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Activity of diadenosine polyphosphates at P2Y receptors stably expressed in 1321N1 cells

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Abstract

The selectivities of the diadenosine polyphosphates (Ap_nAs, $n = 2–6$) at the human $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors stably expressed in 1321N1 human astrocytoma cells was determined using a Fluorescence Imaging Plate Reader (FLIPR) to measure intracellular Ca^{2+} mobilisation. The rank order of agonist potencies at $P2Y_1$ were: ADP > P^1,P^3-diadenosine triphosphate (Ap_3A) > P^1,P^3-diadenosine hexaphosphate (Ap_6A) = P^1,P^3-diadenosine diphosphate (Ap_2A) ≫ P^1,P^3-diadenosine pentaphosphate (Ap_5A). P^1,P^3-diadenosine tetraphosphate (Ap_4A) was inactive up to 1 mM. The rank order of agonist potencies at $P2Y_2$ were: UTP > Ap_4A ≫ Ap_6A > Ap_5A > Ap_3A ≫ Ap_2A. The Ap_4A concentration response curve appeared to be bi-phasic. At $P2Y_4$ all the Ap_nAs tested were inactive as agonists. At $P2Y_6$, only Ap_3A and Ap_5A showed significant agonist activity. At $P2Y_{11}$, only Ap_4A showed significant agonist activity. Ap_nAs were inactive as antagonists of the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors. At $P2Y_4$, however, the Ap_nAs potentiated the UTP response.

Keywords: Diadenosine polyphosphate; P2Y receptor; FLIPR; G-protein coupled receptor

1. Introduction

Diadenosine polyphosphates (Ap_nAs, where $n = 2–7$) belong to a group of dinucleoside polyphosphate molecules. They are derived from ATP and consist of two adenosine molecules linked by two to seven phosphate groups (Baxi and Vishwanatha, 1995). P^1,P^3-diadenosine diphosphate (Ap_2A), P^1,P^3-diadenosine triphosphate (Ap_3A), P^1,P^3-diadenosine tetraphosphate (Ap_4A), P^1,P^3-diadenosine pentaphosphate (Ap_5A), P^1,P^3-diadenosine hexaphosphate (Ap_6A) and P^1,P^3-diadenosine heptaphosphate Ap_7A are all naturally occurring (Jankowski et al., 1999, 2001; Flores et al., 1999).

As well as having intracellular activities, Ap_nAs are thought to be endogenous regulator molecules of P2 receptors (Kisselev et al., 1998) activating both P2Y and P2X receptors. Ap_nAs have been shown to co-localise with ATP and acetylcholine in secretory granules of nerve terminals (Miras-Portugal et al., 1998).

Using heterologously expressed receptors, agonist activity of Ap_3A has been reported at the chick $P2Y_1$ (Pintor et al., 1996), human $P2Y_2$ (Lazarowski et al., 1995) and rat $P2X_1$ and $P2X_3$ receptors (Wildman et al., 1999). Ap_4A has been reported as an agonist at the chick and human $P2Y_1$ (Pintor et al., 1996; Schachter et al., 1996), human $P2Y_2$ (Lazarowski et al., 1995) and rat $P2Y_4$ (Bogdanov et al., 1998), $P2X_2$ (Pintor et al., 1996), $P2X_1$, $P2X_3$, and $P2X_4$ receptors (Wildman et al., 1999).

Ap_5A has agonist activity at rat $P2X_1$ and $P2X_3$ receptors (Wildman et al., 1999) and Ap_6A at rat $P2X_1$, $P2X_3$ and $P2X_4$ receptors (Wildman et al., 1999). Ap_2A is less well studied and Ap_7A has only recently been discovered (Jankowski et al., 1999).

The activity of the Ap_nAs at endogenous receptors expressed in cell lines or isolated tissue preparations is less clear. Results from these experiments are conflicting and can be influenced by: contamination of commercially available Ap_nAs with mononucleotides (Conant et al., 1998); incorrect identification of P2 receptor subtypes

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(Vigne et al., 2000); Ap_nA activity at unidentified novel receptors (Pintor and Miras-Portugal, 2000); and pharmacological differences between orthologues (Kennedy et al., 2000; Wildman et al., 1999).

The pharmacological profiles of ligands at an individual receptor may differ depending on the readout used. This has been seen with the human $P2Y_4$ receptor where production of inositol phosphate shows ATP to be a full agonist with low potency (Nicolas et al., 1996) or a partial agonist (Communi et al., 1995, 1996) relative to UTP. Measurements of intracellular Ca^{2+} mobilisation show ATP to be inactive (Kennedy et al., 2000). Furthermore, different pharmacological profiles of various ligands have been obtained for the same receptor using different expression systems. This has been seen with the $CysLT_2$ receptor where the rank order of ligand potencies for this receptor changes when it is expressed in either *Xenopus laevis* oocytes or human embryonic kidney cells (Heise et al., 2000).

To attempt to circumvent the issues highlighted above, the Ap_nAs have been profiled against five cloned P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$) using a single functional readout, allowing for a more direct comparison of the activity of the Ap_nAs at P2Y receptors. Receptors were stably expressed in 1321N1 human astrocytoma cells and diadenosine polyphosphate-induced increases in cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$) were measured using a Fluorescence Imaging Plate Reader (FLIPR).

2. Material and methods

2.1. Materials

ADP, ATP, UDP, UTP, carbachol, α,β-methyleneATP (α,β-meATP), β,γ-methyleneATP (β,γ-meATP), Ap_2A, Ap_3A, Ap_4A, Ap_5A, Ap_6A, probenicid, and FLUO 3-AM (acetoxymethyl ester derivative of FLUO 3-AM) were purchased from Sigma-Aldrich (Dorset, UK). 2-MethylthioATP (2MeSATP) was purchased from Tocris Cookson (Bristol, UK).

2.2. Cloning of P2Y receptors

The polymerase chain reaction (PCR) was employed to amplify the coding sequences of human $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ from human genomic DNA (Promega, UK), using primers derived from respective Genbank™ accession numbers U42029, U07225, X91852, X97058 and AF030335. For $P2Y_{11}$, the starting codon was added during the PCR amplification. The amplified products were ligated into pCRscript vector (Stratagene, UK) and sequenced.

For mammalian cell expression, the receptors were ligated into either pCIN or pCIH vectors (Rees et al., 1996).

2.3. Expression of P2Y receptors in 1321N1 cells

1321N1 cells were grown in monolayer culture at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 2 mM glutamine and 0.44% glucose. Cells were transfected with 5 μg of DNA using lipofectamine (Life Technologies, UK). Neomycin ($P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$)- or hygromycin ($P2Y_2$)-resistant colonies were selected after 2 weeks using 500 μg ml^{-1} neomycin and 200 μg ml^{-1} hygromycin, respectively (Life Technologies).

2.4. Ca^{2+} mobilisation assay

Cells were plated into black walled, clear bottom, tissue culture-coated 96-well plates 3 days before use in FLIPR. Cells were incubated with 4 μM FLUO-3AM, at 37 °C in 5% CO_2 for 90 min and then washed once in Tyrodes buffer (145 mM NaCl, 10 mM glucose, 2.5 mM KCl, 1.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4) containing 2.5 mM probenicid. Basal cell fluorescence (11,000–15,000 FIU) was determined prior to drug additions. Cell fluorescence was monitored ($\lambda_{Ex} = 488$, $\lambda_{Em} = 540$ nm) immediately in the FLIPR following exposure to increasing concentration of agonist. For antagonist studies, the Ap_nAs were tested at concentrations at which they were shown to be inactive as agonists. This was to ensure that any antagonism observed was not due to depletion of Ca^{2+} from intracellular stores. Cells were pre-incubated with Ap_nAs and cell fluorescence measured for 4 min before addition of agonist.

2.5. Data analysis

For each response the peak increase above basal levels in fluorescence was calculated and iteratively curve-fitted using the ALLFIT model (De Lean et al., 1977). Results were expressed as a percentage of the peak response to the most potent naturally occurring nucleotide at the respective receptors studied. Data were expressed as mean ± S.E.M. or geometric mean with 95% confidence limits for EC_{50} values, from three or more experiments performed in duplicate. The data were considered statistically different at $P \leq 0.01$ using a one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test.

2.6. HPLC procedure

The purity of commercially available nucleotides and Ap_nAs was determined using high performance liquid chromatography (HPLC) techniques. A Dionex (Camberley, UK) PAX-500 4.6 mm i.d × 5 cm HPLC column was used to analyse 10 mM stock solutions of UDP, UTP, ADP, ATP, Ap_2A, Ap_3A, Ap_4A, Ap_5A and Ap_6A. A gradient analysis was performed using the following sol-

vents: solvent A, 2% MeCN; and solvent B, 30% MeCN:1 M Ammonium formate; pH 7.1 (natural pH) (700 mM overall). The flow rate was 2 ml min^{-1} with a 20:1 split, after UV detection $\lambda = 263$ nm, into the mass spectrometer. Each sample was diluted 1:10 with distilled water to give a concentration of 1 mM; 10 μl of each diluted sample was injected on to the HPLC column. The molecular weight of each sample and any related impurities were confirmed in the mass spectrometer. The purity of each sample was determined by integration of the UV signal.

3. Results

3.1. Purity of commercially available nucleotides and diadenosine polyphosphates

UDP and ADP were found to contain approximately 7% and 3% of UMP and AMP (based on peak areas in the UV signal), respectively. No allowance was made for the different UV extinction coefficients of samples (which tends to increase with the lower degree of phosphate substitution), hence, the impurity values are likely to be slightly overestimated. A more detailed analysis was per-

Table 1
Potency of nucleotides and Ap_nAs at $P2Y_1$ and $P2Y_2$ receptors for increasing $[Ca^{2+}]_i$

Compound	$P2Y_1$	n_H	$P2Y_2$	n_H
ADP	7.7 ± 0.08	1.5	–	
UTP			7.1 ± 0.07	1.4
Ap_2A	4.9 ± 0.11	1.2	N.E.	
Ap_3A	7.5 ± 0.03	1.2	4.4 ± 0.05	1.4
Ap_4A	N.E.		6.7 ± 0.06[a]	1.5
Ap_5A	< 4		4.6 ± 0.07	1.2
Ap_6A	5.1 ± 0.26	1.5	4.9 ± 0.10	1.0

Data are expressed as pEC_{50} values (the negative logarithm of the molar concentration of an agonist required to produce 50% the maximal response). Values represent the mean ± S.E.M. from at least $n = 3$ experiments performed in duplicate.
N.E. = no effect at 100 μM. n_H is the curve slope value.
[a] = pEC_{50} of the first phase of bi-phasic curve.

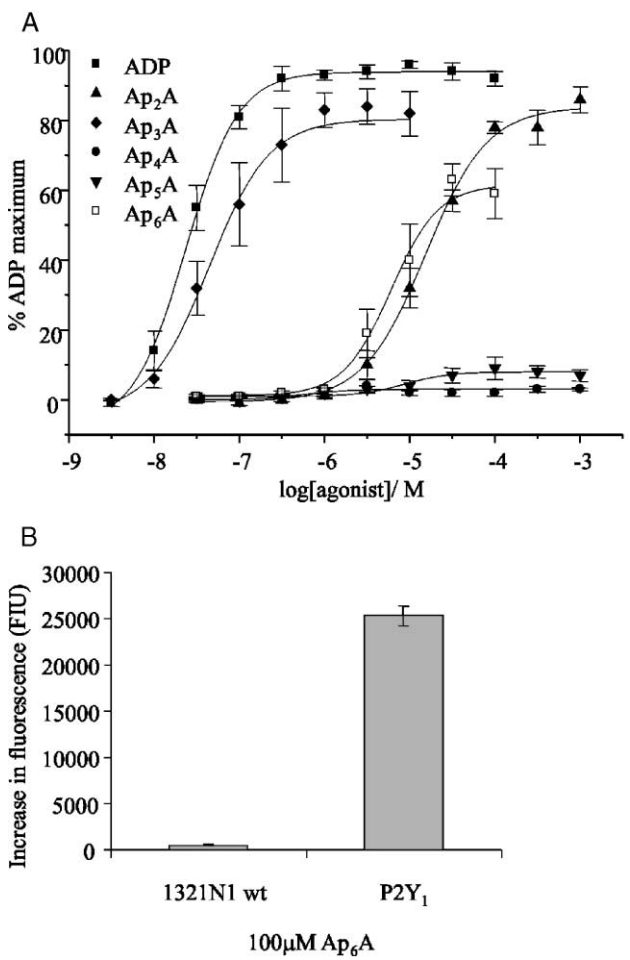

Fig. 1. (A) Concentration effect curves for nucleotides and Ap_nAs stimulating increases in $[Ca^{2+}]_i$ in 1321N1/$P2Y_1$ cells and (B) activity of 100 μM Ap_6A in 1321N1 wild type and 1321N1/$P2Y_1$ cells.

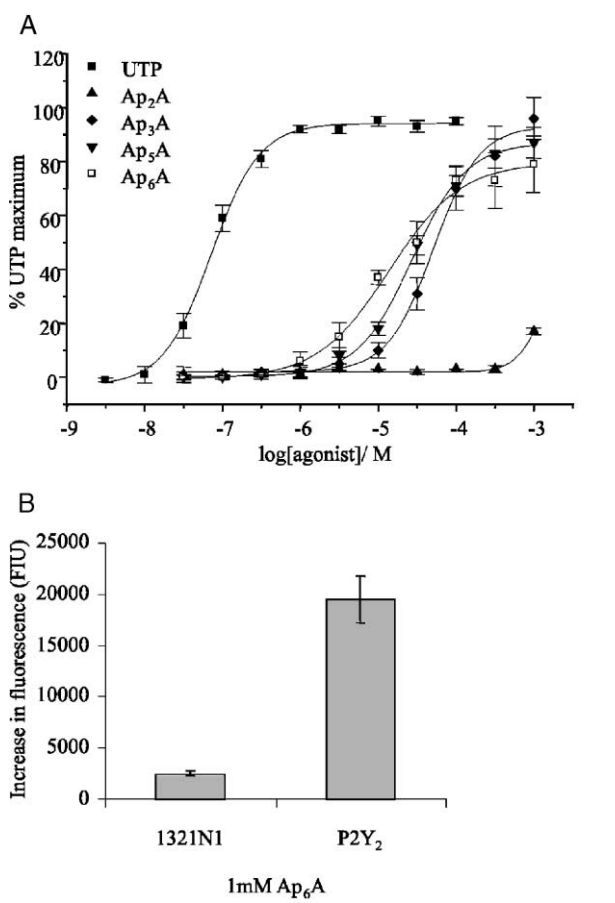

Fig. 2. (A) Concentration effect curves for nucleotides and Ap_nAs stimulating increases in $[Ca^{2+}]_i$ in 1321N1/$P2Y_2$ cells and (B) activity of 1 mM Ap_6A in 1321N1 wild type and 1321N1/$P2Y_2$ cells.

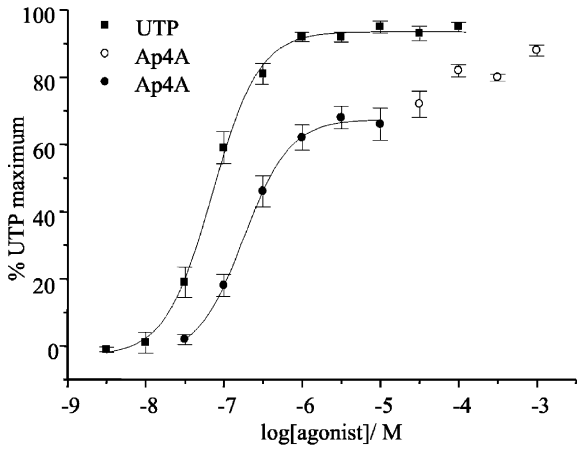

Fig. 3. Concentration effect curve for UTP and Ap_4A at $P2Y_2$ expressed in 1321N1 cells.

formed to specifically assess any low level contamination of ATP in samples of Ap_2A, Ap_3A, Ap_4A, Ap_5A and Ap_6A. By experimentation it was deduced that levels of 0.1% (m/m) were detectable. All Ap_nA samples showed levels of ATP less than 0.5%. There was no detectable evidence of any significant impurities in samples of UTP or ATP.

3.2. Effect of Ap_nAs on 1321N1 cells

No responses to 100 μM uridine (UTP, UDP) or adenine (ATP, ADP, α,β-meATP, β,γ-meATP, 2MeSATP) nucleotides were observed in wild type 1321N1 cells. Activation of the endogenously expressed M3 muscarinic receptor with 1 mM carbachol caused an increase in cell fluorescence of 24,132 ± 2,899 FIU (fluorescence intensity units). Only at a concentration of 1 mM, Ap_3A, Ap_4A and Ap_6A caused a small increase $[Ca^{2+}]_i$. These responses represented 3.55 ± 1.3%, 4.13 ± 0.9% and 11.02 ± 1.3% of the maximal response to carbachol, respectively (Fig. 4A). The nonselective P1 receptor agonist *N*-ethylcarboxamidoadenosine (NECA) (10 μM) was inactive (data not shown).

3.3. Agonist effects of Ap_nAs at human $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors

At $P2Y_1$, Ap_3A was shown to be a full agonist with similar efficacy to ADP. Ap_2A was approximately 1000-fold less potent than ADP. Ap_4A was inactive up to a concentration of 1 mM. The rank order of agonist potencies was: ADP > Ap_3A > Ap_6A > Ap_2A ≫ Ap_5A. Ap_6A behaved as a partial agonist with respect to ADP (Fig. 1A, Table 1), reaching 62 ± 4% of the maximal ADP response;

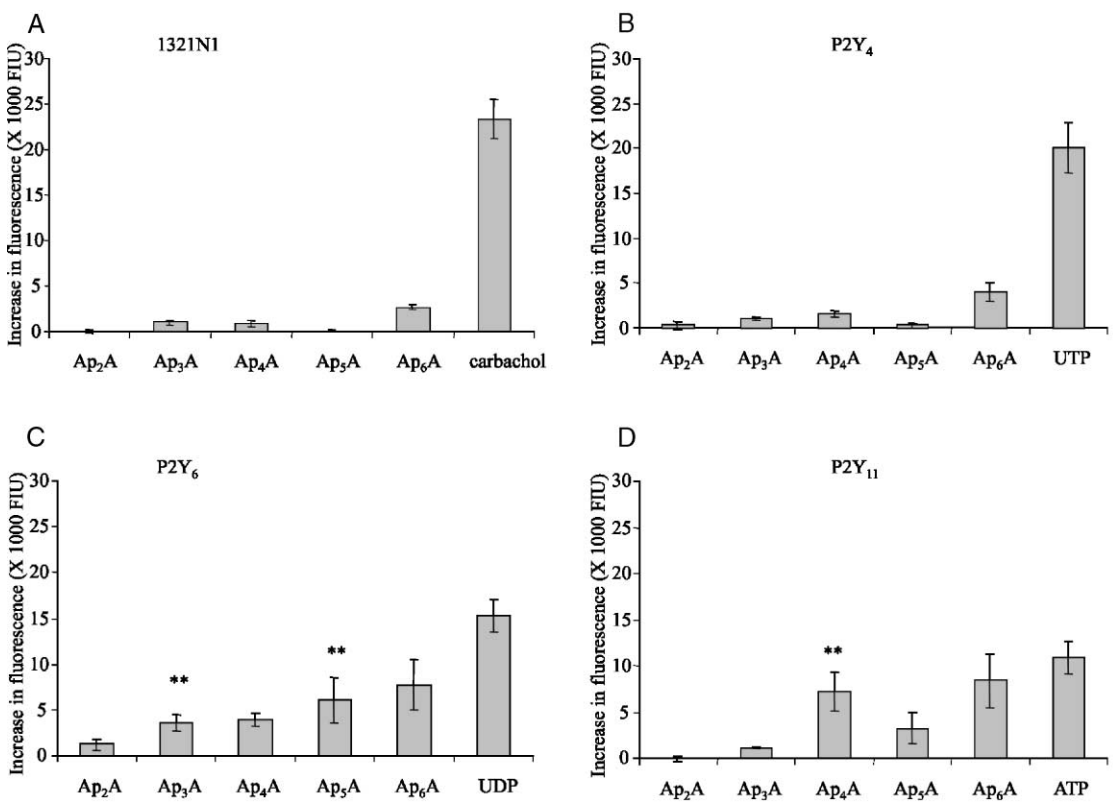

Fig. 4. Effect of 1 mM carbachol and Ap_nAs on increases in $[Ca^{2+}]_i$ in (A) 1321N1 cells. Effect of 100 μM UTP, 100 μM UDP, 1 mM ATP, respectively, and 1 mM Ap_nAs on increases in $[Ca^{2+}]_i$ and 1321N1 cells expressing the (B) $P2Y_4$, (C) $P2Y_6$ and (D) $P2Y_{11}$ receptor. $^{**}P \leq 0.01$ relative to control responses.

100 μM Ap_6A produced an increase in cell fluorescence of 25,345 ± 1057 FIU compared to 517 ± 88 FIU in wild type cells (Fig. 1B).

The rank order of agonist potencies for the $P2Y_2$ receptor was: UTP > Ap_4A > Ap_6A > Ap_5A > Ap_3A ≫ Ap_2A. Ap_3A, Ap_5A and Ap_6A were all full agonists with respect to UTP, giving approximately 90–100% of the maximal UTP response (Fig. 2A, Table 1). Ap_2A had minimal activity, only producing 17 ± 7.4% of the maximum UTP response. The Ap_6A response observed at $P2Y_2$ was greater than responses seen in wild type cells, with 1 mM Ap_6A producing a sevenfold greater response in the $P2Y_2$ line (Fig. 2B). The Ap_4A concentration effect curve appeared to be bi-phasic (Fig. 3). This observation, however, was not consistent and was observed in six out of the nine experiments performed. Fitting the data to a two-site model proved difficult since the second phase of the Ap_4A concentration effect curve did not reach a clear maximum, but in all experiments, 1 mM Ap_4A gave 88 ± 1.2% of the UTP maximal response. The first phase of the Ap_4A curve (10 nM–10 μM) gave an EC_{50} = 0.18 (0.12–0.27) μM with a slope value of 1.5 and reached 66 ± 4.7% of the UTP maximal response. Fitting the whole of the Ap_4A concentration effect curve (30 nM–1 mM) to a single-site model (De Lean et al., 1977) gave a nonsigmoidal curve with a slope value of 0.4.

Pre-treatment of cells with 50 ng ml^{-1} pertussis toxin for 18 h had no effect on either Ap_4A or UTP concentration effect curves (data not shown).

At $P2Y_4$, UTP caused a concentration-dependent increase in $[Ca^{2+}]_i$ (pEC_{50} = 7.16 ± .02, data not shown) while responses to the Ap_nAs were not significantly different from those observed in wild type cells. At $P2Y_6$ (pEC_{50} for UDP = 7.06 ± 0.09, data not shown), only 1 mM Ap_3A and Ap_5A, and at $P2Y_{11}$ (pEC_{50} for ATP = 4.4 ± 0.12, data not shown), only 1 mM Ap_4A responses were significantly greater than those in wild type cells (Fig. 4).

3.4. Antagonist effects of Ap_nAs at human $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors

Ap_nAs that were inactive as agonists at the various P2Y receptors were investigated as possible P2Y receptor antagonists. Cells expressing the $P2Y_1$ receptor were pre-incubated with either 10 μM Ap_4A or Ap_5A followed by ADP (3 nM–10 μM). Both Ap_4A and Ap_5A (10 μM) were unable to antagonise ADP responses; 100 μM Ap_2A did not antagonise UTP responses in the $P2Y_2$ line. At $P2Y_6$, 10 μM Ap_nAs, and at $P2Y_{11}$, 100 μM Ap_nAs did not antagonise a response to an EC_{50} concentration of UDP or ATP, respectively (Fig. 5A,B). At $P2Y_4$, 100 μM of the Ap_nAs caused a potentiation of an EC_{50} response to UTP (Fig. 5C). Priming cells with 10 nM UTP (a concentration which did not cause a response) did not potentiate subsequent increases in $[Ca^{2+}]_i$ evoked by UTP (3 nM–100 μM) (data not shown).

4. Discussion

When attempting to determine the activity of Ap_nAs at different P2Y receptors, comparison of data from different studies is often difficult since different functional readouts have been used and the purity of the ligands can be called into question. The aim of this study was to use the same assay system (mobilisation of intracellular Ca^{2+}) to profile the Ap_nAs at five human P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$) receptors, allowing a more comprehensive comparison between them.

Wild type 1321N1 cells do not respond to adenine nucleotides and are hence commonly used as a host cell line for purinergic receptor expression (Filtz et al., 1994). No responses were seen with either 100 μM ATP, UTP, UDP, ADP, α,β-meATP, β,γ-meATP, 2MeSATP, nucleotides known to act at subtypes of P2 purinoceptors, or NECA, the nonselective P1 receptor agonist, in 1321N1 cells. Responses were seen with 1 mM Ap_6A and, to a lesser extent, 1 mM Ap_3A and Ap_4A. These could not be due to the activation of known P2X receptors since ATP, α,β-meATP and 2MeSATP were inactive. Possible explanations for these responses could involve the interaction of the Ap_nAs with other proteins in the cell (Kisselev et al., 1998), or that the Ap_nAs have activity at an unidentified novel receptor(s) in this cell line.

The Ap_nAs used here were first studied as agonists. None of the Ap_nAs showed agonist activity at $P2Y_4$. This is in agreement with observations seen by others (Kennedy

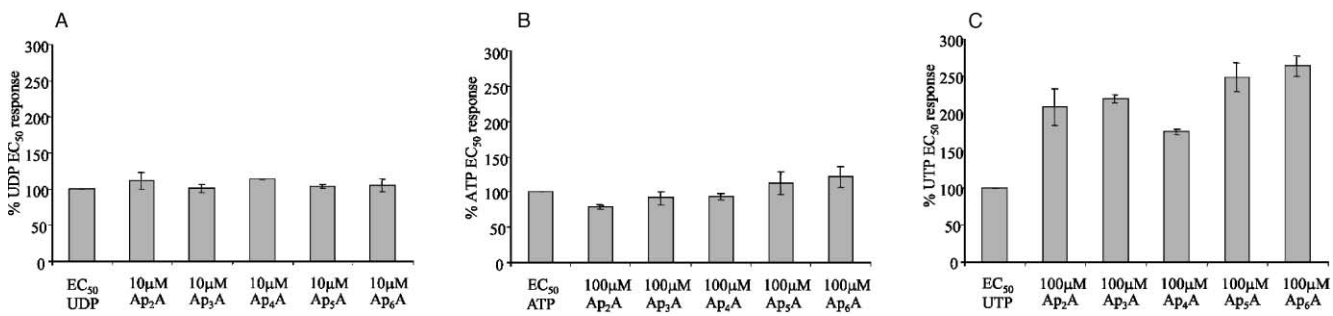

Fig. 5. Effect of Ap_nAs on an EC_{50} agonist response at (A) $P2Y_6$, (B) $P2Y_{11}$ and (C) $P2Y_4$.

et al., 2000; Communi et al., 1995, 1996). Significant responses at $P2Y_6$ were only observed with 1 mM Ap_3A and Ap_5A. At $P2Y_{11}$, previous data have shown Ap_4A and Ap_6A to be inactive, however, these compounds were only tested at 100 μM for their ability to induce an inositol phosphate response (Communi et al., 1997). Our results also show 100 μM Ap_nAs to be inactive as agonists at $P2Y_{11}$ receptors but at 1 mM Ap_4A caused a significant increase in $[Ca^{2+}]_i$ levels.

At $P2Y_1$, the response to Ap_6A was a receptor-mediated event as it was approximately 50 times greater than that observed in wild type cells. In addition, HPLC analysis showed Ap_6A to be pure, so it is unlikely that this response was due to a contaminant. When measuring intracellular Ca^{2+} mobilisation, Ap_6A has previously been shown to be inactive at $P2Y_1$ and $P2Y_2$ receptors endogenously expressed in ECV304 cells (Conant et al., 1998). In addition, nucleotides also have a lower potency value in this cell line, suggesting poor receptor coupling and/or low receptor reserve as explanations for these differences.

At $P2Y_2$, Ap_4A was the most potent of the Ap_nAs tested with a concentration effect curve that appeared to be bi-phasic. This was apparent from the shallow slope value obtained when trying to fit the data to a single site model. There are reports showing that the $P2Y_2$ receptor is able to couple to both G_i and G_q G-proteins, e.g. the mouse $P2Y_2$ receptor expressed in *X. laevis* oocytes (Mosbacher et al., 1998) and the $P2Y_2$ receptor endogenously expressed in cerebral astrocytes (Jimenez et al., 2000). In these experiments, we investigated if the bi-phasic nature of the Ap_4A concentration effect curve was due to the activation of G_i/G_o G-protein coupled receptors. Pertussis toxin had no effect on the Ap_4A response, suggesting that neither phase of the Ap_4A curve resulted from the activation of $P2Y_2$ receptors coupled to G_i/G_o G-proteins.

The second phase of the Ap_4A concentration effect curve may have been due to a nonspecific interaction of Ap_4A with a nonrelated protein in 1321N1 cells; however, the activity of Ap_4A was different at all the P2Y receptors expressed in the same host cell line, making this explanation unlikely. Alternatively, Ap_4A responses could involve the release of Ca^{2+} from two different intracellular Ca^{2+} pools as shown by Holden et al. (2000). Further experiments are required in order to establish if different pools of intracellular Ca^{2+} contribute to the bi-phasic Ap_4A response observed here.

The possible antagonist actions of the Ap_nAs were then investigated. None of the Ap_nAs had antagonist activity at $P2Y_1$, $P2Y_2$, $P2Y_6$ or $P2Y_{11}$ receptors. In the $P2Y_4$ cell line, 100 μM of all the Ap_nAs caused a potentiation of the response to UTP. Priming cells with 10 nM UTP (a concentration which did not cause a response) did not potentiate subsequent increases in intracellular Ca^{2+} evoked by UTP (3 nM–100 μM). In addition, none of the Ap_nAs potentiated subsequent agonist (UDP and ATP respectively) responses at $P2Y_6$ or $P2Y_{11}$. Taken together, these data suggest that the potentiating effect of the Ap_nAs on UTP-induced Ca^{2+} release is $P2Y_4$ specific.

The potentiating effect seen in this study, pharmacologically termed allosteric modulation, has been described to synthetic molecules modulating the actions of other synthetic or endogenous ligands at a number of G-protein coupled receptors (Birdsall et al., 2000; Gao and Ijzerman, 2000; Guyer et al., 1990). Here we have evidence for a series of endogenous ligands, the Ap_nAs, having potential allosteric modulatory effects on the mode of action of another endogenous ligand, UTP. These preliminary findings need to be investigated further in order to determine if the Ap_nAs are truly behaving as allosteric enhancers.

Our observations differ from those reported by Kennedy et al. (2000) who showed that 10 μM Ap_4A was unable to antagonise the increase in $[Ca^{2+}]_i$ evoked by UTP at the human $P2Y_4$ receptor stably expressed in 1321N1 cells. 100 μM Ap_4A, however, caused an approximate 50% inhibition of an EC_{50} UTP response. The differences in the observation from both studies may be attributed to the different experimental protocols used. In this study, cells were pre-treated with Ap_4A for 4 min before co-administration of UTP and FLIPR was used to measure changes in $[Ca^{2+}]_i$. In Kennedy's study, cells were super-fused with Ap_4A for 1 min before co-administration of UTP.

To date, radiolabeled nucleotides for P2 receptors lack specificity. They have been shown not only to bind to P2 receptors but to additional protein(s) that do not correspond to known functional P2 receptor subtypes, and to enzymes and transporters (Laubinger and Reiser, 1998; Yu et al., 1999). Hence, P2 receptor binding assays do not offer a reliable and accurate method for pharmacologically determining receptor number. In this study the cell lines used were not controlled for comparable receptor expression and thus a certain degree of caution must be taken when describing the Ap_nAs as full or partial agonists. However, the potency values for a range of nucleotides at the P2Y receptors expressed here are comparable (i.e. in the same molar range) to those reported by others who have used recombinant systems to pharmacologically characterise P2Y receptors (data not shown) (Schachter et al., 1996; Lazarowski et al., 1995; Kennedy et al., 2000; Communi et al., 1997).

In order to obtain a greater understanding of the role of the Ap_nAs in the pathogenesis of disease, a number of experiments are still required. The experiments here have all involved the use of human receptors. This type of study would also be of benefit using different species orthologues profiling both nucleotides and Ap_nAs, as these often show a different rank order of potency at P2-purinoceptors. In addition, the activity of Ap_7A at both P2Y and P2X receptors needs to be defined and the activity of the Ap_nAs at the recently cloned $P2Y_{12}$ (Zang et al., 2000; Hollopeter et al., 2001) receptor requires investigation. These data would significantly aid with the interpretation of responses to P2 receptor ligands in animal models. The

Ap_nAs profiled here have shown an interesting pattern of selectivity and efficacy amongst the P2Y receptors. From this and the other publications on this series of molecules, it is not unreasonable to suggest that the Ap_nAs are of equal significance to nucleotides as endogenous ligands at P2Y receptors.

The pharmacological characterisation of Ap_nAs at P2Y receptors has been confounded by the use of cell lines expressing endogenous P2 and/or P1 receptors, the use of different isolated tissues, species and assay systems. The purity of commercially available nucleotides has also led to conflicting and confusing data being reported. This study has attempted to eliminate as many inconsistencies as possible using the pharmacological tools available at the time and using a consistent assay system, which determines and compares the selectivity of the Ap_nAs for P2Y receptors.

References

Baxi, M.D., Vishwanatha, J.K., 1995. Diadenosine polyphosphates: their biological significance. J. Pharmacol. Toxicol. Methods 33, 121–128.

Birdsall, N.J.M., Farries, T., Gharagoloo, P., Kobayshi, S., Lazareno, S., Sugimoto, M., 2000. Subtype-selective positive cooperative interactions between brucine analogs and acetylcholine at muscarinic receptors: functional studies. Mol. Pharmacol. 55, 778–786.

Bogdanov, Y.D., Wildman, S.S., Clements, M.P., King, B.F., Burnstock, G., 1998. Molecular cloning and characterization of rat $P2Y_4$ nucleotide receptor. Br. J. Pharmacol. 124, 428–430.

Communi, D., Pirotton, S., Parmentier, M., Boeymaems, J.M., 1995. Cloning and functional expression of a human uridine nucleotide receptor. J. Biol. Chem. 270, 30849–30852.

Communi, D., Motte, S., Boeymanns, J.M., Pirotton, S., 1996. Pharmacological characterisation of the human $P2Y_4$. Eur. J. Pharmacol. 317, 383–389.

Communi, D., Govaerts, C., Parmentier, M., Boeynaems, J.M., 1997. Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. J. Biol. Chem. 272, 31969–31973.

Conant, A.R., Fischer, M.J., McLennan, A.G., Simpson, A.W.M., 1998. Characterisation of P2 receptors on the human umbilical vein endothelial cell line ECV304. Br. J. Pharmacol. 125, 357–364.

De Lean, A., Munson, P.J., Rodbard, D., 1977. Simultaneous analysis of families of sigmoidial curves: applications to bioassay, radio-ligand assay, and physiological dose-response curves. Am. J. Physiol. 235, E97–E102.

Filtz, T.M., Li, Q., Boyer, J.L., Nicholas, R.A., Harden, T.K., 1994. Expression of a cloned P2Y purinergic receptor that couples to phospholipase C. Mol. Pharmacol. 46, 8–14.

Flores, N.A., Stavrou, B.M., Sheridan, D.J., 1999. The effects of diadenosine polyphosphates on the cardiovascular system. Cardiovasc. Res. 42, 15–26.

Gao, Z.G., Ijzerman, A.P., 2000. Allosteric modulation of A_{2A} adenosine receptors by amiloride analogues and sodium ions. Biochem. Pharmacol. 60, 669–676.

Guyer, C.A., Horstman, D.A., Wilson, A.L., Clarke, J.D., Cragoe Jr., E.J., Limbird, L.E., 1990. Cloning, sequencing and expression of the gene encoding the porcine α_2-adrenergic receptor: allosteric modulation by Na^+, H^+, and amiloride analogues. J. Biol. Chem. 265, 17307–17317.

Heise, C.E., O'Dowd, B.F., Figueroa, J.D., Sawyer, N., Nguyen, T., Im, D.-S., Stocco, R., Bellefeuille, J.N., Abramovitz, M., Cheng, R., Williams Jr., D.L., Zeng, Z., Liu, Q., Ma, L., Clements, M.K., Coulombe, N., Liu, Y., Austin, C.P., George, S.R., O'Neill, G.P., Metters, K.M., Lynch, K.R., Evans, J.F., 2000. Characterisation of the human cysteinyl leukotriene 2 receptor. J. Biol. Chem. 275, 30531–30536.

Holden, C.P., Haughey, N.J., Dolhun, B., Shepel, P.N., Nath, A., Geiger, J.D., 2000. Diadenosine pentaphosphate increases levels of intracellular calcium in astrocytes by a mechanism involving release from caffeine/ryanodine- and IP3- sensitive stores. J. Nuerosci. Res. 59, 276–282.

Hollopeter, G., Jantzen, H.M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R.B., Nurden, P., Nurden, A., Julius, D., Conley, P.B., 2001. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409, 202–207.

Jankowski, J., Tepel, M., Van De Giet, M., Tente, I.M., Henning, L., Junker, R., Zidek, W., Schluter, H., 1999. Identification and Characterisation of P^1,P^7-Di(adenosine-5′)-heptaphosphate from human platelets. J. Biol. Chem. 274, 23926–23931.

Jankowski, J., Hagememann, J., Tepel, M., Van de Giett, M., Stephan, N., Henning, L., Gouni-Berthold, H., Sachinidis, A., Zidek, W., Schluter, H., 2001. Dinucleotides as growth-promoting extracellular mediators. J. Biol. Chem. 276, 8904–8909.

Jimenez, A.I., Castro, E., Communi, D., Boeynaems, J.-M., Delicado, E.G., Miras-Portugal, M.T., 2000. Coexpression of several types of metabotrophic nucleotide receptors in single cerebellar astrocytes. J. Neurochem. 75, 2071–2079.

Kennedy, C., Qi, A.-D., Herold, C., Harden, K., Nicholas, R., 2000. ATP, an agonist at the rat $P2Y_4$ receptor, is an antagonist at the human $P2Y_4$ receptor. Mol. Pharmacol. 57, 926–931.

Kisselev, L.L., Justesen, J., Wolfson, A.D., Frolova, L.Y., 1998. Diadenosine oligophosphates (Ap_nA) a class of signalling molecules? FEBS Lett. 427, 157–163.

Lazarowski, E.R., Watt, W.C., Stutts, M.J., Boucher, R.C., Harden, T.K., 1995. Pharmacological selectivity of clones human P2YU-purinoceptor: potent activation by diadenosine tetraphosphate. Br. J. Pharmacol. 116, 1619–1627.

Laubinger, W., Reiser, G., 1998. Differential characterisation of binding sites for adenine and uridine nucleotides in membranes from rat lung as possible tools for studying P2 receptors in lung. Biochem. Pharmacol. 55, 687–695.

Miras-Portugal, M.T., Gualix, J., Pintor, J., 1998. The neurotransmitter role of diadenosine polyphosphates. FEBS Lett. 430, 78–82.

Mosbacher, J., Maier, R., Fakler, B., Glatz, A., Crespo, J., Bilbe, G., 1998. P2Y receptor subtypes differentially couple to inwardly-rectifying potassium channels. FEBS Lett. 436, 104–110.

Nicolas, R.A., Watt, W.C., Lazarowski, E.R., Li, Q., Harden, T.K., 1996. Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP and UTP-specific receptor. Mol. Pharmacol. 50, 224–229.

Pintor, J., Miras-Portugal, M.T., 2000. Receptors for diadenosine polyphosphates $P2_D$, $P2Y_{ApnA}$, P_4 and dinucleotide receptors: are there too many? TIPS 21, 135.

Pintor, J., King, B.F., Miras-Portugual, M.T., Burnstock, G., 1996. Selectivity and activity of adenine dinucleotides at recombinant $P2X_2$ and $P2Y_1$ purinoceptors. Br. J. Pharmacol. 119, 1006–1012.

Rees, E.S., Coote, J., Stables, J., Goodson, S., Harris, S., Lee, M.G., 1996. Bicistronic vector for the creation of stable cell lines that predisposes all antibiotic-resistant cells to express recombinant protein. BioTechniques 20, 102–110.

Schachter, J.B., Li, Q., Boyer, J.L., Nicholas, R.A., Harden, T.K., 1996. Second messenger cascade specificity and pharmacological selectivity of the human P2Y1-purinoceptor. Br. J. Pharmacol. 118, 167–173.

Vigne, P., Breittmayer, J.P., Frelin, C., 2000. Diadenosine polyphosphates as antagonists of the endogenous $P2Y_1$ receptor in rat brain capillary endothelial cells of the B7 and B10 clones. Br. J. Pharmacol. 129, 1506–1512.

Wildman, S.S., Brown, S.G., King, B.F., Burnstock, G., 1999. Selectivity

of diadenosine polyphosphates for the rat P2X receptor subunits. Eur. J. Pharmacol. 367, 119–123.

Yu, H., Bianchi, B., Metzger, R., Lynch, K.J., Kowaluk, E.A., Jarvis, M.F., Van Biesen, T., 1999. Lack of specificity of [^{35}S]-ATP(S and [^{35}S]-ADPβS as radioligands for ionotropic and metabotropic P2 receptor binding. Drug Dev. Res. 48, 84–93.

Zang, F.L., Luo, L., Gustafson, E., Lachowicz, J., Smith, M., Qiao, X., Liu, Y.-H., Chen, G., Pramanik, B., Laz, T.M., Palmer, K., Bayne, M., Monsma, F., 2000. ADP is a cognate ligand for the orphan G-protein coupled receptor SP1999. J. Biol. Chem. 276, 8608–8615.