap_4A and Ap_5A are agonists of P_{2X} purinoceptors in guinea-pig vas deferens, the latter dinucleotide being 100 fold more potent than ATP (MacKenzie *et al.*, 1988). Ap_4A, Ap_5A and Ap_6A elicit vaso-constriction via P_{2X} purinoceptors in rat mesenteric artery, these dinucleotides being 30–130 fold more potent than ATP (Ralevic *et al.*, 1995). P_{2X} purinoceptors in rat urinary bladder are activated by Ap_6A alone (Hoyle, 1990). The P_{2X} purinoceptors on nodose ganglion neurones are activated by Ap_4A and Ap_5A (Marchenko *et al.*, 1988), but the activity of Ap_4A is less than 15% of the maximal activity of ATP. An ionotropic purinoceptor described as a dinucleotide receptor and P_4 purinoceptor is stimulated fully by Ap_5A (Pintor & Miras-Portugal, 1995a; Schubert *et al.*, 1995). However, none of the above native P_{2X} purinoceptors show the same selectivity for adenine dinucleotides as seen in the present study of the recombinant $P2X_2$ purinoceptor. This diversity in dinucleotide selectivity, potency and activity also may prove useful in the future characterization of members of the P_{2X} purinoceptor family, whether studied *in situ* or as recombinant receptors.

Potentiation of ATP activity and suramin blockade by Ap_5A

Nanomolar concentrations of Ap_5A potentiated ATP-responses at the recombinant $P2X_2$ purinoceptor by increasing agonist potency. These low concentrations of Ap_5A failed to alter extracellular pH and, so, this action of Ap_5A differed from the reported effects of acidicifiction on agonist potency at $P2X_2$ (King *et al.*, 1996c). The Hill co-efficient for ATP indicated two molecules are necessary to activate $P2X_2$. The Hill co-efficient for ATP was not significantly altered in presence of

Ap_5A, indicating Ap_5A does not occupy either of the two binding sites. Ap_5A also enhanced the efficacy of suramin, a competitive antagonist at $P2X_2$ purinoceptors, causing a parallel and leftwards shift of the suramin inhibition-curve. Thus, Ap_5A appears to affect allosterically the binding of ATP and suramin at $P2X_2$.

The observed potentiation of ATP-responses by Ap_5A may have a functional bearing on purinergic signalling in the CNS, since both ATP and Ap_5A are constituents of storage vesicles in brain synaptosomes (Pintor *et al.*, 1992a). The present findings indicate that exocytotically-released Ap_5A would enhance fast ATP-transmission at $P2X_2$ receptors which, from *in situ* hybridization experiments, appears to be distributed widely throughout the brain and spinal cord (Collo *et al.*, 1996). Interestingly, a potentiating effect also has been described for periodate-oxidized ATP at P_{2X} purinoceptors in vas deferens (Fedan & Lamport, 1990; Fedan & Grant, 1995). However, periodate-oxidized ATP is a synthetic compound and potentiates only at millimolar concentrations while Ap_5A is a naturally-occurring substance and potentiates at nanomolar concentrations. Thus, the physiological role for adenine dinucleotides (at nanomolar concentrations) in the CNS may rest more with potentiation of ATP-activity at $P2X_2$ purinoceptors than their selective activation (at micromolar concentrations) of P2X and P2Y purinoceptors as discussed above.

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References

ABBRACCHIO, M.P. & BURNSTOCK, G. (1995). Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol. Ther.*, **64,** 445–475.

AKBAR, G.K.M., DASARI, V.R., WEBB, T.E., AYYANATHAN, K., PILLARISETTI, K., SANDHU, A., ATHWAL, R.S., DANIEL, J.L., ASHBY, B., BARNARD, E.A. & KUNAPULI, S.P. (1996). Molecular cloning of a novel P_2 purinoceptor from human erythroleukaemic cells. *J. Biol. Chem.*, **271,** 18363–18367.

BARISH, M.E. (1983). A transient calcium-dependent chloride current in immature *Xenopus* oocyte. *J. Physiol.*, **342,** 309–325.

BO, X., ZHANG, Y., NASSAR, M., BURNSTOCK, G. & SCHÖEPFER, R. (1995). A P2X purinoceptor cDNA conferring a novel pharmacological profile. *FEBS Lett.*, **375,** 129–133.

BRAKE, A.J., WAGENBACH, M.J. & JULIUS, D. (1994). New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature*, **371,** 519–523.

CASTRO, E., TORRES, M., MIRAS-PORTUGAL, M.T. & GONZALEZ, M.P. (1990). Effect of diadenosine polyphosphates on catecholamine secretion from isolated chromaffin cells. *Br. J. Pharmacol.*, **100,** 360–364.

CHANG, K., HANAOKA, K., KUMADA, M. & TAKUWA, Y. (1995). Molecular cloning and functional analysis of a novel P_2 nucleotide receptor. *J. Biol. Chem.*, **271,** 26152–26158.

CHEN, C., AKOPIAN, A.N., SIVILOTTI, L., COLQUHOUN, D., BURNSTOCK, G. & WOOD, J.N. (1995). A P2X purinoceptor expressed by a subset of sensory neurons. *Nature*, **377,** 428–431.

COLLO, G., NORTH, R.A., KAWASHIMA, E., MERLO-PICH, E., NEIDHART, S., SURPRENANT, A. & BUELL, G. (1996). Cloning of $P2X_5$ and $P2X_6$ receptors, and the distribution and properties of an extended family of ATP-gated ion channels. *J. Neurosci.*, **16,** 2495–2507.

COMMUNI, D., PIROTTON, S., PARMENTIER, M. & BOEYNAEMS, J.-M. (1995). Cloning and functional expression of a human uridine nucleotide receptor. *J. Biol. Chem.*, **270,** 30849–30852.

DENISENKO, O.N. (1984). Synthesis of diadenosine 5′,5‴-P^1,P^3-triphosphate in yeast at heat shock. *FEBS Lett.*, **178,** 149–152.

FEDAN, J.S. & GRANT, L.J.R. (1995). Potentiating and inhibitory effects of periodate-oxydized ATP analogs on contractions of vas deferens to ATP. *Eur. J. Pharmacol.*, **281,** 213–217.

FEDAN, J.S. & LAMPORT, S.J. (1990). Two dissociable phases in the contractile response of the guinea-pig vas deferens to adenosine triphosphate. *J. Pharmacol. Exp. Ther.*, **253,** 993–998.

GREEN, A.K., COBBOLD, P.H. & DIXON, C.J. (1995). Cytosolic free Ca^{2+} oscillations by diadenosine 5′,5‴-P^1,P^3-triphosphate and diadenosine 5′,5‴-P^1,P^4-tetraphosphate in single rat hepatocytes are indistinguishable from those induced by ADP and ATP respectively. *Biochem. J.*, **310,** 629–635.

GRUMMT, F. (1978). Diadenosine 5′,5‴-P^1,P^4-tetraphosphate triggers initiation of in vitro DNA replication in baby hamster kidney cells. *Proc. Natl. Acad. Sci. U.S.A.*, **75,** 371–377.

HOYLE, C.H.V. (1990). Pharmacological activity of adenine dinucleotides in the periphery: possible receptor classes and transmitter function. *Gen. Pharmacol.*, **21,** 827–831.

HOYLE, C.H.V. & BURNSTOCK, G. (1992). Actions of purine nucleotides on isolated circular muscle from the human sigmoid colon: evidence for a novel class of purinoceptor. *Int. J. Purine Pyrimidine Res.*, **3,** 13.

KING, B.F., PINTOR, J., WANG, S., ZIGANSHIN, A.U., ZIGANSHINA, L.E. & BURNSTOCK, G. (1996a). A novel P_1 purinoceptor activates an outward K^+ current in follicular oocytes of *Xenopus laevis*. *J. Pharmacol. Exp. Ther.*, **276,** 93–100.

KING, B.F., WANG, S. & BURNSTOCK, G. (1995). Two P_2 purinoceptor subtypes activate inward current in folliculated oocytes of *Xenopus laevis*. *Br. J. Pharmacol.*, **116,** 50P.

KING, B.F., WANG, S. & BURNSTOCK, G. (1996b). P_2 purinoceptor-activated inward currents in follicular oocytes of *Xenopus laevis*. *J. Physiol.*, **494,** 17–28.

KING, B.F., ZIGANSHINA, L.E., PINTOR, J. & BURNSTOCK, G. (1996c). Full sensitivity of $P2X_2$ purinoceptor revealed by changing extracellular pH. *Br. J. Pharmacol.*, **117,** 1371–1373.

KLISHIN, A., LOZOVAYA, N., PINTOR, J., MIRAS-PORTUGAL, M.T. & KRISHTAL, O.A. (1994). Possible functional role of diadenosine polyphosphates: negative feedback for excitation in hippocampus. *Neuroscience*, **58,** 235–236.

KRISHTAL, O.A., MARCHENKO, S.M., OBUKHOV, A.G. & VOLKOVA, T.M. (1988). Receptors for ATP in rat sensory neurones: the structure-function relationship for ligands. *Br. J. Pharmacol.*, **95,** 1057–1067.

LANDAU, E.M. & BLITZER, R.D. (1994). Chloride current assay for phospholipase C in *Xenopus* oocytes. *Methods Enzymol.*, **238,** 140–154.

LAZAROWSKI, E.R., WATT, W.C., STUTTS, M.J., BOUCHER, R.C. & HARDEN, T.K. (1995). Pharmacological selectivity of the human P_{2U} purinoceptor: potent activation by diadenosine tetraphosphate. *Br. J. Pharmacol.*, **116,** 1619–1627.

LEWIS, C., NEIDHART, S., HOLY, C., NORTH, R.A., BUELL, G. & SURPRENANT, A. (1995). Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory nuerons. *Nature*, **377,** 432–435.

LUSTIG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90,** 5113–5117.

LUTHJE, J. & OGILVIE, A. (1983). Diadenosine triphosphate (Ap_3A) mediates human platelet aggregation by liberation of ADP. *Biochem. Biophys. Res. Commun.*, **115,** 253–260.

MACKENZIE, I., KIRKPATRICK, K.A. & BURNSTOCK, G. (1988). Comparative study of the action of Ap_5A and α,β-methylene ATP on non-adrenergic, non-cholinergic neurogenic excitation in the guinea pig vas deferens. *Br. J. Pharmacol.*, **94,** 699–706.

MARCHENKO, S.M., OBUKHOV, A.G., VOLKOVA, T.M. & TARUSOVA, N.B. (1988). Bis (5′adenosyl) tetraphosphate a partial agonist of the ATP receptors on sensory neurons of the rat. *Neurofiziologia*, **20,** 427–431.

MCLENNAN, A.G. & ZAMECNIK, P.C. (1992). Dinucleoside polyphosphates - an introduction. In *Ap_4A and other Dinucleoside Polyphosphates*. McLennan, A.G. pp. 1–7. Florida: CRC Press.

PINTOR, J., DIAZ-REY, M.A. & MIRAS-PORTUGAL, M.T. (1993). Ap_4A and ADP-β-S binding to P_2 purinoceptors present on rat brain synaptic terminals. *Br. J. Pharmacol.*, **108,** 1094–1099.

PINTOR, J., DIAZ-REY, M.A., TORRES, M. & MIRAS-PORTUGAL, M.T. (1992a). Presence of diadenosine polyphosphates - Ap_4A and Ap_5A - in rat brain synaptic terminals. Ca^{2+} dependent release evoked by 4-aminopyridine and veratridine. *Neurosci. Lett.*, **136,** 141–144.

PINTOR, J., KOWALEWSKI, H.J., TORRES, M., MIRAS-PORTUGAL, M.T. & ZIMMERMANN, H. (1992b). Synaptic vesicle storage of diadenosine polyphosphates in the *Torpedo* electric organ. *Neurosci. Res. Commun.*, **10,** 9–14.

PINTOR, J. & MIRAS-PORTUGAL, M.T. (1993). Diadenosine polyphosphates -ApnA- as new neurotransmitters. *Drug. Devel. Res.*, **28,** 259–262.

PINTOR, J. & MIRAS-PORTUGAL, M.T. (1995a). A novel receptor for diadenosine polyphosphates coupled to the Ca^{2+} increase in rat brain synaptosomes. *Br. J. Pharmacol.*, **115,** 895–902.

PINTOR, J. & MIRAS-PORTUGAL, M.T. (1995b). P_2 purinoceptors for diadenosine polyphosphates in the nervous system. *Gen. Pharmacol.*, **26,** 229–235.

PINTOR, J., ROTLLÁN, P., TORRES, M. & MIRAS-PORTUGAL, M.T. (1992c). Characterization and quantification of diadenosine hexaphosphate in chromaffin cells: granular storage and secretagogue-induced release. *Anal. Biochem.*, **200,** 296–300.

PINTOR, J., TORRES, M., CASTRO, E. & MIRAS-PORTUGAL, M.T. (1991). Characterization of diadenosine tetraphosphate (Ap_4A) binding sites in cultured chromaffin cells: evidence for a P_{2Y} site. *Br. J. Pharmacol.*, **103,** 1980–1984.

RALEVIC, V., HOYLE, C.H.V. & BURNSTOCK, G. (1995). Pivotal role of phosphate chain length in vasoconstrictor versus vasodilator actions of adenine dinucleotides in rat mesenteric arteries. *J. Physiol.*, **483,** 703–713.

RODRIGUEZ DEL CASTILLO, A., TORRES, M., DELICADO, E.G. & MIRAS-PORTUGAL, M.T. (1988). Subcellular distribution studies of diadenosine polyphosphates - Ap_4A and Ap_5A- in bovine adrenal medulla: presence in chromaffin granules. *J. Neurochem.*, **51,** 1696–1703.

SCHLÜTER, H., OFFERS, E., BRUGGEMANN, G., VAN DER GLET, M., TEPEL, M., NORDHOFF, E., KARAS, M., SPIEKER, C., WITZEL, H. & ZIDEK, W. (1994). Diadenosine polyphosphate and the physiological control of blood pressure. *Nature*, **367,** 186–188.

SCHUBERT, P., PINTOR, J. & MIRAS-PORTUGAL, M.T. (1995). Inhibitor actions of adenosine and adenine dinucleotides in the central nervous system. In *Adenosine and Adenine Nucleotides: from Molecular Biology to Integrative Physiology*. ed. Belardinelli, L. & Pelleg, A. pp. 181–288. Boston, Mass.: Kluwer Academic Publishers.

SIMON, J., WEBB, T.E., KING, B.F., BURNSTOCK, G. & BARNARD, E.A. (1995). Characterisation of a recombinant P2Y purinoceptor. *Eur. J. Pharmacol. (Mol. Pharmacol. Sect.)*, **291,** 281–289.

STONE, T.W. & PERKINS, M.N. (1981). Adenine dinucleotide effect on rat cortical neurones. *Brain Res.*, **229,** 241–245.

SURPRENANT, A., RASSENDREN, F., KAWASHIMA, E., NORTH, R.A. & BUELL, G. (1996). The cytolytic P_{2Z} receptor for extracellular ATP identified as a P_{2X} receptor (P_{2X7}). *Science*, **272,** 735–738.

TEPEL, M., BACHMANN, J., SCHLÜTER, H. & ZIDEK, W. (1996). Diadenosine polyphosphates increase cytosolic calcium and attenuate angiotensin-II-induced changes in calcium in vascular smooth muscle. *J. Vasc. Res.*, **33,** 132–138.

VALERA, S., HUSSY, N., EVANS, R.J., ADAMI, N., NORTH, R.A., SURPRENANT, A. & BUELL, G. (1994). A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature*, **371,** 516–519.

WEBB, T.E., HENDERSON, D., KING, B.F., WANG, S., SIMON, J., BATESON, A.N., BURNSTOCK, G. & BARNARD, E.A. (1996a). A novel G protein-coupled P_2 purinoceptor ($P2Y_3$) activated preferentially by nucleoside diphosphates. *Mol. Pharmacol.*, (in press).

WEBB, T.E., KAPLAN, M.G. & BARNARD, E.A. (1996b). Identification of 6H1 as a P2Y purinoceptor: $P2Y_5$. *Biochem. Biophys. Res. Commun.*, **219,** 105–110.

WEBB, T.E., SIMON, J., KRISHEK, B.J., BATESON, A.N., SMART, T.G., KING, B.F., BURNSTOCK, G. & BARNARD, E.A. (1993). Cloning and functional expression of a brain G-protein coupled ATP receptor. *FEBS Lett.*, **324,** 219–225.

WILLIAMS, M. (1994). Purinoceptors nomenclature: challenges for the future. In *Adenosine and Adenine Nucleotides: from Molecular Biology to Integrative Physiology*. ed. Belardinelli, L. & Pelleg, A. pp. 39–48. Boston, Mass.: Kluwer Academic Publishers.

ZIGANSHIN, A.U., ZIGANSHINA, L.E., KING, B.F. & BURNSTOCK, G. (1995). Characteristics of ecto-ATPase of *Xenopus* oocytes and the inhibitory actions of suramin on ATP breakdown. *Pflügers Arch.*, **429,** 412–418.

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