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Diinosine pentaphosphate (IP_5I) is a potent antagonist at recombinant rat $P2X_1$ receptors

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1 The antagonist activity of a series of diinosine polyphosphates (Ip_nI, where $n=3$, 4, 5) was assessed against ATP-activated inward currents at rat $P2X_{1-4}$ receptors expressed in *Xenopus* oocytes and studied under voltage-clamp conditions.

2 Diinosine polyphosphates were prepared by the enzymatic degradation of their corresponding diadenosine polyphosphates (e.g., Ap_5A into Ip_5I) using 5′-adenylic deaminase, and purified using reverse-phase chromatography.

3 Against ATP-responses at $rP2X_1$ receptors, the potency order for antagonism was (pIC_{50}): Ip_5I (8.5) > Ip_4I (6.3) > Ip_3I (>4.5). Ip_5I (10–100 nM) caused a concentration-dependent rightwards displacement of the ATP concentration-response curve without reducing the maximum ATP effect. However, the Schild plot was non-linear which indicated Ip_5I is not a competitive antagonist. Blockade by micromolar concentrations of Ip_5I was not surmountable. Ip_4I also behaved as a non-surmountable antagonist.

4 Against ATP-responses at $rP2X_3$ receptors, the potency order for antagonism was (pIC_{50}): Ip_4I (6.0) > Ip_5I (5.6) > Ip_3I (>4.5). Blockade by Ip_4I (pA_2, 6.75) and Ip_5I (pA_2, 6.27) was surmountable at micromolar concentrations.

5 Diinosine polyphosphates failed to inhibit ATP-responses at $rP2X_2$ receptors, whereas agonist responses at $rP2X_4$ were reversibly potentiated by Ip_4I and Ip_5I. None of the parent diadenosine polyphosphates behave as antagonists at $rP2X_{1-4}$ receptors.

6 Thus, Ip_5I acted as a potent and relatively-selective antagonist at the $rP2X_1$ receptor. This dinucleotide pentaphosphate represents a high-affinity antagonist for the $P2X_1$ receptor, at which it acts in a competitive manner at low (≤100 nM) concentrations but has more complex actions at higher (>100 nM) concentrations.

Keywords: P2X receptor; ion channel; ATP; dinucleotide; Ip_5I; diinosine polyphosphate; antagonist; *Xenopus* oocyte

Abbreviations: ATP, adenosine 5′-triphosphate; Ap_5A, diadenosine pentaphosphate; D-β,γ-meATP, D isomer of β,γ-methylene ATP; L-β,γ-meATP, L isomer of β,γ-methylene ATP; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; I_{ATP}, ATP-activated membrane current; IC_{50}, concentration causing 50% reduction of agonist response; Ip_3I, diinosine triphosphate; Ip_4I, diinosine tetraphosphate; Ip_5I, diinosine pentaphosphate; KN-62, 1-[N,O-bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; MRS 2220, cyclic pyroxidine-$\alpha^{4,5}$-monophosphate-6-azophenyl-2′,5′-disulphonic acid; NF023, 8,8′-(carbonylbis(imino-3,1-phenylene carbonyliminobis-(1,3,5-napthalenetrisulphonic acid); n_H Hill coefficient; pA_2, negative log of antagonist concentration causing a 2 fold decrease in agonist potency; pEC_{50}, negative log of agonist concentration causing 50% of maximum effect; pIC_{50}, negative log of antagonist concentration causing 50% reduction of agonist response; PPADS, pyridoxal-α^5-phosphate-6-azophenyl-2′,4′-disulphonic acid; $rP2X_{1-4}$, rat $P2X_{1-4}$ receptor subtypes, RB-2, Reactive blue 2; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenol)-ATP; V_h, holding potential

Introduction

P2X receptors are ATP-gated cation channels composed of oligomeric assemblies of three, or possibly four, receptor protein subunits (Kim *et al*., 1997; Nicke *et al*., 1998; Torres et al., 1999). Seven P2X subunits ($P2X_{1-7}$) have been cloned thus far, the operational profiles and pharmacological characteristics of homomeric assemblies of $P2X_{1-7}$ receptors having been well documented (for reviews, see: Evans *et al*., 1998; Humphrey *et al*., 1998; King, 1998). Transcripts for $P2X_{1-4}$ receptor proteins are abundant in excitable tissues (neurons, smooth muscle, cardiac muscle) and in secretory epithelia, while the distribution of $P2X_5$ and $P2X_7$ mRNA is highly restricted. $P2X_6$ transcripts are especially abundant in the neuraxis (Collo *et al*., 1996), but it remains controversial that the $P2X_6$ subunit protein is capable of forming homomeric assemblies (Torres *et al*., 1999).

The correspondence between homomeric P2X receptors and native P2X receptors has been hampered by a paucity of selective ligands for P2X subunits. Operational profiles of homomeric P2X receptors have been matched loosely to some examples of native P2X receptors (Evans & Surprenant, 1996), but the possibility of subpopulations of homomeric and heteromeric P2X receptors in any pool of native P2X receptors cannot be discounted. Some progress has been made with agonists showing reasonable P2X subunit selectivity, e.g. L-β,γ-meATP at $P2X_1$ receptors (Evans et al., 1995), D-β,γ-meATP at $P2X_3$ receptors (Rae et al., 1998), and activity series of the diadenosine polyphosphates (Ap_nA, where $n=2-6$) at $P2X_{1-4}$ receptors (Wildman *et al*., 1999a). However, differentiating homomeric P2X receptors by agonist activity alone is a lengthy and laborious process (Humphrey *et al*., 1998). Further

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progress has been made with P2X subunit-selective antagonists: the anion transport inhibitor, DIDS, is relatively selective for $P2X_1$ receptors at micromolar concentrations (Evans *et al*., 1995); the PPADS derivative, MRS 2220, is wholly selective for $P2X_1$ receptors at micromolar concentrations (Jacobson *et al*., 1998); the suramin analogue, NF023, is a potent antagonist at $P2X_1$ receptors at submicromolar concentrations, whilst 35–138 fold less potent at species orthologues of $P2X_3$ receptors (Soto *et al*., 1999); TNP-ATP is a potent antagonist at both $P2X_1$ and $P2X_3$ receptors at nanomolar concentrations (Virginio *et al*., 1998); KN-62 is a potent antagonist of $P2X_7$ receptors, although it shows differential activity at species orthologues of the $P2X_7$ receptor (Humphreys *et al*., 1998). Neither suramin, PPADS nor Reactive blue 2 (RB-2) readily discriminate between P2X subunits (Evans *et al*., 1998; King, 1998; Ralevic & Burnstock, 1998).

Diinosine polyphosphates (abbreviated Ip_nI, where *n* is the number of phosphates) comprise two ribosylated inosine molecules bridged by a phosphate chain. These dinucleotides are synthesized by deaminating diadenosine polyphosphates with the non-specific AMP-deaminase of *Aspergillus sp*. (Guranowski *et al*., 1995; Pintor *et al*., 1997). One member of this family, P^1,P^5-bis(5′-inosyl) pentaphosphate (Ip_5I), has already shown interesting pharmacological properties, being a potent antagonist at: (i) a specific dinucleotide receptor for diadenosine polyphosphates in rat brain synaptosomes (IC_{50} value, 4 nM); (ii) a P2X receptor in the same preparation (IC_{50} value, 30 μM); (iii) the $P2X_1$-like receptor in guinea-pig isolated vas deferens (pA_2 value, 6.5) (Hoyle *et al*., 1997; Pintor *et al*., 1997; 1999). In this paper, we describe the antagonist properties of Ip_5I and two related dinucleotides, Ip_4I and Ip_3I, on homomeric $P2X_{1-4}$ receptors. The activity profile of dinucleotide pentaphosphate reveals selectivity for the $P2X_1$ receptor subtype at nanomolar concentrations.

Methods

Diinosine polyphosphate synthesis

Diinosine pentaphosphate (Ip_5I) was prepared by enzymatic degradation of diadenosine pentaphosphate (Ap_5A). Ip_4I and Ip_3I were also prepared in the same manner from Ap_4A and Ap_3A, respectively. 5′-adenylic acid deaminase (0.12 U) from *Aspergillus sp*. was incubated with 10 mM Ap_5A in a final volume of 1 ml of 50 mM HEPES (pH 6.5) for 90 min (at 37°C). Aliquots (10 μl) were taken at different times, placed in 100°C water bath for 5 min to stop the enzymatic reaction, and diluted 1 : 100 with distilled water to monitor the production of Ip_5I by HPLC techniques. After 90 min, the reaction was stopped by boiling the incubation medium at 100°C for 5 min, after which protein debris was removed by filtration through a Millex-G5 filter (0.22 μm; from Millipore). The reaction product was confirmed as Ip_5I by HPLC detection. Samples were treated with phosphodiesterase (3 mU, at 37°C) from *Crotalus durissus* (EC.3.1.15.1) (for rationale, see Results) then diluted 1 : 100 with distilled water for HPLC separation and detection of Ip_5I breakdown products.

Chromatographic procedures

The chromatographic equipment consisted of a Waters 600E delivery system, a Waters 717+ autosampler and a Waters 2487 dual wavelength absorbance detector, which were managed by Millenium 2010 software. Analyses were performed under reverse-phase chromatography conditions, equilibrating the system with 100 mM KH_2PO_4, 4% methanol, pH 6.0, at 1.5 ml min^{-1}. The column was a Spherisorb ODS-2 (25 cm length, 0.4 cm diameter; from Waters). Detection was monitored at 260 nm wavelength. For phosphodiesterase measurements, ion-pair chromatography was performed. The mobile phase conditions were 10 mM KH_2PO_4, 2 mM tetrabutyl ammonium, 15% acetonitrile, pH 7.5, at 2 ml min^{-1}. The column was a Spherisorb ODS-2. Detection was performed as above.

Oocyte preparation

Xenopus laevis frogs were anaesthetized in Tricaine (0.2% w v^{-1}), killed by decapitation, and the ovarian lobes removed surgically. Oocytes (stages V and VI) were defolliculated by a 2-step process involving collagenase treatment (Type IA, 2 mg ml^{-1} in a Ca^{2+}-free Ringer's solution, for 2–3 h) followed by stripping away the follicular layer with fine forceps. Defolliculated oocytes were stored in Barth's solution (pH 7.5, at 4°C) 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; gentamycin sulphate, 50 μg l^{-1}. Separate batches of defolliculated oocytes were injected cytosolically (40 nl, 1 μg ml^{-1}) with cRNAs for rat $P2X_{1-4}$ receptors (see Acknowledgements), incubated for 24–48 h at 18°C in Barth's solution and, thereafter, kept at 4°C for up to 12 days until used in electrophysiological experiments.

Electrophysiology

ATP-evoked membrane currents (I_{ATP}) ($V_h = -60$ to -90 mV) were recorded from cRNA-injected oocytes using a twin-electrode voltage-clamp amplifier (Axoclamp 2B). The voltage-recording and current-recording microelectrodes (1–5 MΩ tip resistance) were filled with 3.0 M KCl. Oocytes were superfused with a Ringer's solution (5 ml min^{-1}, at 18°C) containing (mM): NaCl, 110; KCl, 2.5; HEPES, 5; $BaCl_2$, 1.8, adjusted to pH 7.5. The extracellular pH was maintained at pH 7.5 in all experiments, since the potency of ATP at $P2X_{1-4}$ receptors is affected by H^+ ions (King *et al*., 1996; Wildman *et al*., 1999b,c).

Solutions and drugs

All solutions were nominally Ca^{2+}-free to avoid the activation of a Ca^{2+}-dependent Cl^- current ($I_{Cl,Ca}$) in oocytes. ATP was prepared in Ca^{2+}-free Ringer's solution at the concentrations stated, and superfused *via* a gravity-feed continuous flow system which allowed rapid addition and washout. Ip_nI compounds were dissolved in a buffer solution (HEPES 50 mM, pH 6.5 with KOH) to give a 10 mM stock solution, then diluted further using Ringer's solution and readjusted to pH 7.5. For inhibition curves, ATP (at the EC_{70} value at pH 7.5 (in μM): $P2X_1$, 1; $P2X_2$, 20; $P2X_3$, 3; $P2X_4$, 10) was added to the superfusate for 60–120 s, then washed off with Ringer's solution for 30 min. After obtaining agonist responses of consistent amplitude (Figure 1A), diinosine polyphosphates (Ip_nI, 0.1–100,000 nM) were added to the superfusate for 30 min before and during re-application of ATP. The blocking activity of Ip_nI compounds did not improve with pre-incubation periods longer than 10 min (Figure 1B) which suggested that Ip_nI blockade was not use-dependent, although Ip_nI compounds were routinely applied throughout the full 30 min of the ATP-washout period. For Schild analyses, the concentration/ response (C/R) relationship was determined for ATP (0.01–300 μM), then cRNA-injected oocytes were

incubated with Ip_nI compounds for 30 min, after which ATP C/R curves constructed again in the continued presence of Ip_nI. EC_{50} values and slopes of C/R curves were taken from Hill plots, using the transform log $(I/I_{max}-I)$ where I is the current evoked by each concentration of ATP. pA_2 values were determined by Schild analysis, using the equation {log (DR − 1) − log [Ip_nI]}.

Statistics

Data are presented as means ± s.e.mean of 4–6 sets of data from different oocyte batches. Significant differences were determined by either unpaired or paired Student's *t*-test using commercially-available software (Instat v2.05a, GraphPad).

Materials

All common salts were AnalaR grade (Aldrich Chemicals, U.K.). Adenosine 5′-triphosphate, diadenosine polyphosphates (Ap_3A, Ap_4A, Ap_5A) and phosphodiesterase (EC.3.1.15.1) (from *Crotalus durissus*) were purchased from Boehringer (Mannheim, Germany). 5′-adenylic acid deaminase (from *Aspergillus sp.*) was purchased from Sigma Chemical Co. (St. Louis, U.S.A.).

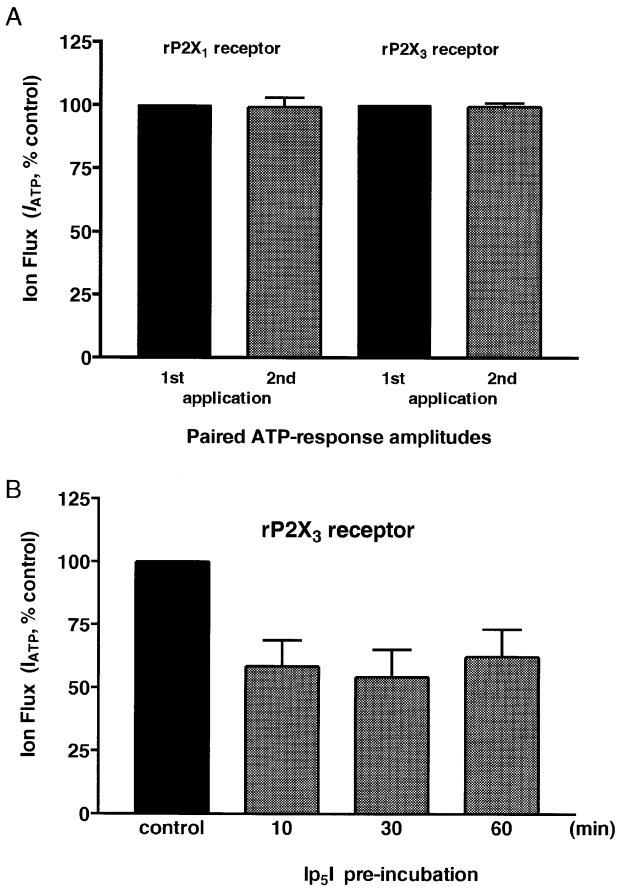

Figure 1 Consistency of agonist and antagonist activity. (A) histograms of the amplitudes of paired agonist-responses at homomeric rP2X$_1$ and rP2X$_3$ receptors (using ATP: rP2X$_1$, 1 μM; rP2X$_3$, 3 μM). Washout periods of 30 min were used between first and second applications of ATP, a periodicity sufficient to yield I_{ATP} responses of consistent amplitude. Thus, Ip_nI-related antagonism could not be explained in terms of a rundown of ATP-responses at P2X$_1$ and P2X$_3$ receptors. (B) the effect of increasing the pre-incubation period (10, 30 and 60 min) to Ip_5I (3 μM) on the level of antagonism of ATP-responses (using 3 μM) at homomeric rP2X$_3$ receptors. The blocking activity of Ip_5I neither improved nor waned over 60 min pre-incubation, indicating that blockade was not use-dependent. Data: mean ± s.e.mean ($n=4$) in A and B.

Results

Production of Ip_nI

5′-adenylic acid deaminase of *Aspergillus sp.* efficiently transformed Ap_5A into Ip_5I, fully converting the substrate into product over 90 min without further transformation of Ip_5I into other by-products. During intermediate times, HPLC analysis of the reaction medium revealed an additional peak with a retention time between that of the initial Ap_5A peak and final Ip_5I peak (Figure 2A). This additional peak gradually disappeared over 90 min and was attributed to the intermediary reaction product (Ip_5A), where only one of two adenosine moieties had been deaminated. Like results were obtained when producing Ip_4I from Ap_4A and Ip_3I from Ap_3A.

The final reaction product was confirmed as Ip_5I (as opposed to related breakdown products, i.e. IMP and Ip_4) in experiments where phosphodiesterase was used. This enzyme cleaves Ip_5I to yield inosine monophosphate (IMP) and the mononucleotide inosine 5′-tetraphosphate (Ip_4) (Figure 2B). The retention times for these breakdown products was compared and matched against times for standard solutions of IMP and Ip_4. The absence of IMP and Ip_4 peaks (prior to enzymatic treatment at zero time) in HPLC analysis indicated

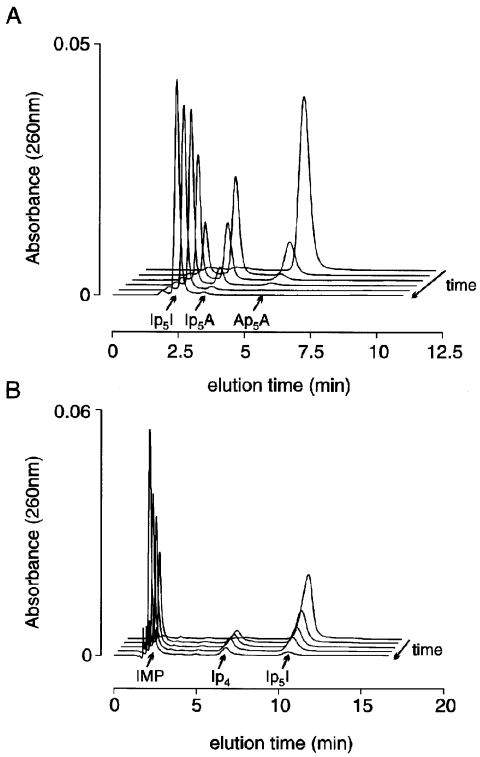

Figure 2 HPLC analysis of Ip_5I production. (A) a series of chromatographic profiles for the time-dependent enzymatic conversion of Ap_5A into the intermediary product Ip_5A and final product Ip_5I by adenylate deaminase (*Aspergillus sp.*) over a period of 90 min (chromatograms at: T = 0, 15′, 30′, 45′, 60′ and 90′). (B) a series of chromatographic profiles for the time-dependent production of IMP and Ip_4 by phosphodiesterase breakdown of the reaction product Ip_5I (chromatograms at: T = 0, 5′, 10′, 15′ and 30′). Ordinate scalars (A and B) as AUFS (absorbance units full scale), as defined by Millennium 2010 software (Waters).

Ip_5I had not been broken down during its synthesis. Similar results were observed when testing the purity of Ip_4I and Ip_3I.

Blockade of P2X receptors by Ip_5I

The pentaphosphate Ip_5I (1 μM) was a potent inhibitor of ATP-responses at rP2X$_1$ receptors, and weak inhibitor of ATP-responses at rP2X$_3$ receptors (Figure 3A,B and Table 1). However, Ip_5I failed to block rP2X$_2$ receptors and potentiated ATP-responses at rP2X$_4$ receptors (Figure 3C,D and Table 1). Thus, Ip_5I is an antagonist at Group 1 P2X receptors (as defined by Humphrey *et al*., 1998), yet approximately 900 fold more potent at rP2X$_1$ than rP2X$_3$ receptors. The inhibitory and facilitatory effects of Ip_5I were reversed on washout. Micromolar levels of Ip_5I had no effect on the holding current of either *Xenopus* oocytes injected with cRNA for rP2X$_{1-4}$ receptors or water-injected (control) oocytes. Thus, Ip_5I inhibition or facilitation was not due to a partial agonistic effect.

Blockade of P2X receptors by Ip_nI series

The blocking activity Ip_4I and Ip_3I was also investigated at Group 1 P2X receptors (Figure 4A,B and Table 1). Ip_4I was 180 fold less potent than Ip_5I as an antagonist of rP2X$_1$ receptors yet, in contrast, 3 fold more potent than Ip_5I as an antagonist of P2X$_3$ receptors. The triphosphate Ip_3I was a weak antagonist of rP2X$_1$ and rP2X$_3$ receptors, only showing significant levels of blockade at high concentrations ($>10\mu$M). The blocking actions of Ip_4I and Ip_3I were reversed on washout.

The actions Ip_4I and Ip_3I were also studied at rP2X$_2$ and rP2X$_4$ receptors (Figure 5A,B and Table 1). Both Ip_nI compounds, like Ip_5I, were inactive as either antagonists or potentiators at rP2X$_2$ receptors (Figure 5A). At rP2X$_4$

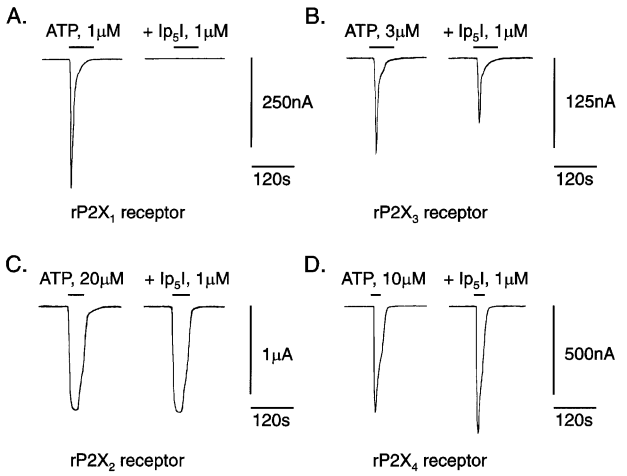

Figure 3 Ip_5I antagonism of ATP-responses at rP2X$_{1-4}$ receptors. ATP-activated whole-cell inward currents (I_{ATP}), before (first record) and during (second record) superfusion of micromolar Ip_5I (1 μM, 30 min pre-incubation). Paired I_{ATP} records were taken from single cRNA-injected oocytes expressing homomeric rP2X$_1$ (in A), rP2X$_3$ (in B), rP2X$_2$ (in C) and rP2X$_4$ receptors (in D). ATP was applied at a concentration equivalent to the EC_{70} value for each recombinant rP2X$_{1-4}$ receptor (see Methods). $V_h = -60$ mV in A–D.

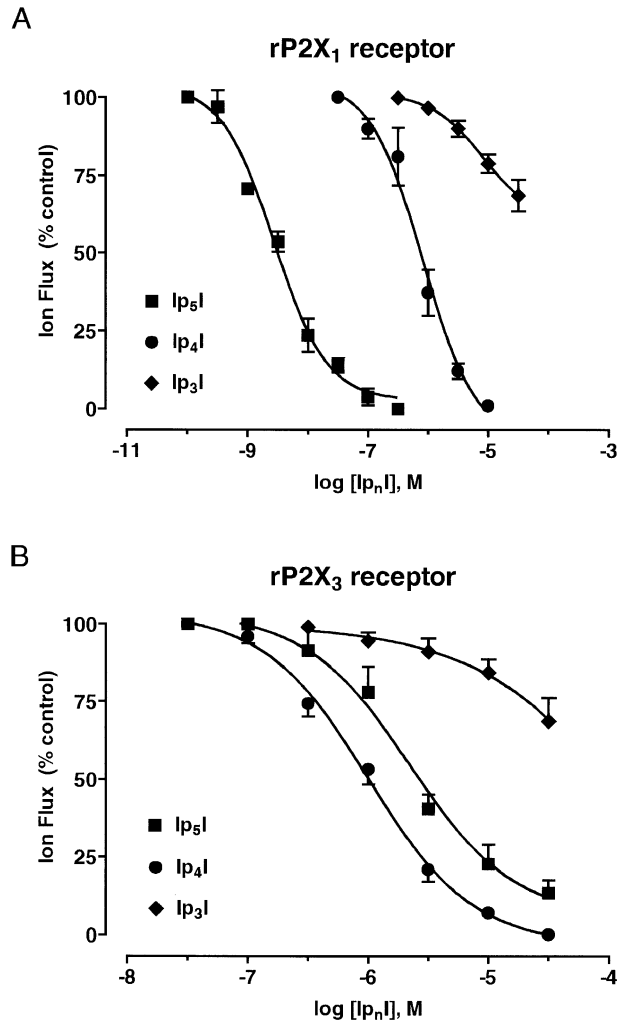

Figure 4 Inhibition curves for Ip_nI series at Group 1 P2X receptors. (A) Concentration-dependent inhibition of I_{ATP} (ATP, 1 μM) at homomeric rP2X$_1$ receptors by Ip_5I, Ip_4I and Ip_3I. (B) Concentration-dependent inhibition of I_{ATP} (ATP, 3 μM) at homomeric rP2X$_3$ receptors by the same diinosine polyphosphates. IC_{50} values and slopes of inhibition curves are given in Table 1. Data: mean $\pm$s.e.mean ($n=5$) in A and B.

Table 1 Activity indices of Ip_nI compounds at rP2X$_{1-4}$ receptors

Compound	*P2X$_1$ IC_{50} value*	*P2X$_2$*	*P2X$_3$ IC_{50} value*	*P2X$_4$ EC_{50} value*
Ip_5I	0.0031$\pm$0.0004 μM (n_H, -0.96 ± 0.08)	inactive (0.1–30 μM)	2.8$\pm$0.7 μM (n_H, -1.02 ± 0.14)	0.0020$\pm$0.0004 μM (n_H1.33$\pm$0.09)
Ip_4I	0.560$\pm$0.080 μM (n_H, -1.47 ± 0.20)	inactive (0.1–30 μM)	1.0$\pm$0.3 μM (n_H, -1.16 ± 0.10	1.69$\pm$0.4 μM (n_H, 2.29$\pm$0.17)
Ip_3I	>30 μM	inactive 0.1–30 μM)	>30 μM	inactive (0.3–10 μM)

IC_{50} and EC_{50} values for the inhibitory and facilitatory effects of Ip_nI compounds on ATP-responses at recombinant P2X$_{1-4}$ receptors. Slopes of inhibition and facilitation curves are given in brackets. Data: mean$\pm$s.e.mean ($n=5$).

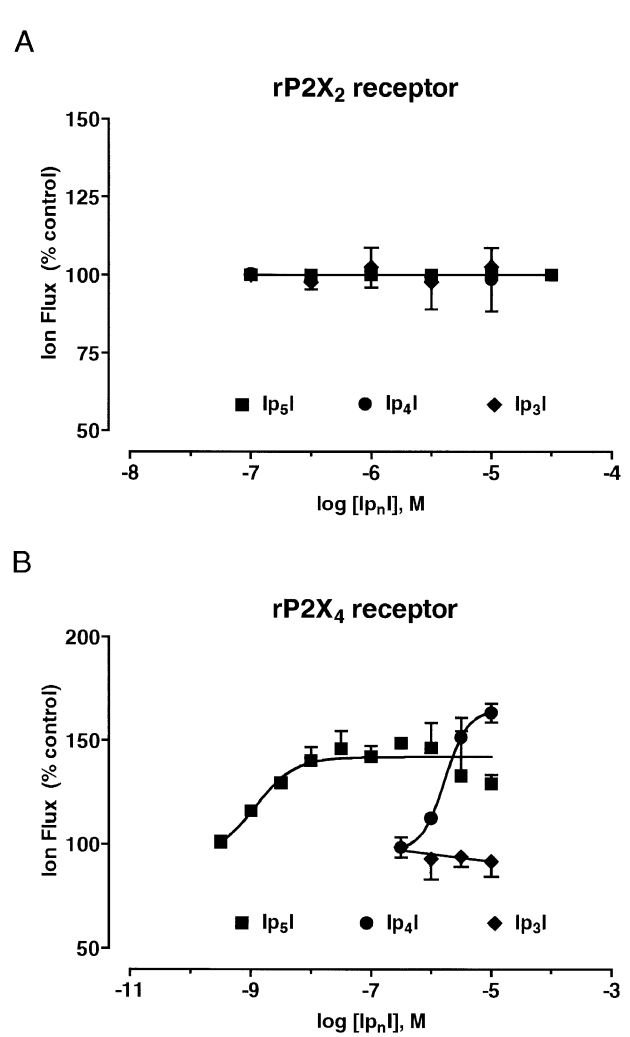

Figure 5 Activity of Ip_nI series at rP2X$_2$ and rP2X$_4$ receptors. (A) The Ip_nI series neither inhibited nor potentiated ATP-responses (using 20 μM) at rP2X$_2$ receptors. (B) Ip_5I and Ip_4I potentiated ATP-responses (using 10 μM) at rP2X$_4$ receptors. Activity indices for Ip_nI series given in Table 1. Data: mean$\pm$s.e.mean ($n=5$) in A and B.

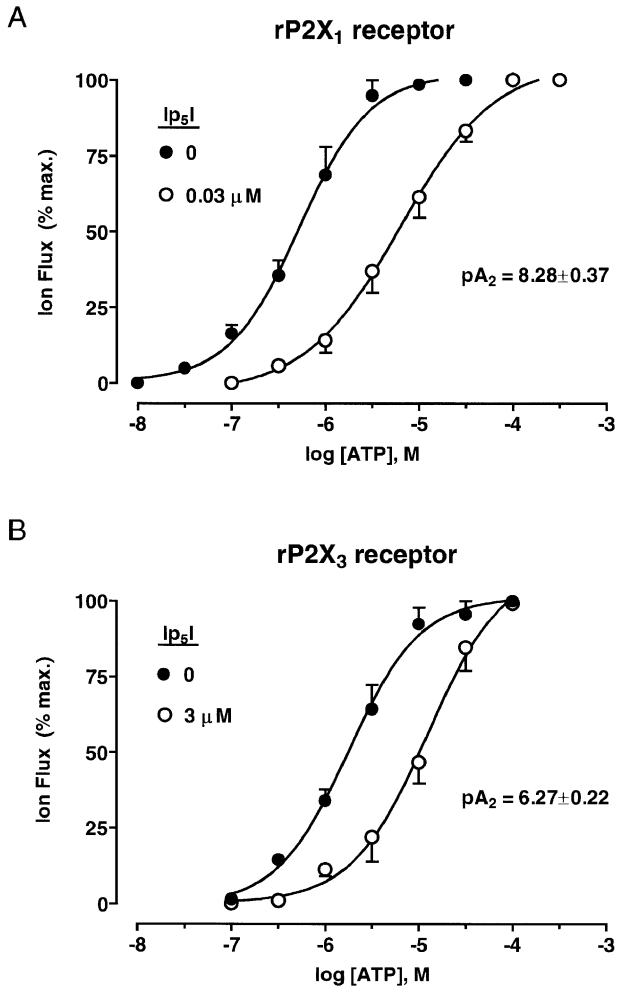

Figure 6 Ip_5I antagonism of ATP-responses at Group 1 P2X receptors. (A) Concentration-response curves for ATP (0.01–300 μM) at homomeric rP2X$_1$ receptors, before and during the presence of Ip_5I (30 nM). (B) Concentration-response curves for ATP (0.1–100 μM) at homomeric rP2X$_3$ receptors, before and during the presence of Ip_5I (3 μM). EC_{50} values, Hill coefficients and pA_2 values given in Table 2. Data: mean$\pm$s.e.mean ($n=4$) for paired C/R curves, in A and B.

Table 2 Schild analysis of Ip_5I displacement of ATP C/R curves at rP2X$_1$ receptors

Ip_5I (nM)	*EC_{50} values and Hill coefficients*		*pA_2*	n
	Control	*+Ip_5I*		
10	0.38$\pm$0.07 μM	1.89$\pm$0.87 μM	8.43$\pm$0.29	4
	(n_H, 0.96$\pm$0.06)	(n_H, 0.89$\pm$0.08)	(range, 7.99–8.89)	
30	0.42$\pm$0.06 μM	3.56$\pm$2.05 μM	8.28$\pm$0.37	4
	(n_H, 0.99$\pm$0.14)	(n_H, 0.82$\pm$0.09)	(range, 7.70–8.86)	
100	0.70$\pm$0.15 μM	92.1$\pm$15.7 μM	8.99$\pm$0.19	4
	(n_H, 1.02$\pm$0.15)	(n_H, 0.86$\pm$0.16)	(range, 8.49–9.23)	

EC_{50} values and Hill coefficients (n_H) for the ATP C/R relationship in the absence (control) and presence (+Ip_5I) of diinosine pentaphosphate (10–100 nM). Data: mean$\pm$s.e.mean of paired sets of observations.

receptors, Ip_4I was over 800 fold less potent than Ip_5I at potentiating ATP-responses (an effect reversed on washout), while Ip_3I had no effect (Figure 5B and Table 1).

Schild analysis of Ip_5I blockade

Ip_5I (10, 30 and 100 nM) caused a rightwards displacement of the ATP concentration-response (C/R) curve, without altering the maximum agonist effect (Figures 6A and 8 and Table 2). However, higher concentrations of Ip_5I (1 and 10 μM) completely blocked the agonist effects of ATP ($\leqslant$1 mM) (Figure 8), beyond which ATP exerts non-specific excitatory effects on *Xenopus* oocytes (Kupitz & Atlas, 1993). Thus, Ip_5I blockade was surmountable at low Ip_5I concentrations ($\leqslant$100 nM), and nonsurmountable at micromolar concentrations. The antagonistic effects of Ip_5I (10–100 nM) on ATP C/R curves (EC_{50} values, Hill coefficients and pA_2 values) are summarized in Table 2. Schild analysis of paired C/R curves revealed that pA_2 values were not uniform, varying between 7.70–9.23 (range of 12 determinations) for the mean pA_2

values listed (Table 2). This variability, in combination with nonsurmountable blockade with micromolar Ip_5I, indicated that this dinucleotide did not antagonize ATP-mediated membrane currents at rP2X$_1$ receptors in a simple competitive manner.

On the other hand, high concentrations of Ip_5I (3 μM, approximate IC_{50} value) did cause a surmountable antagonism of ATP-responses at rP2X$_3$ receptors (Figure 6B). EC_{50} values and Hill coefficients for ATP were (for the following Ip_5I concentrations): 0 μM, 1.4 ± 0.4 μM (n_H, 1.13 ± 0.12); 3 μM, 9.6 ± 2.5 μM (n_H: 1.08 ± 0.18) ($n=4$). The pA_2 value for Ip_5I antagonism at rP2X$_3$ receptors was 6.27 ± 0.22 ($n=4$).

Schild analysis of Ip_4I blockade

Ip_4I (3 μM, approximate IC_{50} value) caused a nonsurmountable inhibition of ATP-response at rP2X$_1$ receptors, in the manner of non-competitive antagonist (Figure 7A). EC_{50} values and Hill coefficients for ATP were (for the following Ip_4I concentrations) : 0 μM, 0.38 ± 0.06 μM (n_HH, 0.84 ± 0.17); 3 μM, 1.91 ± 0.43 μM (n_H, 0.63 ± 0.46) ($n=4$). The maximum ATP effect was reduced by $56\pm7\%$ ($n=4$) by the dinucleotide. Thus, Ip_4I caused a reduction in both agonist potency and efficacy.

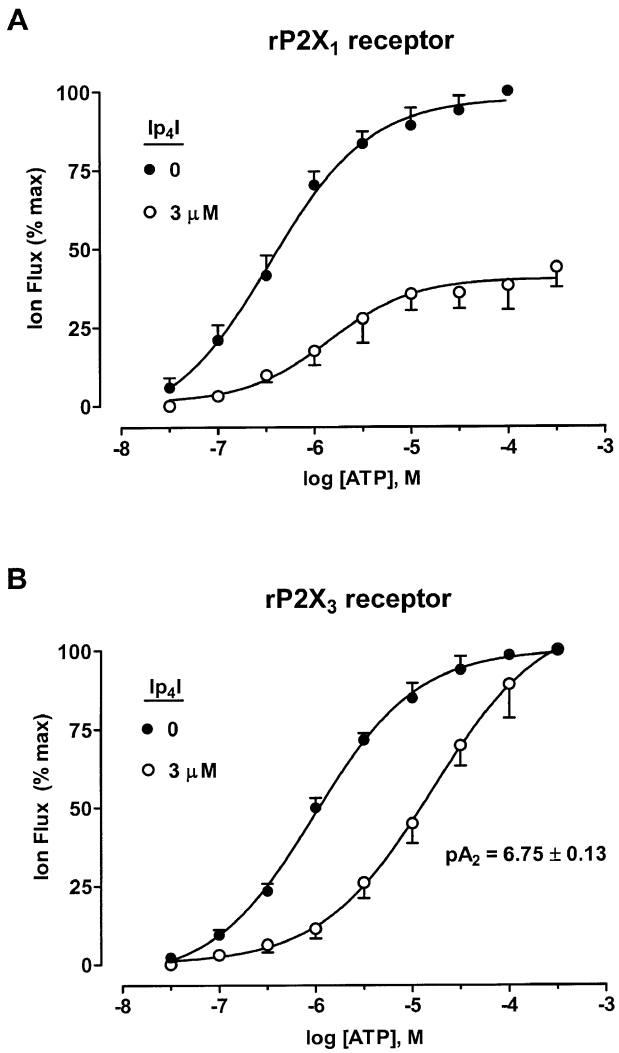

Figure 7 Ip_4I antagonism of ATP-responses at Group 1 P2X receptors. (A) Concentration-response curves for ATP (0.03–300 μM) at homomeric rP2X$_1$ receptors, before and during the presence of Ip_4I (3 μM). (B) Concentration-response curves for ATP (0.03–300 μM) at homomeric rP2X$_3$ receptors, before and during the presence of Ip_4I (3 μM). EC_{50} values, Hill coefficients and pA_2 values given in the text. Data: means$\pm$s.e.mean ($n=4$) for paired C/R curves, in A and B.

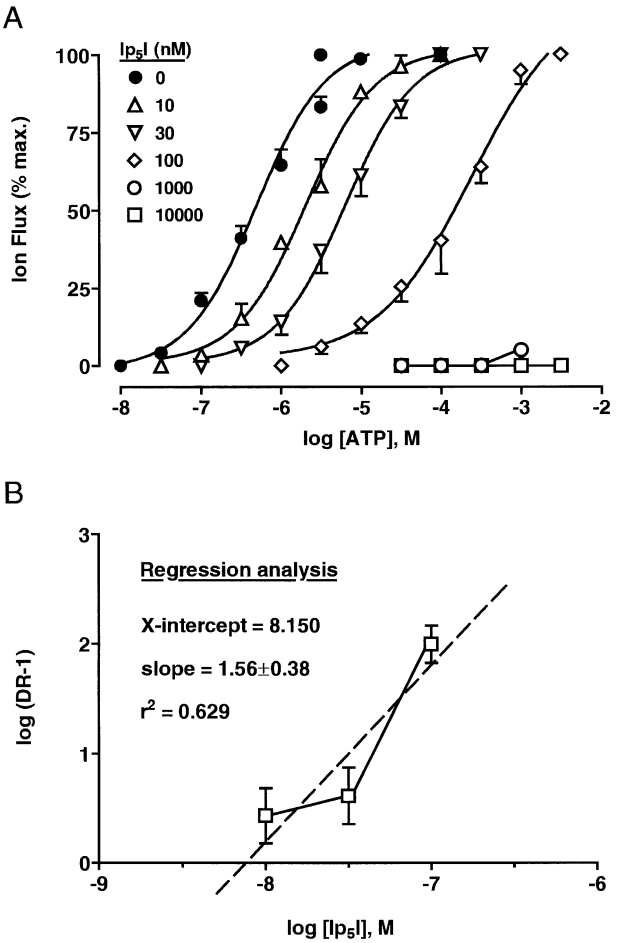

Figure 8 Schild analysis of Ip_5I blockade of rP2X$_1$ receptors. (A) Concentration/response (C/R) curves for the ATP-activated rP2X$_1$ receptor, in the absence and presence of Ip_5I (10–10,000 nM). The control C/R curve represented pooled data of 20 determinations, and test C/R curves determined for four experiments for each of the five concentrations of Ip_5I used (10, 30, 100, 1000 and 10,000 nM). (B) The Schild plot of C/R data according to EC_{50} values stated in Table 2. Linear regression (Prism v2.0, GraphPad) gave an estimated pA_2 of 8.150, mean slope of 1.56 (i.e., greater than unity), and a low correlation coefficient (r^2) of 0.629 for linearity. The Schild plot was clearly biphasic.

At rP2X$_3$ receptors, Ip_4I (3 μM, approximate IC_{75} value) caused a surmountable inhibition of ATP responses (Figure 7B). EC_{50} values and Hill coefficients for ATP were (for the following Ip_4I concentrations): 0 μM, 1.0 ± 0.3 μM (n_H, 0.82 ± 0.11); 3 μM, 17.8 ± 5.3 μM (n_H, 0.76 ± 0.19) ($n=4$). The pA_2 value for Ip_4I antagonism at rP2X$_3$ receptors was 6.75 ± 0.13 ($n=4$).

Discussion

The present results showed that Ip_5I was an effective antagonist of ATP-responses at Group 1 P2X receptors (P2X$_1$ and P2X$_3$), being selective for rP2X$_1$ receptors at low ($\leqslant$100 nM) concentrations. This pentaphosphate was 900 fold less potent at P2X$_3$ receptors (comparing IC_{50} values), at which micromolar concentrations were required to block ATP-activated inward currents. The blocking actions of Ip_5I at P2X$_1$ receptors at nanomolar concentrations initially seemed

consistent with a competitive antagonism. However, several features suggested that the mechanism of Ip_5I blockade was more complex than originally thought. First, micromolar concentrations of Ip_5I caused a nonsurmountable inhibition of ATP-responses (Figure 8A). Second, determinations of the pA_2 value were dependent on the Ip_5I concentration used (see Table 2). Third, a Schild plot of combined Ip_5I data (10, 30 and 100 nM) was non-linear and the slope (by regression analysis) was greater than unity (Figure 8B). Taken together, these features suggested that Ip_5I is not a simple competitive antagonist at rP2X$_1$ receptors. Also, the parent compound Ap_5A is a partial agonist at rP2X$_1$ receptors (Wildman *et al*., 1999a), suggesting that the binding site for the pentaphosphate (as either Ap_5A or Ip_5I) might not be the same position as the ATP docking site.

For P2X$_1$ receptors, the blocking activity of the Ip_nI series decreased as the phosphate chain was reduced in length. Thus, Ip_5I was 180 fold more potent than Ip_4I and greater than 10,000 fold more potent than Ip_3I. This potency order for antagonism clearly contrasted with the agonist potency order of their parent compounds at rP2X$_1$ receptors, where Ap_4A (7.4) > Ap_5A (6.0) > Ap_3A (>4) (pEC_{50} values) (Wildman *et al*., 1999a). Like Ip_5I, Ip_4I was a non-competitive antagonist at rP2X$_1$ receptors and significantly reduced the maximum ATP effect. Its parent compound, Ap_4A, is a partial agonist at rP2X$_1$ receptors, suggesting the binding site for the tetraphosphate (as either Ap_4A or Ip_4I) might again differ from the ATP docking site. Interestingly, neither dinucleotide triphosphate (Ip_3I and Ap_3A) interacted well with rP2X$_1$ receptors in terms of antagonist and agonist activities, yet rP2X$_1$ receptor is activated by a number of mononucleoside triphosphates.

The rP2X$_3$ receptor showed a slight preference for Ip_4I over Ip_5I, their pA_2 values being 6.75 and 6.27, respectively. Both diinosine compounds appeared to act as competitive antagonists, causing a parallel rightwards shift of the ATP C/R curve without reducing the maximum ATP effect (Figures 6B and 7B). Their parent compounds, Ap_4A and Ap_5A, are both full agonists at rP2X$_3$ receptors, at which the tetraphosphate (pEC_{50}, 6.10) is slightly more potent than the pentaphosphate (pEC_{50}, 5.88) (Wildman *et al*., 1999a). As far as the triphosphate is concerned, Ip_3I is a weak antagonist and Ap_3A a partial agonist at rP2X$_3$ receptors.

The Ip_nI series lacked activity at rP2X$_2$ receptors at which the parent Ap_nA compounds otherwise showed interesting effects. Ap_4A is a full agonist at rP2X$_2$ receptors, although 4 fold less potent than ATP, while nanomolar Ap_5A is a potent potentiator of ATP-responses (Pintor *et al*., 1996). Thus, the inability of Ip_4I and Ip_5I to interact with rP2X$_2$ receptors contrasted sharply with our earlier results with their parent compounds (Ap_4A and Ap_5A), although the lack of activity of the diinosine polyphosphates at least reflects the efficiency of the enzyme degradation process to make Ip_nI compounds. Ip_5I was reported to be inactive against ATP-responses at P2X$_2$-like receptors in neonatal rat cerebellar Purkinje neurons, although Ip_3I and Ip_4I have not yet been tested in this model (Garcia-Lecea *et al*., 1999).

For rP2X$_4$ receptors, the potentiating effects of Ip_5I and Ip_4I struck a chord with similar potentiating effects of other compounds (e.g. suramin, PPADS and Reactive blue 2) tested as P2 receptor antagonists at the rat P2X$_4$ receptor (Bo *et al*., 1995) and mouse P2X$_4$ receptor (Townsend-Nicholson *et al*., 1999). The Ip_nI compounds proved just as ineffective as antagonists at rP2X$_4$ receptors as other compounds tested so far. The parent dinucleotide of Ip_4I is a partial agonist at rP2X$_4$ receptors (pEC_{50}, 5.5) (Wildman *et al*., 1999a). However, the potentiating effect of Ip_4I was not accompanied by any change in holding currents and was not believed to be due to Ap_4A contamination.

Of the Ip_nI series, only Ip_5I has been tested at native P2X receptors. Ip_5I is considerably less potent as an antagonist at the P2X$_1$-like receptor in guinea-pig vas deferens (pA_2, 6.5 ± 0.1) (Hoyle *et al*., 1997), compared to its blocking activity at the recombinant rP2X$_1$ receptor in the present study (pA_2 range, 7.70–9.23). This reduction in activity may be due to Ip_5I breakdown by ecto-nucleotidases in guinea-pig vas deferens, or perhaps due to a difference between guinea-pig and rat P2X$_1$ receptors. The guinea-pig P2X$_1$ receptor has not yet been cloned and, furthermore, Ip_5I has not yet been tested on the human P2X$_1$ receptor. Thus far, the blocking activity of Ip_5I has only been characterized at the rat P2X$_1$ receptor. Potential species differences in blocking activity notwithstanding, the observed Ip_5I activity at native P2X$_1$ receptors in guinea-pig still compares favourably with the non-competitive blocking activity of suramin (pK_b, 5.3 ± 0.2), iso-PPADS (pK_b, 6.6 ± 0.2) and Reactive blue 2 (pK_b, 5.8 ± 0.2) at P2X$_1$-like receptors in rat vas deferens (Khakh *et al*., 1994). To this end, Ip_5I may yet prove to be a useful pharmacological tool in bioassays of naturally-occurring P2X$_1$ receptors, for example human HL60 cells (Buell *et al*., 1996) and murine thymocytes (Chavtcho *et al*., 1996) as well as P2X$_1$-like receptors in vas deferens and vascular smooth muscle of various mammalian species (Humphrey *et al*., 1998).

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References

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

BUELL, G., MICHEL, A.D., LEWIS, C., COLLO, G., HUMPHREY, P.P. & SURPRENANT, A. (1996). P2X$_1$ receptor activation in HL60 cells. *Blood,* **87,** 2659–2664.

CHAVTCHO, Y., VALERA, S., AUBRY, J.P., RENNO, T., BUELL, G. & BONNEFOY, J.Y. (1996). The involvement of an ATP-gated ion channel, P(2X1), in thymocyte apoptosis. *Immunity,* **5,** 275–283.

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.

EVANS, R.J., LEWIS, C., BUELL, G., VALERA, S., NORTH, R.A. & SURPRENANT, A. (1995). Pharmacological characterization of heterolously expressed ATP-gated cation channels (P_{2X} purinoceptors). *Mol. Pharmacol.,* **48,** 178–183.

EVANS, R.J. & SURPRENANT, A. (1996). P2X receptors in autonomic and sensory neurons. *Semin. Neurosci.,* **8,** 217–223.

EVANS, R.J., SURPRENANT, A. & NORTH, R.A. (1998). P2X receptors: Cloned and Expressed. In: Turner J.T., Weisman G.A. & Fedan J.S. (eds). *The P2 Nucleotide Receptors*. Humana Press, New Jersey, Ch. 2, pp 43–62.

GARCIA-LECEA, M., DELICADO, E.G., MiRAS-PORTUGAL, M.T. & CASTRO, E. (1999). P2X$_2$ characteristics of the ATP receptor coupled to $[Ca^{2+}]_i$ increases in cultured Purkinje neurons from neonatal rat cerebellum. *Neuropharmacol.,* **38,** 699–706.

GURANOWSKI, A.E., STARZYNSKA, E., GÜNTHER SILLERO, M.A. & SILLERO, A. (1995). Conversion of adenosine(5′)oligophospho(5′)adenosines into inosine(5′)oligophospho(5′)inosines by non-specific adenylate deaminase from the snail *Helix pomatia*. *Biochim. Biophys. Acta,* **1243,** 78–84.

HOYLE, C.H.V., PINTOR, J., GUALIX, J. & MIRAS-PORTUGAL, M.T. (1997). Antagonism of P2X receptors in guinea-pig vas deferens by diinosine pentaphosphate. *Eur. J. Pharmacol.,* **333,** R1–R2.

HUMPHREY, P.P.A., KHAKH, B.S., KENNEDY, C., KING, B.F. & BURNSTOCK, G. (1998). Nucleotide receptors: P2X receptors. In: *IUPHAR Compendium of Receptor Characterization and Classification*. IUPHAR Media Publications, pp 195–208.

HUMPHREYS, B.D., VIRGINIO, C., SURPRENANT, A., RICE, J. & DUBYAK, G.R. (1998). Isoquinolines as antagonists of the P2X$_7$ nucleotide receptor: high selectivity for the human versus rat receptor homologues. *Mol. Pharmacol.,* **54,** 22–32.

JACOBSON, K.A., KIM, Y.C., WILDMAN, S.S., MOHANRAM, A., HARDEN, K., BOYER, J.L., KING, B.F. & BURNSTOCK, G. (1998). A pyridoxine cyclic phosphate and its 6-azoaryl-derivative selectively potentiate and antagonize activation of P2X$_1$ receptors. *J. Med. Chem.,* **41,** 2201–2206.

KHAKH, B.S., MICHEL, A. & HUMPHREY, P.P.A. (1994). Estimates of antagonist affinities at P_{2X} purinoceptors in rat vas deferens. *Eur. J. Pharmacol.,* **263,** 301–309.

KIM, M., YOO, O.J. & CHOE, S. (1997). Molecular assembly of the extracellular domain of P2X$_2$, an ATP-gated ion channel. *Biochem. Biophys. Res. Comm.,* **240,** 618–622.

KING, B.F. (1998). Molecular Biology of P2X Purinoceptors. In: Burnstock G., Dobson J.G., Liang B.T. & Linden J. (eds). *Cardiovascular Biology of Purines*. Kluwer Academic Publications, Massachusetts, Ch. 10, pp 159–186.

KING, B.F., ZIGANSHINA, L.E., PINTOR, J. & BURNSTOCK, G. (1996). Full sensitivity of P2X$_2$ purinoceptor to ATP revealed by changing extracellular pH. *Br. J. Pharmacol.,* **117,** 1371–1373.

KUPITZ, Y. & ATLAS, D. (1993). A putative ATP-activated Na^+ channel involved in sperm-induced fertilization. *Science,* **261,** 484–486.

NICKE, A., BÄUMERT, H.G., RETTINGER, J., EICHELE, A., LAMBRECHT, G., MUTSCHLER, E. & SCHMALZING, G. (1998). P2X$_1$ and P2X$_3$ receptors form stable trimers: a novel structural motif of ligand-gated ion channels. *EMBO J.,* **17,** 3016–3028.

PINTOR, J., DÍAZ-HERNÁNDEZ, M., BUSTAMANTE, C., GUALIX, J., DE TERREROS, F.J. & MIRAS-PORTUGAL, M.T. (1999). Presence of dinucleotide and ATP receptors in human cerebrocortical synaptic terminals. *Eur. J. Pharmacol.,* **366,** 159–165.

PINTOR, J., GUALIX, J. & MIRAS-PORTUGAL, M.T. (1997). Diinosine polyphosphates, a group of dinucleotides with antagonistic effects on diadenosine polyphosphate receptor. *Mol. Pharmacol.,* **51,** 277–284.

PINTOR, J., KING, B.F., MIRAS-PORTUGAL, M.T. & BURNSTOCK, G. (1996). Selectivity and activity of adenine dinucleotides at recombinant P2X$_2$ and P2Y$_1$ receptors. *Br. J. Pharmacol.,* **119,** 1006–1012.

RAE, M.G., ROWAN, E.G. & KENNEDY, C. (1998). Pharmacological properties of P2X$_3$-receptors present in neurones of the rat dorsal root ganglia. *Br. J. Pharmacol.,* **124,** 176–180.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413–492.

SOTO, F., LAMBRECHT, G., NICKEL, P., STUHMER, W. & BUSCH, A.E. (1999). Antagonistic properties of the suramin analogue NF023 at heterologously expressed P2X receptors. *Neuropharmacol.,* **38,** 141–149.

TORRES, G.E., EGAN, T.M. & VOIGT, M.M. (1999). Hetero-oligomeric assembly of P2X receptor subunits. *J. Biol. Chem.,* **274,** 6653–6659.

TOWNSEND-NICHOLSON, A., KING, B.F., WILDMAN, S.S. & BURNSTOCK, G. (1999). Molecular cloning, functional characterization and possible cooperativity between the murine P2X$_4$ and P2X$_{4a}$ receptors. *Mol. Brain. Res.,* **64,** 246–254.

VIRGINIO, C., ROBERTSON, G., SURPRENANT, A. & NORTH, R.A. (1998). Trinitrophenyl-substituted nucleotides are potent antagonists selective for P2X$_1$, P2X$_3$, and heteromeric P2X receptors. *Mol. Pharmacol.,* **53,** 969–973.

WILDMAN, S.S., BROWN, S.G., KING, B.F. & BURNSTOCK, G. (1999a). Selectivity of diadenosine polyphosphates for rat P2X receptor subunits. *Eur. J. Pharmacol.,* **367,** 119–123.

WILDMAN, S.S., KING, B.F. & BURNSTOCK, G. (1999b). Modulation of ATP-responses at recombinant rP2X$_4$ receptors by extracellular pH and Zinc. *Br. J. Pharmacol.,* **126,** 762–768.

WILDMAN, S.S., KING, B.F. & BURNSTOCK, G. (1999c). Modulatory activity of extracellular H^+ and Zn^{2+} on ATP-responses at rP2X$_1$ and rP2X$_3$ receptors. *Br. J. Pharmacol.,* **128,** 486–492.

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