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Selectivity of diadenosine polyphosphates for rat P2X receptor subunits

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Abstract

The pharmacological activity of diadenosine polyphosphates was investigated at three recombinant P2X receptors (rat $P2X_1$, rat $P2X_3$, rat $P2X_4$) expressed in *Xenopus* oocytes and studied under voltage-clamp conditions. For the rat $P2X_1$ receptor, only P^1,P^6-diadenosine hexaphosphate (Ap_6A) was a full agonist yet 2–3 folds less potent than ATP. At rat $P2X_3$, P^1,P^4-diadenosine tetraphosphate (Ap_4A), P^1,P^5-diadenosine pentaphosphate (Ap_5A) and Ap_6A were full agonists and more potent than ATP. Ap_4A alone was equipotent with ATP at rat $P2X_4$, but only as a partial agonist. Compared to known data for rat $P2X_2$ and human $P2X_1$ receptors, our findings contrast with rat $P2X_2$ where only Ap_4A is a full agonist although four folds less potent than ATP. At rat and human orthologues of $P2X_1$, Ap_5A was a partial agonist with similar potency. These data provide a useful basis for selective agonists of P2X receptor subunits.

Keywords: Adenine dinucleotide; Diadenosine polyphosphate; P2X receptor; ATP; *Xenopus* oocyte

1. Introduction

Diadenosine polyphosphates (Ap_nA, $n = 2–6$) are naturally-occurring adenine dinucleotides, where two adenine molecules are linked at the 5′ position of their ribose moieties by a chain of phosphates varying from 2 to 6 in length. These adenine dinucleotides possess both intracellular and extracellular actions, are concentrated in central synaptosomes, and are released in a Ca^{2+}-dependent process from brain slices after nerve stimulation (for a review, see Pintor et al., 1997).

Extracellular diadenosine polyphosphates bind to and activate P2X and P2Y receptor subtypes for ATP in a number of mammalian tissues and with a variety of effects (Hoyle, 1990; Abbracchio and Burnstock, 1994; Pintor and Miras-Portugal, 1995; Ogilvie et al., 1996). For the P2X receptor family, seven subtypes ($P2X_{1-7}$) have been cloned thus far (North and Barnard, 1997), although the pharmacological activity of the diadenosine polyphosphate family has only been studied at human $P2X_1$ ($hP2X_1$) receptors expressed in *Xenopus* oocytes and human embryonic kidney (HEK 293) cells (Evans et al., 1995) and at rat $P2X_2$ ($rP2X_2$) receptors expressed in *Xenopus* oocytes (Pintor et al., 1996).

In the present study, we have extended this pharmacological survey of adenine dinucleotide activity to include three recombinant P2X receptors from rat tissue ($rP2X_1$, $rP2X_3$ and $rP2X_4$) and have commented on their agonist and modulatory activities to reveal an emerging picture of selectivity for diadenosine polyphosphates at P2X subunits.

2. Materials and methods

2.1. Electrophysiology

Xenopus oocytes were harvested and prepared, as previously described (King et al., 1996, 1997). Defolliculated oocytes were injected cytosolically with capped ribose nucleic acid (cRNA) encoding either rat $P2X_1$ (Valera et al., 1995), rat $P2X_3$ (Chen et al., 1995) or rat $P2X_4$ receptors (Bo et al., 1995). RNA-injected oocytes and sham-injected control oocytes were incubated for 48 h at 18°C in Barth's solution and kept for 5 to 10 days at 4°C until used in electrophysiological experiments. ATP-activated inward-currents (I_{ATP}) were recorded from RNA-injected oocytes using a twin-electrode voltage-clamp amplifier (Axoclamp 2B; holding potential (V_h) = −60 to

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−90 mV). Control oocytes did not respond to either ATP nor adenine dinucleotides (the Ap_nA series). Voltage-recording and current-recording microelectrodes (1–5 MΩ tip resistance) were filled with 3.0 M KCl. Oocytes were placed in a Perspex recording chamber and superfused with modified Ringer's solution containing (mM) NaCl, 110; KCl, 2.5; HEPES, 5; $BaCl_2$, 1.8, adjusted to pH 7.5.

2.2. Drug solutions

All solutions were made up in modified Ringer's solution which was nominally Ca^{2+}-free. Omission of extracellular Ca^{2+} prevented the activation of Ca^{2+}-dependent Cl^--channels in *Xenopus* oocytes secondary to Ca^{2+} influx through ATP-activated ion-channels. ATP and adenine dinucleotides (the Ap_nA series) were prepared in Ca^{2+}-free Ringer's solution (at the concentrations stated in the text). Agonists were added for 120 s or until membrane currents peaked, then washed out for a period of 20 min. This extended washout period was necessary to ensure successive ATP-responses (applied at the EC_{50} value) were of constant amplitude. Adenine dinucleotides, when used as modulators, were superfused for 20 min prior to, and during, superfusion of submaximal concentrations of ATP ($rP2X_1$, 0.1 μM; $rP2X_3$, 0.3 μM; $rP2X_4$, 3 μM). The pH of the Ringer's solution, and all drugs used, was routinely adjusted to pH 7.5, by adding either 1.0 N HCl or 1.0 N NaOH, since agonist activity at P2X receptors is sensitive to changes in extracellular pH (King et al., 1996, 1997; Stoop et al., 1997; Wildman et al., 1998).

2.3. Statistics

Data are presented as mean ± SEM of four sets of data from different oocyte batches. Significant differences were determined by Student's *t*-test, using a commercial software package (Instat, v2.05A; GraphPad).

2.4. Chemicals

All common salts were AnalaR grade (BDH, UK). ATP disodium salt was purchased from Boehringer Mannheim (Germany) while all adenine dinucleotides (Ap_3A, Ap_4A and Ap_6A ammonium salts, Ap_2A and Ap_5A sodium salts) were purchased from Sigma (Poole, Dorset UK). We have commented previously on the purity of commercially-prepared diadenosine polyphosphates and found little contamination (< 1%) with ATP (Pintor et al., 1996).

3. Results

3.1. Agonist activity of adenine dinucleotides at rat $P2X_1$ receptor

ATP (0.01–30 μM) evoked inward membrane currents in defolliculated *Xenopus* oocytes expressing $rP2X_1$ receptors (EC_{50} value, 0.30 ± 0.01 μM; Hill coefficient (n_H), 1.5 ± 0.1; $n = 4$). Of the dinucleotide series tested, only Ap_6A was a full agonist (EC_{50}, 0.72 ± 0.08 μM; n_H, 1.2 ± 0.2; $n = 4$) yet 2–3 fold less potent than ATP. Both Ap_4A and Ap_5A were partial agonists with maximal responses as low as 40% of the maximal ATP effect (Fig. 1A). Ap_4A (EC_{50}, 38 ± 11 nM; n_H, 1.2 ± 0.1; $n = 4$) was eight fold more potent than ATP, while Ap_5A (EC_{50},

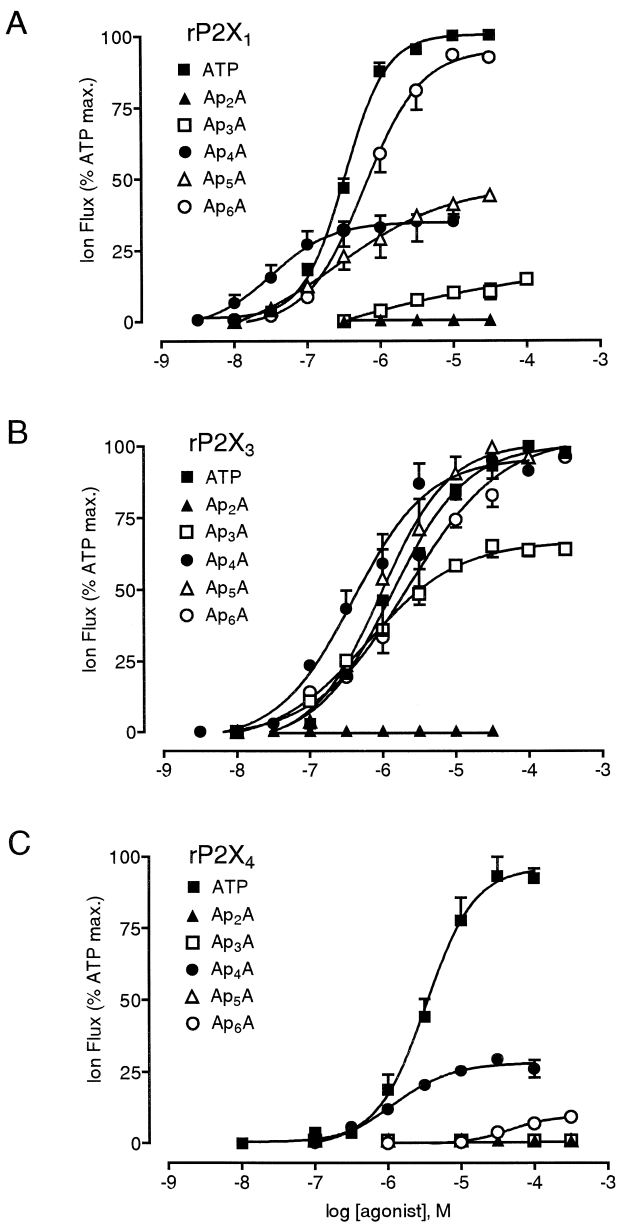

Fig. 1. Adenine dinucleotide activity at rat $P2X_1$, $P2X_3$ and $P2X_4$ receptors. Concentration–responses curves for ATP and the adenine dinucleotide series (Ap_nA, $n = 2$–6) at $P2X_1$ (A), $P2X_3$ (B) and $P2X_4$ (C). Agonist activity was normalized to the maximal response to ATP in each experiment. Agonist potency was determined as the EC_{50} value for each curve, for four determinations per agonist. EC_{50} values are given in Table 1 and in the text. Hill coefficients for agonists are also given in the text. Curves were fitted using the Hill equation, as defined by Prism v2.0 (GraphPad).

0.90 ± 0.1 μM; n_H, 1.0 ± 0.1; $n = 4$) was 2–3 fold less potent. P^1,P^3-diadenosine triphosphate (Ap_3A; 0.3–100 μM) showed weak agonist activity and, at the highest concentrations used, elicited inward currents as low as 10% of the maximal ATP effect. P^1,P^2-diadenosine pyrophosphate (Ap_2A; 0.1–30 μM) was inactive as an agonist and, furthermore, neither antagonized nor potentiated ATP-responses.

3.2. Agonist activity of adenine dinucleotides at rat $P2X_3$ receptor

ATP (0.01–100 μM) evoked inward membrane currents in oocytes expressing $rP2X_3$ receptors (EC_{50}, 1.8 ± 0.3 μM; n_H, 0.7 ± 0.05; $n = 4$). Three dinucleotides were full agonists and more potent than ATP: Ap_4A (EC_{50}, 0.80 ± 0.12 μM; n_H, 0.9 ± 0.1; $n = 4$); Ap_5A (EC_{50}, 1.3 ± 0.3 μM, n_H, 0.7 ± 0.1; $n = 4$); Ap_6A (1.6 ± 0.4 μM; n_H, 0.8 ± 0.1; $n = 4$). Ap_3A was a partial agonist with maximal responses as low as 60% of the maximal ATP effect (Fig. 1B), although this dinucleotide (EC_{50}, 1.0 ± 0.5 μM; n_H, 0.8 ± 0.1; $n = 4$) was more potent than ATP. Ap_2A (0.1–30 μM) was inactive as an agonist. However, Ap_2A (100 μM) caused a modest potentiation (175 ± 26%) of control responses (taken as 100%) to submaximal concentrations of ATP (0.3 μM, approximately EC_{25}), and this effect was reversed on washout. The EC_{50} value for this potentiation was 8.3 ± 0.7 μM ($n = 4$).

3.3. Agonist activity of adenine dinucleotides at rat $P2X_4$ receptor

ATP (0.1–100 μM) evoked inward membrane currents in oocytes expressing $rP2X_4$ receptors (EC_{50}, 4.1 ± 1.0 μM; n_H, 1.2 ± 0.1; $n = 4$). Ap_4A was as potent as ATP at $rP2X_4$ (EC_{50}, 3.0 ± 0.4 μM; n_H, 1.1 ± 0.2; $n = 4$), although a partial agonist with maximal responses as low as 30% of the maximal ATP effect (Fig. 1C). Ap_6A (30–300 μM) was much less active than either ATP or Ap_4A, evoking maximal responses as low as 10% of the maximal ATP effect ($n = 4$). Ap_2A, Ap_3A and Ap_5A (10–300 μM) were inactive as agonists. However, Ap_2A and Ap_3A (1–100 μM) potentiated ATP-activated currents at $rP2X_4$ receptors in a concentration-dependent manner. ATP-responses (to 3 μM, approximately EC_{40}) were maximally increased by 146 ± 7% (Ap_2A: EC_{50}, 1.6 ± 0.8 μM) and 154 ± 13% (Ap_3A: EC_{50}, 0.93 ± 0.12 μM). The potentiating effects of Ap_2A and Ap_3A were reversed on washout. Ap_5A (1–30 μM) was inactive, either as a modulator or an antagonist, against ATP-activated currents.

4. Discussion

In the present study, we found that the diadenosine polyphosphate series (Ap_nA, $n = 2–6$) showed different patterns of pharmacological activity at three rat P2X receptors ($rP2X_1$, $rP2X_3$, $rP2X_4$). These data, when compared to like studies of human $P2X_1$ (Evans et al., 1995) and rat $P2X_2$ receptors (Pintor et al., 1996), reveal agonist selectivity for dinucleotides at the $P2X_{1-4}$ subunits (see Table 1).

Only Ap_6A was a full agonist at $rP2X_1$, although the potency order of dinucleotides showing agonist properties was $Ap_4A > ATP > Ap_6A = Ap_5A$ (on the basis of EC_{50} values). Ap_5A and Ap_4A were partial agonists at $rP2X_1$. Ap_5A is also a partial agonist at the human orthologue ($hP2X_1$) (Evans et al., 1995), although its potency is similar at the rat (EC_{50}, 0.9 μM) and human (EC_{50}, 0.8 μM) orthologues of the $P2X_1$ receptor. Four dinucleotides were as potent as ATP, or more so, at $rP2X_3$ with a potency order $Ap_4A \geq Ap_3A > Ap_5A = Ap_6A \geq ATP$ (based on EC_{50} values). However, Ap_3A was a partial agonist while the other three dinucleotides were full agonists. Ap_4A and Ap_6A activated the $rP2X_4$ receptor with a potency order $Ap_4A \geq ATP \gg Ap_6A$ (based on EC_{50} values), although neither dinucleotide was a full agonist. Ap_4A gave about 30% of the maximal activity of ATP, while Ap_6A was weaker (10% maximal activity). The results for $rP2X_1$, $rP2X_3$ and $rP2X_4$ contrast with known data for $rP2X_2$, where Ap_4A is the only dinucleotide to activate this P2X subunit and is less potent than ATP (Pintor et al., 1996). However, a consistent finding with all

Table 1
Agonist activity of ATP and adenine dinucleotides at rat $P2X_{1-4}$ receptors

	$rP2X_1$	$rP2X_2$	$rP2X_3$	$rP2X_4$
ATP	full agonist (0.30 ± 0.01 μM)	full agonist (3.7 ± 0.7 μM)	full agonist (1.8 ± 0.3 μM)	full agonist (4.1 ± 1.0 μM)
Ap_2A	inactive (0.1–30 μM)	inactive (1–100 μM)	inactive (0.1–30 μM)	inactive (0.1–30 μM)
Ap_3A	partial agonist (EC_{50} > 100 μM)	inactive (1–100 μM)	partial agonist (1.0 ± 0.5 μM)	inactive (0.1–30 μM)
Ap_4A	partial agonist (0.04 ± 0.01 μM)	full agonist (15.2 ± 1.0 μM)	full agonist (0.80 ± 0.12 μM)	partial agonist (3.0 ± 0.4 μM)
Ap_5A	partial agonist (0.9 ± 0.1 μM)	inactive (0.1–100 μM)	full agonist (1.3 ± 0.3 μM)	inactive (0.1–30 μM)
Ap_6A	full agonist (0.72 ± 0.08 μM)	inactive (0.1–100 μM)	full agonist (1.6 ± 0.4 μM)	partial agonist (EC_{50} > 100 μM)

Agonist activity and potency of ATP and the adenine dinucleotide series (Ap_nA, $n = 2–6$) at homomeric rat $P2X_1$–$P2X_4$ receptors expressed in *Xenopus* oocytes. Potency indices are expressed as EC_{50} values (mean ± SEM; $n = 4$). Dinucleotides were full agonists if they matched the maximal activity of ATP; partial agonists failed to do so. Data for rat $P2X_2$ taken from the work of Pintor et al. (1996). Ap_5A is a partial agonist (~ 50% of maximal ATP activity, with an EC_{50} value of 0.8 μM) at human $P2X_1$ receptors (Evans et al., 1995).

four P2X subunits was an inability by P^1,P^2-diadenosine pyrophosphate (Ap_2A) to act as an agonist. $P2X_{1-4}$ subunits are relatively insensitive to ADP and related adenine diphosphates (e.g., adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS) and 2-methylthio ADP (2-MeSADP)) compared to ATP and related adenine triphosphates (e.g., adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) and 2-methylthio ATP (2-MeSATP) (King, 1998), and this trend appears to hold true for the diphosphate represented by Ap_2A.

The combined results shown in Table 1 reveal the potential to discriminate between certain P2X subunits on the basis of the activity and potency of diadenosine polyphosphates relative to ATP. Ap_6A is a full agonist at both fast desensitizing P2X subunits ($P2X_1$ and $P2X_3$), but $rP2X_3$ is also activated fully by Ap_4A and Ap_5A while $rP2X_1$ is not. Also, Ap_3A is a potent agonist at $rP2X_3$ but relatively inactive at $rP2X_1$. For the slowly-desensitizing P2X subunits ($P2X_2$ and $P2X_4$), Ap_4A is a full agonist at $rP2X_2$ but not at $rP2X_4$. These two slowly-desensitizing P2X subunits are found throughout the peripheral and central nervous systems (Vulchanova et al., 1996; Lê et al., 1998) and their different pharmacological profiles to dinucleotides may provide the means to identify these functional P2X subunits in native P2X receptors of neural tissues.

Transcripts for $rP2X_1$, $rP2X_2$ and $rP2X_4$ co-localise in blood vessels (Nori et al., 1998). Potentially, the relative activities of Ap_6A and Ap_4A at these three P2X subunits ($rP2X_1$, $rP2X_2$, $rP2X_4$) could help reveal the presence of homomeric and heteromeric P2X receptor subtypes in different vascular beds. Transcripts for $rP2X_2$ and $rP2X_4$ also co-localise with $rP2X_3$ in neurons of sensory ganglia (Collo et al., 1996). The relative activities of diadenosine polyphosphates at native P2X receptors in rat dorsal root ganglia (DRG) is of some interest, since it is believed that the pharmacological and biophysical profiles of the P2X receptors on sensory neurons change with age. The P2X receptor in neonatal rat DRG tissue appears to show a $P2X_3$ phenotype (Robertson et al., 1996) whereas, in older DRG, the P2X receptor appears to change its phenotype to that of $P2X_{2/3}$ heteromultimers (Evans and Surprenant, 1996). Ap_5A (EC_{50}, 3.2 μM) is a full agonist, but Ap_4A (EC_{50}, 5.2 μM) a partial agonist, at the P2X receptor in neonatal rat DRG (Rae et al., 1998). In adult rat nodose ganglia, where the P2X receptor is thought to be a heteromultimeric assembly of $P2X_2$ and $P2X_3$ (Lewis et al., 1995), Ap_4A is a weak partial agonist while Ap_2A, Ap_3A and Ap_5A are considered to be antagonists (Krishtal et al., 1988). Neither of the above pharmacological profiles matched the agonist activities of dinucleotides at the recombinant $rP2X_3$ receptor (see Table 1).

We also found that several adenine dinucleotides devoid of agonist activity were modulators of ATP-activity at P2X subunits. Ap_2A was not an agonist at the $rP2X_3$ receptor but, instead, reversibly potentiated ATP-responses. Similarly, Ap_2A and Ap_3A were not agonists at $rP2X_4$ yet reversibly potentiated ATP-activated currents at this subtype. These modulatory actions are reminiscent of the actions of Ap_5A which selectively potentiates ATP-responses at $rP2X_2$ receptors by shifting the concentration–response curve for the agonist leftwards (Pintor et al., 1996). In the present study, Ap_2A and Ap_3A caused a similar displacement of ATP concentration–response curves without altering the maximum ATP effect. The degree of Ap_2A potentiation at $rP2X_3$ and $rP2X_4$ receptors was modest, increasing the amplitude of ATP-responses by 1–2 fold. Ap_5A potentiation at $rP2X_2$ receptor was modest also, showing a 1–2 fold increase in agonist activity (Pintor et al., 1996). Recently, Ap_5A has been shown to potentiate ATP-responses in rat cerebellar astrocytes, although such ATP-responses appear to be mediated by a metabotropic ATP receptor (Jimenez et al., 1998). If true, these data indicate that some diadenosine polyphosphates have the capacity to potentiate ATP responses at both P2X (ionotropic) and P2Y (metabotropic) receptor subtypes.

5. Conclusion

In conclusion, it is evident that there is some selectivity in the actions of the adenine dinucleotides for those P2X subunits tested thus far ($hP2X_1$, $rP2X_1$, $rP2X_2$, $rP2X_3$ and $rP2X_4$ receptors). A picture is emerging that these P2X subunits are affected by certain adenine dinucleotides in different ways. Of the dinucleotides tested, Ap_6A seems the best choice as an agonist for $rP2X_1$ although Ap_6A will also fully activate $rP2X_3$. However, Ap_5A and Ap_4A are full agonists at $rP2X_3$ but not $rP2X_1$ and, accordingly, they can help discriminate between $rP2X_3$ and $rP2X_1$. Ap_4A is the best agonist for $P2X_2$ although it will also fully activate $P2X_3$ and partially activate $P2X_1$. However, all agonist responses at $P2X_2$ are enhanced under acidic conditions, whereas they are either unaffected or reduced under similar conditions at $P2X_3$ and $P2X_1$ (King et al., 1996, 1997; Stoop et al., 1997; Wildman et al., 1998). Also, $P2X_2$ is a slow desensitizing receptor while the kinetics of activation and inactivation are faster for $P2X_3$ and $P2X_1$ (King, 1998). $P2X_3$ is identified by full agonist activity of Ap_4A, Ap_5A and Ap_6A and by Ap_2A potentiation of ATP-responses at this fast desensitizing receptor. $rP2X_4$ is the most difficult to identify by the actions of dinucleotides, but potentiation of ATP-responses by Ap_2A and Ap_3A appears to be signatory. However, $rP2X_4$ is also marked by its insensitivity to blockade by suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (for reviews, see North and Barnard, 1997; King, 1998). Thus, adenine dinucleotides may prove to be useful tools in identifying P2X subtypes in endogenous P2X receptors in whole tissues when, currently, there is a lack of selective agonists and antagonists for these ligand-gated ion channels.

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