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Pharmacological selectivity of the cloned human P_{2U}-purinoceptor: potent activation by diadenosine tetraphosphate

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1 The human P_{2U}-purinoceptor was stably expressed in 1321N1 human astrocytoma cells and the pharmacological selectivity of the expressed receptor was studied by measurement of inositol lipid hydrolysis.

2 High basal levels of inositol phosphates occurred in P_{2U}-purinoceptor-expressing cells. This phenomenon was shown to be due to release of large amounts of ATP from 1321N1 cells, and could be circumvented by adoption of an assay protocol that did not involve medium changes.

3 UTP, ATP and ATPγS were full and potent agonists for activation of phospholipase C with EC_{50} values of 140 nM, 230 nM, and 1.72 μM, respectively. 5BrUTP, 2ClATP and 8BrATP were also full agonists although less potent than their natural congeners. Little or no effect was observed with the selective P_{2Y}-, P_{2X}-, and P_{2T}-purinoceptor agonists, 2MeSATP, α,β-MeATP, and 2MeSADP, respectively.

4 Diadenosine tetraphosphate, Ap_4A, was a surprisingly potent agonist at the expressed P_{2U}-purinoceptor with an EC_{50} (720 nM) in the range of the most potent P_{2U}-purinoceptor agonists. Ap_4A may be a physiologically important activator of P_{2U}-purinoceptors.

Keywords: P_{2U}-purinoceptor; extracellular nucleotides; diadenosine polyphosphates; ATP release; inositol lipid hydrolysis

Introduction

P_{2U}-purinoceptors are a subclass of P_2-purinoceptors that are found in a broad range of tissues including circulating monocytes and airway epithelial cells (Dubyak & El-Moatassim, 1993; Harden *et al.*, 1995). These receptors were originally distinguished (Dubyak *et al.*, 1988; Fine *et al.*, 1989; Okajima *et al.*, 1989; Stutchfield & Cockcroft, 1990; Brown *et al.*, 1991) as phospholipase C-activating Ca^{2+}-mobilizing receptors that exhibited a pharmacological selectivity that differentiated them from P_{2Y}-purinoceptors, which previously were considered the major phospholipase C-activating P_2-purinoceptor subtype. The P_{2U}-purinoceptor is the only member of the identified P_2-purinoceptors that is activated by both UTP and ATP (Dubyak & El-Moatassim, 1993; Fredholm *et al.*, 1994).

Insight into molecular aspects of P_2-purinoceptors has begun to accrue recently, and cDNAs encoding P_{2U}-, P_{2Y}-, and P_{2X}-purinoceptors have been isolated (Lustig *et al.*, 1993; Webb *et al.*, 1993; Brake *et al.*, 1994; Filtz *et al.*, 1994; Parr *et al.*, 1994; Valera *et al.*, 1994). The availability of cDNA for the human P_{2U}-purinoceptors (Parr *et al.*, 1994) has allowed us to undertake a detailed pharmacological analysis of this receptor stably expressed in a cell line, 1321N1 human astrocytoma cells, that when transfected with vector alone displays no intracellular second messenger response to any nucleotide analogue that has been tested. In addition to defining the pharmacological selectivity of the cloned human receptor, two additional observations have been made. First, 1321N1 cells release ATP. The properties and magnitude of this phenomenon suggest that the release of endogenous ATP and its metabolic products needs to be considered carefully in all studies of P_2-purinoceptors with intact cells. Second, diadenosine polyphosphates previously have been shown to be synthesized and released from a variety of tissues including chromaffin cells, neurones, and platelets (Foldgaard & Klenow, 1982; del Castillo *et al.*, 1988; Pintor & Miras-Portugal, 1993). The effects of extracellular diadenosine polyphosphates and the observation of high affinity binding sites for these compounds have led to the suggestion that diadenosine polyphosphates are activators of physiologically relevant cell surface receptors, i.e. the putative P_{2D}-purinoceptors (Hilderman *et al.*, 1991; Pintor *et al.*, 1993). Here we show that several diadenosine polyphosphates are agonists at human P_{2U}-purinoceptors. Moreover, one of these compounds, diadenosine tetraphosphate, is an agonist that exhibits a potency essentially equivalent to that of ATP and UTP for P_{2U}-purinoceptor activation.

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Methods

Expression of the human P_{2U}-purinoceptor in 1321N1 human astrocytoma cells

1321N1 human astrocytoma cells were infected with either a retroviral vector containing the human P_{2U}-receptor cDNA (LHP2USN plasmid) or the control vector containing only the neomycin-resistance gene (LN), and vector-bearing cells were selected with G418 as described (Parr *et al.*, 1994). UTP-stimulated inositol phosphate formation was measured in homogeneous cell populations derived from single cloned cells. Clone 17K (H2PU-1321N1 cells), which showed the greatest response to UTP, was selected for this study.

Inositol phosphate formation

Confluent cells grown on 12-well dishes were incubated for 18 h in 0.5 ml inositol-free DMEM containing 1-2 μCi [^{3}H]-*myo*-inositol. In initial assays the cells were washed free of unincorporated [^{3}H]-inositol and preincubated with 10 mM LiCl for 10 min before challenge with agonists. This procedure was later modified by including apyrase in the wash solution or by assaying inositol phosphate accumulation in cells that were not washed after the labelling period, as detailed in the text and in the figure legends. Incubations were terminated by addition of 5% trichloroacetic acid (TCA) followed by extraction with ethyl ether. Individual inositol phosphates were separated on Dowex AG1-X8 columns as described (Brown *et al.*, 1991). All assays were performed with triplicate samples that differed by less than 20% from the mean. Error bars were omitted from some figures for clarity.

Table 1 Retention time of adenine related bases, nucleosides, and nucleotides on ion pairing (a) and ion exchange (b) h.p.l.c

A *Ion-pairing h.p.l.c.*	*Elution time* (min)
Hypoxanthine	5.5
Xanthine	5.7
Inosine	7.6
Adenine	8.5
Cyclic ADP-ribose	8.7
NADP	9.1
NAD	11.4
Adenosine	14.3
AMP	19.6
2-3 Cyclic AMP	31.7
ADP	38.2
ADP-ribose	37.2
Cyclic AMP	45.8
A_2P_2	46.1
NADH	46.1
ATP	50.3
dATP	50.9
A_2P_3	51.5
A_2P_5	53.3
NADPH	63.0

B *Ion exchange h.p.l.c.*	*Elution time* (min)
Adenosine	4.3
AMP	9.1
Cyclic AMP	13
A_2P_2	14.5
ADP-ribose	14.9
ADP	16
NADH	17.1
ATP	22.3
A_2P_4	26
A_2P_5	32.2
A_2P_6	35.5

Time values were determined from the peak of (maximal) absorbance at 260 nm of known standard (100–300 μM) as indicated in Methods.

Release of adenine ^{3}H-nucleosides and ^{3}H-nucleotides

Confluent cells grown on 12-well dishes were labelled with 10 μCi ml^{-1} [^{3}H]-adenine for 2–3 h. The unincorporated radioactivity was removed and aliquots of the incubation medium were taken at various times for measurement of released radioactivity. To measure intracellular radioactivity, cells were lysed with 5% TCA and the extracts neutralized as above. H.p.l.c. analysis indicated that more than 80% of the incorporated radioactivity eluted as [^{3}H]-ATP.

Separation of nucleotides by h.p.l.c.

Individual ^{3}H species present in the medium or in cell lysates were separated by reverse phase ion-pairing h.p.l.c. as described previously (Webster *et al.*, 1985; Ryll & Wagner, 1991) with modifications. The separation system consisted of a hypersil-C18 column (Metachem Tech., Torrens, CA) and a mobile phase developed with buffer A (100 mM potassium phosphate, pH 5.3, 8 mM TBAHS) from 0 to 11 min, buffer B (90% A, 10% methanol) from 11 to 55 min and returning to 100% buffer A from 55 to 86 min. Nucleotides also were separated by an ion-exchange system utilizing a VYDAC nucleotide-analysis column (VYDAC, Hesperia, CA, U.S.A.) and a linear ammonium phosphate gradient. The gradient was from 100% buffer A (0.45 M NH_4COOH, pH 4.8) to 100% buffer B (0.5 M NaH_2PO_4, pH 2.7) over 25 min, maintaining 100% buffer B from 25 to 40 min, whereupon initial conditions (100% A) were resumed from 40–60 min. The absorbance at 260 nm of the eluent was monitored with an on-line Model 490 multiwavelength detector (Waters Division, Millipore, Milford, MA, U.S.A.). Radioactivity was monitored on-line with 25% efficiency using a Radiomatic Flo-one detector (Radiomatic Instruments, Tampa, FL, U.S.A.). ^{3}H-nucleotides were identified by their co-elution with known standards. Retention times for various standards of adenine-related bases, nucleosides and nucleotides are indicated in Table 1.

Luciferin-luciferase assay

Cells were incubated in DMEM for various times and ATP was measured in either the incubation medium or in cell sonicates utilizing the luciferin-luciferase reaction as described by Yang *et al.* (1994). Reactions were quantified with an Auto Lumat LB 953 luminometer (Berthold Systems Inc., Aliguippa, PA, U.S.A.).

Measurement of lactate dehydrogenase activity (LDH)

LDH activity was quantified in cell medium and lysates obtained as described above by assaying changes in the NADH/NAD ratio at 340 nm as described by Wroblewski & LaDue (1955).

Nucleotide hydrolysis

Samples were incubated for 5 to 30 min at 30°C with 0.2–5 iu ml^{-1} of either NADase, apyrase, alkaline phosphatase, cyclic AMP-phosphodiesterase (PDE), oligonucleotide 5′-nucleotidohydrolase (3′-PDE) or with oligonucleotide 3′-nucleotidohydrolase (5′-PDE). Incubations were terminated by addition of 5% TCA followed by ether extraction. Reaction products were separated by h.p.l.c. as above. Enzyme activities were monitored in parallel with specific substrates.

Materials

All uridine nucleotides and nucleosides, ATP, ADP, AMP, adenosine, 3′-PDE (from *Crotalus durissus*), 5′-PDE (from calf spleen), bovine cyclic AMP-PDE, and alkaline phosphatase were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.); adenosine 3′:5′-cyclic monophosphate, 2′-3′ cyclic AMP, nicotinamide-adenine dinucleotides, ADP-ribose, diadenosine polyphosphates, inosine, xanthine, hypoxanthine, apyrase, and NADase were purchased from Sigma (St. Louis, MO, U.S.A.); α,βMe-ATP, 2MeSATP, 2ClATP and 2MeSADP were from Research Biochemicals (Natick, MA, U.S.A.); cyclic ADP ribose was purchased from Research Products International Corp. (Mount Prospect, IL, U.S.A.); *Myo*-[2-^{3}H]-inositol (20 Ci mmol^{-1}), [8-^{3}H]-adenine (10-25 Ci mmol^{-1}), and [2,8-^{3}H]-ATP (15-30 Ci mmol^{-1}) were from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.).

Data analysis

Unless stated otherwise, experiments were performed with triplicate determinations that differed <20% from the mean. The data were considered statistically different at $P<0.05$ using Student's paired *t* test.

Results

The overall goal of this research was to define in detail the pharmacological specificity of the cloned human P_{2U}-purinoceptor. We previously have cloned and stably expressed human P_{2U}- and avian P_{2Y}-purinoceptors in a cell line, 1321N1 human astrocytoma cells, that in the absence of transfection with P_2-purinoceptor cDNA exhibits no second messenger response to extracellular purine or pyrimidine nucleotides (Brown, 1992; Filtz *et al.*, 1994; Parr *et al.*, 1994). Previous studies had suggested that release of nucleotides occurs from 1321N1 cells with a resultant stimulation of the expressed re-

ceptors (Filtz *et al.*, 1994; Parr *et al.*, 1994). Therefore, to examine reliably the pharmacological specificity of the human P_{2U}-purinoceptor, the characteristics of the release of endogenous receptor-activating molecules were examined.

Medium from [^{3}H]-adenine-prelabelled control, vector-infected, or HP2U-1321N1 cells was collected at various times following removal of unincorporated [^{3}H]-adenine, and released nucleotide and nucleosides were resolved by h.p.l.c. (Figure 1 and Table 2). Results obtained with HP2U-1321N1 cells, which were identical to those observed with control cells, are described. [^{3}H]-ATP accumulated transiently with maximal levels obtained during the first 5 min. Longer incubations resulted in a gradual decrease of [^{3}H]-ATP and transient formation of [^{3}H]-ADP. There was a negligible accumulation of [^{3}H]-AMP and [^{3}H]-adenosine, but a sustained accumulation of their metabolic products, [^{3}H]-inosine, [^{3}H]-xanthine, and [^{3}H]-hypoxanthine, was observed. Extracellular [^{3}H]-ATP was not detectable after 3 h (Table 2); in contrast, intracellular [^{3}H]-ATP levels were essentially unchanged over the 3 h incubation (data not shown). Addition of 1 mM carbachol, which activates muscarinic cholinoceptors and mobilizes Ca^{2+} in 1321N1 cells (Masters *et al.*, 1984; Nakahata & Harden, 1987), had no effect on the pattern of appearance of [^{3}H]-ATP and its degradation products suggesting that release of nucleotides is not regulated by inositol lipid hydrolysis or by intracellular calcium levels (data not shown). In addition to the compounds identified above, an unidentified ^{3}H-labelled species (retention time = 46.1 min, Figure 1) gradually accumulated in the medium (Table 2). This species co-eluted with standards of NADH and Ap_2A on a reverse-phase ion-pairing h.p.l.c. system (Figure 1 and Table 1), but was resistant to hydrolysis during a 30 min incubation with NADase or 3'-PDE, enzymes that hydrolyze nicotinamide adenine dinucleotides and diadenosine polyphosphates (del Castillo *et al.*, 1988), respectively. This species also remained essentially intact after incubation with apyrase and alkaline phosphatase. The unidentified ^{3}H-species exhibited a unique retention time of 8.5 min on ion-exchange h.p.l.c. (see Table 1). Partial hydrolysis and accumulation of a species that co-elutes with ADP was observed during incubation of the unknown species with cyclic AMP-PDE and with 5'-PDE (data not shown). Although these results are consistent with the potential presence of a 3'5'-phosphodiester linkage, the identity of this compound remains unknown. The unidentified species did not accumulate

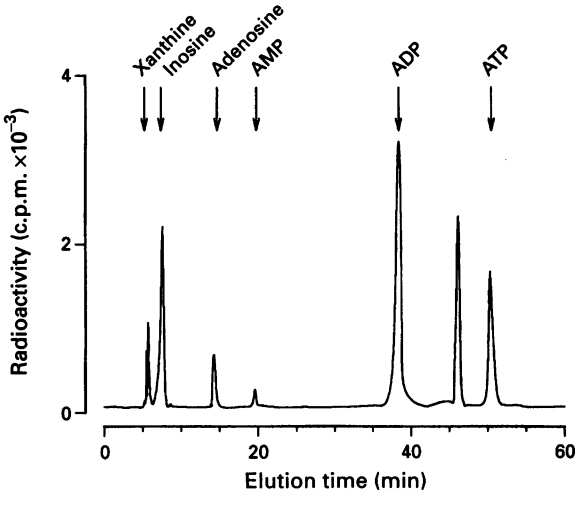

Figure 1 H.p.l.c. separation of ^{3}H species released from [^{3}H]-adenine labelled HP2U-1321N1 cells: confluent HP2U-1321N1 cells were labelled with 10 μCi [^{3}H]-adenine as described in Methods. After 1 h the unincorporated radioactivity was removed by aspiration and the cells were incubated for 30 min with 0.3 ml of fresh medium. The medium was collected and any contaminating cells were removed by rapid centrifugation. ^{3}H species present in the supernatant were resolved by ion-pairing h.p.l.c. Elution times for standards are indicated with arrows on top of the figure. The data are representative of results from three experiments performed with duplicate samples.

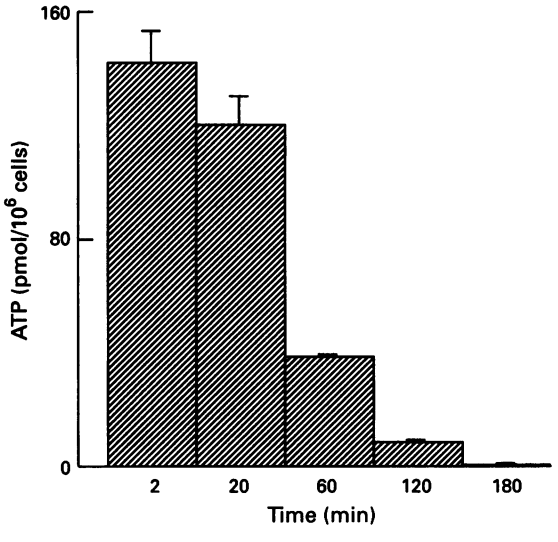

Figure 2 Measurement of extracellular ATP by a luciferin-luciferase assay: HP2U-1321N1 cells were grown to a density of $0.5-0.7 \times 10^6$ cells per well in 12-well tissue culture plates. The medium was removed and the cells were incubated with 0.3 ml of fresh, prewarmed DMEM. At the times indicated the medium was collected, any contaminating cells were removed by centrifugation, and 0.1 ml was combined with a luciferin-luciferase reaction cocktail for quantitation of ATP. The data are the mean value ± s.d. from quadruplicate samples. The results are representative of two experiments.

Table 2 Time course of accumulation of ^{3}H species released from [^{3}H]-adenine labelled HP2U-1321N1 cells

Time	*Nucleosides*[1]	*AMP*	*ADP*	*Unknown*	*ATP*
30 s	4.9 ± 0.7	5.6 ± 0.4	11.2 ± 2.3	–	21.6 ± 1.9
5 min	57.3 ± 6.8	11.4 ± 0.3	27.9 ± 0.6	8.7 ± 2.8	37.8 ± 4.4
30 min	71.9 ± 11.1	5.1 ± 0.7	37.9 ± 2.6	25.7 ± 4.2	21.8 ± 2.0
4 h	47.2 ± 9.9	4.4 ± 0.5	15.0 ± 1.2	76.6 ± 6.6	–

Confluent HP2U-1321N1 cells were labelled with 10 μCi [^{3}H]-adenine as described in Methods. After 1 h the unincorporated radioactivity was removed by aspiration and the cells incubated with 0.3 ml of fresh medium. At the indicated times the medium was collected and any contaminated cells were removed by rapid centrifugation. ^{3}H species present in the supernatant were resolved by ion-pairing h.p.l.c. The data are expressed as c.p.m. $\times 10^{-3}$ and represent the mean ± s.d. of results from three experiments performed with duplicate samples.

[1] The data represent the peak areas corresponding to adenosine, inosine, xanthine and hypoxanthine pooled together.

in cell medium that was incubated with exogenous [^{3}H]-ATP, indicating that it did not represent a breakdown product of extracellular ATP (data not shown).

The release of ATP from 1321N1 cells also was directly quantified by the luciferin-luciferase assay. Up to 150 pmol ATP/10^6 cells (approximately 9% of the cell content of ATP) was released upon replacing the incubation medium with fresh medium (Figure 2), resulting in a concentration of ATP of 200–300 nM for confluent cells in a well of a 12-well culture dish containing 0.5 ml of medium. Released ATP was degraded and no ATP was detected after 3 h. Thus, the pattern of extracellular ATP accumulation measured with the luciferin-luciferase assay followed that of [^{3}H]-ATP. Accumulation of extracellular ATP was not associated with detectable extracellular LDH activity, suggesting that a lytic mechanism of nucleotide release does not occur (the sensitivity of the assay allowed detection of LDH activity with up to a 1:300 dilution of cell lysates). Similar patterns of extracellular accumulation of ATP were observed with control and vector-infected 1321N1 cells (data not shown).

1321N1 human astrocytoma cells do not express endogenous receptors for ATP (Brown, 1992; Filtz *et al.*, 1994; Parr *et al.*, 1994), and incubation of control or vector-infected (data not shown) 1321N1 cells with ATP, or with UTP, ADP, 2MeSATP, α,βMeATP, $A_{P4}A$, or UDP, all of which are nucleotides known to interact selectively with subtypes of P_2-purinoceptors (Fredholm *et al.*, 1994), resulted in no detectable accumulation of inositol phosphates. In contrast, enhancement of inositol phosphate accumulation was observed with 10 to 1,000 μM of the muscarinic cholinoceptor agonist, carbachol (not shown).

Stable expression of the human P_{2U}-purinoceptor in 1321N1 cells resulted in basal levels of [^{3}H]-inositol phosphates that were markedly greater than levels obtained with vector-transfected cells, and a 30–50% increase in the accumulation of inositol phosphates was observed after addition of UTP or ATP to HP2U-1321N1 cells (Figure 3a). Thus, the release of endogenous ATP as illustrated above apparently confounds any quantitative pharmacological analysis of drug specificities at P_{2U}-purinoceptors expressed in these cells. To address this problem more directly, experiments were carried out under conditions where extracellular accumulation of ATP was minimized by inclusion of apyrase, a non-specific ATPase, in both the medium used to wash cells and in the assay medium. Addition of apyrase resulted in reduction of basal inositol phosphate levels in HP2U-1321N1 cells to values that were essentially identical to those obtained with vector cells. Only small responses to ATP and UTP were observed in the presence of apyrase (Figure 3b and data not shown). In contrast, a marked inositol phosphate response that was 50–70% higher than accumulation observed with carbachol (Figure 3b) was obtained with the hydrolysis-resistant ATP analogue, ATPγS.

Although incubation with apyrase proved useful for reduction of basal [^{3}H]-inositol phosphate levels in HP2U-1321N1 cells, presumably by hydrolyzing any released ATP, the hydrolytic activity of this enzyme introduces another set of concerns for pharmacological studies of P_2-purinoceptors. As such, methodology was pursued that would circumvent the need to use apyrase. The most successful approach was simply to quantitate receptor-promoted inositol phosphate accumulation in cells that received no washes. Thus, after labelling cells for 18 h with [^{3}H]-inositol, LiCl was added without a change of medium; after an additional 15 min incubation the cells were challenged with agonists. Extracellular [^{3}H]-ATP did not accumulate during incubation of [^{3}H]-adenine-labelled cells under these conditions, and as with apyrase treatment, low basal levels of inositol phosphates were observed (Figure 3c). More importantly, and unlike the data obtained with apyrase, responses to ATP and UTP were comparable to those obtained with ATPγS (Figures 3c and 4). These data illustrate that phosphoinositide hydrolysis is triggered during cell medium changes, apparently by the release of substantial amounts of ATP. Based on these results, inositol phosphate formation was subsequently assayed in cells that were not subjected (except where indicated) to any washes after the labelling period.

Addition of ATP, UTP or ATPγS to HP2U-1321N1 cells resulted in a time-dependent accumulation of inositol phosphates (Figure 4). Initial rates of inositol phosphate accumulation were identical for each of the nucleotides. The rate of nucleotide-stimulated inositol phosphate formation decreased slightly after the first 2–4 min, but accumulation continued for at least 1 h. Carbachol-stimulated inositol phosphate accumulation proceeded at a slower rate with time, suggesting that purinoceptor-mediated responses were only partially desensitized relative to the endogenous muscarinic receptor-mediated response.

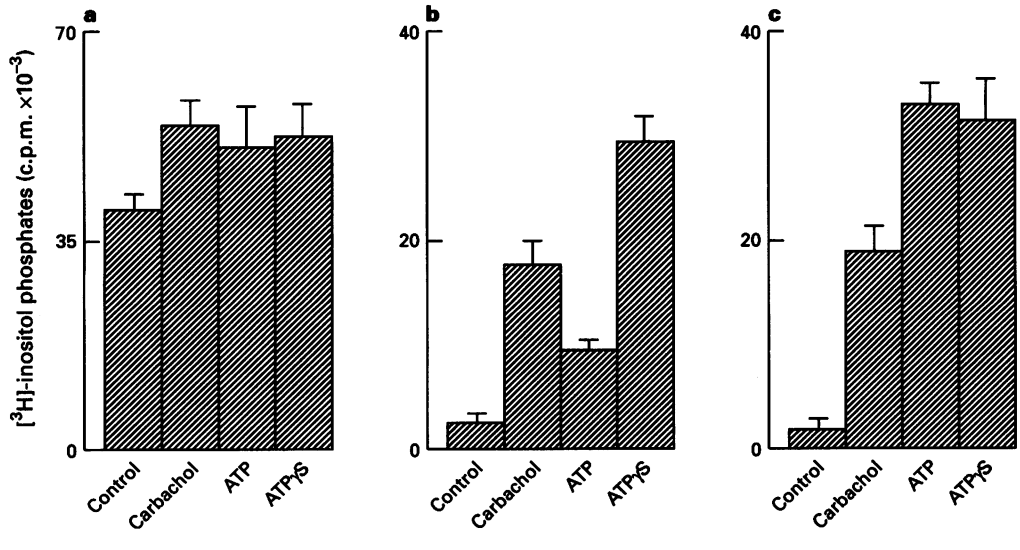

Figure 3 Formation of [^{3}H]-inositol phosphates in HP2U-1321N1 cells. Confluent cells were labelled for 18 h with 2 μCi ml^{-1} [^{3}H]-inositol. The cells were washed with medium (a) or with medium containing 1 u ml^{-1} apyrase (b) and 10 mM LiCl was added for 15 min before challenge with agonists for an additional 20 min. (c) LiCl and agonist were added to cells without changing the medium. [^{3}H]-inositol phosphates were quantitated as described in Methods. The data represent the mean ± s.d. of three experiments performed with triplicate samples.

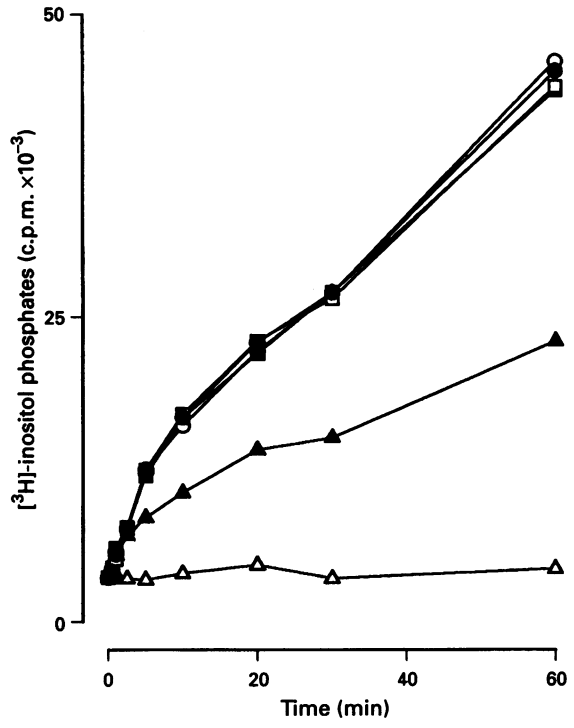

Figure 4 Time course of agonist-stimulated [^{3}H]-inositol phosphate formation in HP2U-1321N1 cells. Confluent cells were labelled for 18 h with 2 μCi ml^{-1} [^{3}H]-inositol, LiCl was added without a change of medium, and after an additional 15 min the cells were incubated without any addition (△) or with 100 μM of either ATP (○), UTP (●), A_2P_4 (□), or ATPγS (■), or with 1 mM carbachol (▲). Incubations were terminated at the times indicated by addition of 5% trichloroacetic acid and the resulting [^{3}H]-inositol phosphates were quantified as indicated in Methods. The data are the mean value from two experiments performed with triplicate samples.

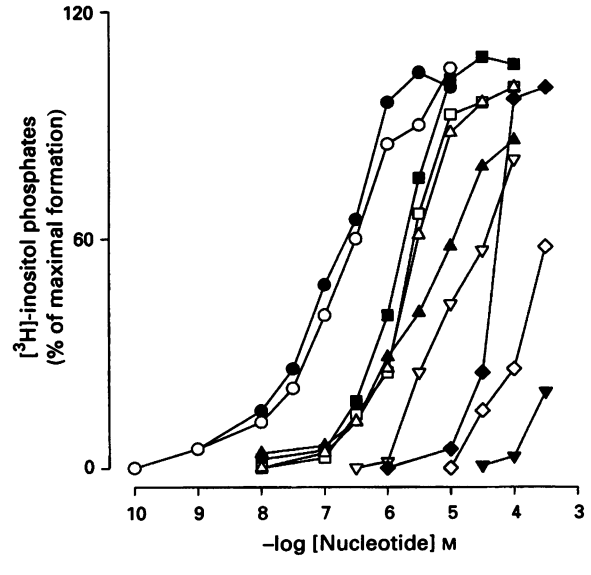

Figure 5 Concentration-effect relationship for nucleotide-stimulated [^{3}H]-inositol phosphate formation in HP2U-1321N1 cells. Confluent cells were labelled with [^{3}H]-inositol and preincubated with LiCl as described in the legend to Figure 4. The cells were incubated for 20 min with the indicated concentrations of UTP (●), ATP (○), ATPγS (■), 5BrUTP (□), 2ClATP (△), AppNHp (▲), 8BrATP (▽), GTPγS (◆), 2MeSATP (◇) or $\alpha\beta$,MeATP (▼). Individual curves represent the mean of 4 to 10 experiments performed with triplicate samples. Data are expressed as percentage of the maximal response obtained with UTP in the same experiment. [^{3}H]-inositol phosphates were quantified as indicated in Methods.

To characterize pharmacologically the human P_{2U}-purinoceptor expressed in 1321N1 cells, concentration-response curves were generated with various nucleotides. The relative potency order for agonist-stimulated inositol phosphate formation was consistent with that previously reported for P_{2U}-purinoceptors. UTP and ATP were the most potent agonists (Figure 5 and Table 3), and the EC_{50} values for these agonists were 10–30 fold lower than those previously reported for the native receptor expressed in various cell types (Dubyak & El-

Table 3 Potency of nucleotides for stimulation of inositol phosphate formation in HP2U-1321N1 cells

Agonist	*EC_{50}* (μM)
UTP	0.14±0.02
ATP	0.23±0.01
Ap_4A	0.72±0.02
ATPγS	1.72±0.15
5BrUTP	2.06±0.04
2ClATP	2.30±0.3
AppNHp	5.66±0.7
GTP	12.3±0.9
UDP	16.5±1.2
8BrATP	23.0±1.6
GTPγS	26.5±1.5
GDP	66.0±3.0

The data are the mean±s.d. from at least three different experiments where concentration-effect curves for each nucleotide were determined with triplicate samples as in Figure 5.

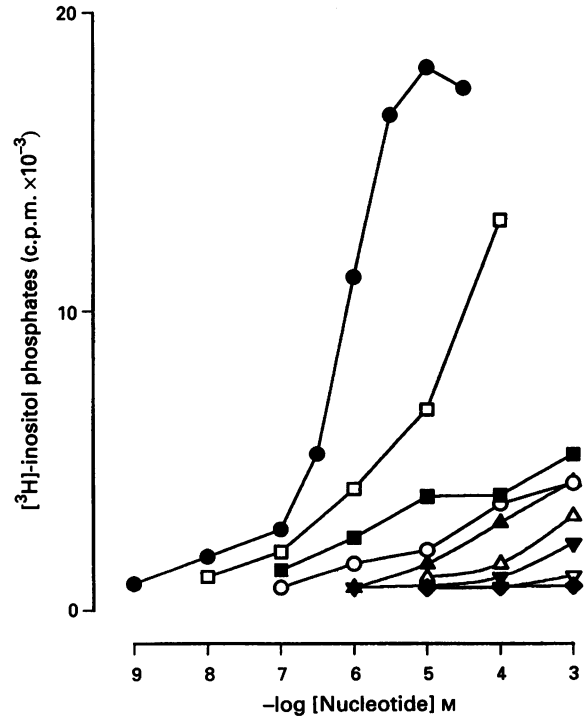

Figure 6 Effect of adenosine dinucleotides on [^{3}H]-inositol phosphate formation in HP2U-1321N1 cells. Cells were labelled and preincubated with LiCl as indicated in the legend of Figure 4. The cells were challenged for 20 min with either Ap_4A (●), Ap_3A (□), Ap_5A (■), Ap_2A (○), NADH (▲), NAD (△), NADP (▼) Ap_6A (▽), or NADH (◆). [^{3}H]-inositol phosphates were quantified as described in Methods. The data represent the mean of either two or three different experiments performed with triplicate samples.

Moatassim, 1993; Fredholm *et al.*, 1994). The hydrolysis-resistant ATP analogues, ATPγS and AppNHp, were also full agonists although 10 and 50 times less potent, respectively, than UTP. The subtype-selective agonists 2MeSADP (P_{2T}-purinoceptors), 2MeSATP (P_{2Y}-purinoceptors), and α,β-MeATP (P_{2X}-purinoceptors) had little (2MeSATP) or no (α,β-MeATP and 2MeSADP) effect on inositol phosphate accumulation at concentrations of 100 μM or less. UDP, which has been shown to stimulate potently a pyrimidine-selective phospholipase C-linked receptor in C6-2B rat glioma cells (Lazarowski & Harden, 1994), also had only a weak effect on HP2U-1321N1 cells. 5BrUTP, which is a full agonist at C6-2B cell uridine nucleotide receptors, and 2ClATP, which is a potent full agonist at P_{2Y}-purinoceptors, were full agonists at the expressed P_{2U}-purinoceptor. 8BrATP, GTP, GTPγS, and GDP were also low potency full agonists (Figure 5 and Table 3). Little or no effect was observed with 1 to 1000 μM AMP, UMP, adenosine, or uridine (data not shown).

Diadenosine polyphosphates are stored and released by a broad range of cell types (Foldgaard & Klenow, 1982; del Castillo *et al.*, 1988; Pintor & Miras-Portugal, 1993). A putative purinoceptor, termed the P_{2D}-purinoceptor, has been reported to display high affinity for diadenosine tetraphosphate and other diadenosine polyphosphates and to be present on chromaffin cells and neural tissue (Hilderman *et al.*, 1991; Pintor *et al.*, 1993; Pintor & Miras-Portugal, 1993). Ap_4A had no effect on inositol phosphate formation in wild type 1321N1 cells or in vector-infected cells (data not shown). However, addition of diadenosine polyphosphates to HP2U-1321N1 cells resulted in a time (Figure 4) and concentration- (Figure 6) dependent stimulation of inositol phosphate formation. Ap_4A (EC_{50} value = 720 ± 20 nM) was the most potent agonist of the adenine-containing dinucleotides tested, and was also more potent than ATPγS and only slightly less pontent than ATP. The effects of Ap_4A were not modified in the presence of apyrase (data not shown). Thus, Ap_4A is not converted to breakdown products, e.g., ATP, to stimulate inositol phosphate formation, and ATP is not present as an activating contaminant in the preparation of Ap_4A. The effects of a maximally effective concentration of Ap_4A (100 μM) were not additive with those of UTP (control, 4748 ± 622; UTP, 39365 ± 7635; Ap_4A, 38765 ± 1741; UTP + Ap_4A, 37061 ± 2161; c.p.m. ± s.d., $n = 3$). Ap_3A appeared to be a full agonist but 100 times less potent than Ap_4A. Ap_5A had relatively weak effects on inositol phosphate accumulation and only 25% of the maximal response to UTP was obtained with as high as 1 mM Ap_5A (Figure 6). No effect of Ap_5A was observed on either UTP or Ap_4A-stimulated inositol phosphate formation in HP2U-1321N1 cells (data not shown), although the low potency of Ap_5A made difficult any rigorous analysis of this diadenosine polyphosphate as a potential partial agonist on P_{2U}-purinoceptors.

Experiments were carried out to examine whether desensitization occurred with the heterologously expressed human P_{2U}-purinoceptor. HP2U-1321N1 cells were preincubated for 30 min with either ATPγS or Ap_4A. The initial incubation was carried out in the absence of LiCl to prevent accumulation of inositol phosphates. The cells were rapidly washed twice with medium containing apyrase and the inositol phosphate response to a second challenge was subsequently measured after co-addition of agonist and LiCl at various times. Preincubation of the cells with ATPγS or Ap_4A resulted in a 50% reduction in inositol phosphate formation in response to subsequent challenge with either ATPγS or Ap_4A (Figure 7). Thus, cross-desensitization occurred between ATPγS and Ap_4A-promoted responses, which further illustrates that Ap_4A interacts with the P_{2U}-purinoceptor. Responsiveness to nucleotides recovered rapidly in HP2U-1321N1 cells following removal of agonists and values near control cell responses were observed between 10 and 30 min after removal of the desensitizing nucleotide.

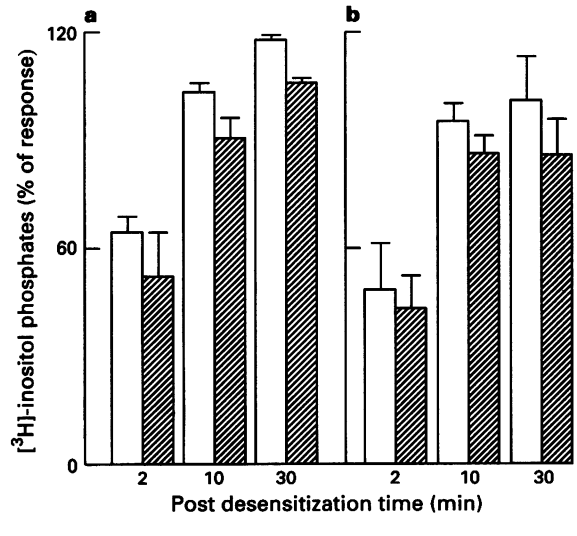

Figure 7 Desensitization and recovery of agonist-stimulated [^{3}H]-inositol phosphate formation in HP2U-1321N1 cells. Confluent cells were labelled for 18 h with [^{3}H]-inositol, washed and incubated for 30 min with 0.5 ml DMEM/HEPES containing 1 iu ml^{-1} of apyrase and 100 μM of either ATPγS (a) or Ap_4A (b). The cells were rapidly washed twice with medium containing apyrase and at the times indicated 100 μM of either ATPγS (open columns) or Ap_4A (hatched columns) was added in combination with 10 mM LiCl. After an additional 20 min incubation, trichloroacetic acid was added, and [^{3}H]-inositol phosphates were quantified as indicated in Methods. The data are expressed as a percentage of response obtained with each agonist in control cells, and they represent the mean value ± s.d. of two different experiments performed with triplicate samples.

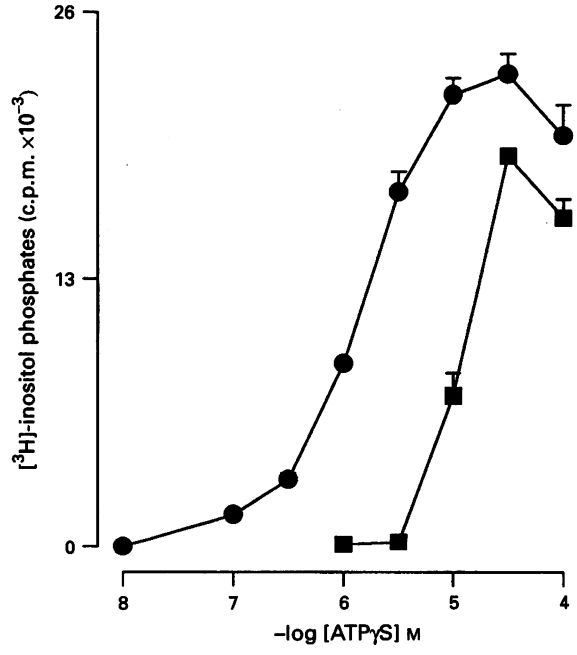

Figure 8 Concentration-effect relationship for ATPγS-stimulated [^{3}H]-inositol phosphate formation in desensitized HP2U-1321N1 cells. Confluent cells were labelled with [^{3}H]-inositol for 18 h in the presence (■) or absence (●) of ATPγS (100 μM) during the last 8 h of labelling. The cells were washed twice with medium containing apyrase and subsequently challenged for 20 min with the indicated concentration of ATPγS in combination with 10 mM LiCl. The data represent the mean of two experiments performed with triplicate samples. [^{3}H]-inositol phosphates were measured as indicated in Methods.

One potential explanation for the high potency of agonists observed in this study relative to potencies reported previously for the same agonists at natively expressed P_{2U}-purinoceptors receptors, is that overexpression of P_{2U}-purinoceptors results in receptor reserve in HP2U-1321N1 cells. To address this possibility, concentration-effect curves for ATPγS-stimulated inositol phosphate formation were generated with cells previously incubated with 100 μM ATPγS for a time (8 h) that ostensibly should lead to receptor down-regulation. A marked shift to the right of the concentration-effect curve for ATPγS-stimulated inositol phosphate formation was observed in cells subjected to an 8 h preincubation (Figure 8). This effect was accompanied by a small decrease in the maximal effect observed with ATPγS; preincubation for 24 h with ATPγS resulted in an essentially complete loss of responsiveness to agonist (data not shown). These results are consistent with the presence of considerable receptor reserve for P_{2U}-purinoceptors in HP2U cells.

Discussion

The stable expression of P_{2U}-purinoceptors in a null cell line has allowed a detailed pharmacological analysis of a receptor of defined structure that potentially represents a therapeutically important target in human airway disease and in pathophysiological conditions associated with other tissues. Two major observations were made in this study. The first involves release of endogenous ATP from 1321N1 human astrocytoma cells and the second involves the surprising potency of the diadenosine polyphosphate compound, Ap_4A for activation of the human P_{2U}-purinoceptor.

Changes of medium resulted in release of ATP from 1321N1 human astrocytoma cells in quantities sufficient to activate the heterologously expressed P_{2U}-purinoceptor. These results are consistent with previous reports illustrating that shear or mechanical stimulation causes release of ATP from a number of tissues (Forrester, 1972; Pearson & Gordon, 1979; Bodin *et al.*, 1991; Osipchuk & Cahalan, 1992). Release of ATP from 1321N1 cells is apparently independent of mobilization of Ca^{2+} since the muscarinic receptor agonist, carbachol, did not promote nucleotide release. This conclusion is in contrast to previous suggestions that non-cytolytic release of nucleotides from cells is linked to changes in second messenger molecules such as Ca^{2+} (Gordon, 1986; Yang *et al.*, 1994). The data do not rule out the possibility that an unknown receptor-active molecule is released during medium changes that promotes nucleotide release through a mechanism not involving Ca^{2+}.

The physiological roles of astrocytes in brain have not been clearly established although 'buffering' of extracellular medium and release of neuroactive substances appear to be important (Walz, 1989; Martin, 1992). Primary cultures of astrocytes express P_2-purinoceptors (Bruner & Murphy, 1990; Kastritsis *et al.*, 1992). Whether autocrine release of ATP and activation of endogenous P_2-purinoceptors on astrocytes is of physiological significance represents a potentially important biological question.

The release of endogenous ATP has not been extensively considered in most pharmacological and biochemical studies of P_2-purinoceptors, and therefore, the extent to which this phenomenon has been a confounding factor in previous studies of P_2-purinoceptors is unclear. We have tested this possibility in a number of cell lines, i.e. CF/T43 human airway epithelial cells and HT29 human colon carcinoma cells, that express endogenous P_{2U}-purinoceptors. Little or no release of ATP occurs during medium change or other manipulation of these cells (E.R. Lazarowski, unpublished observations). However, a sustained basal accumulation of inositol phosphates has been reported to occur in bovine aortic endothelial cells following a medium change. This effect, together with the fact that the potency of nucleotides for stimulation of inositol phosphate formation was low in these cells (Purkiss *et al.*, 1994) suggests that release of endogenous nucleotides and a consequential modification of P_2-purinoceptor responsiveness may have occurred.

Activation of the human P_{2U}-purinoceptor stably expressed in 1321N1 human astrocytoma cells occurred with a potency order that was consistent with that observed for P_{2U}-purinoceptors in tissues ranging from airway epithelial cells to circulating monocytes to pituitary lactotrophs. However, the apparent potencies for activation of the expressed human receptor were 10–30 fold higher than those published previously for the native receptor, i.e. much lower EC_{50} values were observed for each of the agonists tested. One possibility is that the endogenous receptors previously studied on cell lines existed in a desensitized state due to receptor activation as a consequence of release of nucleotide. This possibility has not been ruled out for all previously used test systems, although the receptor on CF/T43 human airway epithelial cells and on HT29 human colon carcinoma cells is not under the influence of endogenously released ATP (E.R. Lazarowski, unpublished data). Thus, other explanations are needed for the increase in potency of agonists in the HP2U-1321N1 cells.

The existence of receptor reserve would account for the increased apparent potency of agonists in 1321N1 cells stably expressing the human P_{2U}-purinoceptor. Ample precedent exists for attainment of very high levels of heterologously expressed receptors under typical protocols with mammalian expression vectors. The corresponding agonist potencies for activation of these receptors lie well to the left of those of the same agonists for activation of the native receptors (Kenakin *et al.*, 1992). Unfortunately, a radioligand binding assay is not available for quantification of P_{2U}-purinoceptor levels in HP2U-1321N1 cells. Nevertheless, extended incubation with ATPγS prior to challenge with agonist produced results that are consistent with the occurrence of 'spare' receptors in these cells. Concentration-effect curves for agonists were shifted by 10–20 fold to the right with a minor change in maximal effect as a consequence of an 8 h agonist preincubation and presumably down-regulation of P_{2U}-purinoceptors. The potencies of agonists observed in ATPγS-preincubated cells were in the range of those for these same agonists determined in cells expressing native receptors. These results can be contrasted with similar experiments previously reported with native P_{2U}-purinoceptors on CF/T43 airway epithelial cells (Brown *et al.*, 1991). Agonist-preincubation resulted in a decrease in maximal effect observed during subsequent agonist rechallenge with no appreciable effect occurring in the apparent potency of agonist for receptor stimulation. That is, there was no evidence of receptor reserve in CF/T43 cells, and EC_{50} values for stimulation of inositol phosphate accumulation by P_{2U}-purinoceptor agonists were 30 fold greater than the values determined for the corresponding agonists in HP2U-1321N1 cells. The availability of a receptor-selective irreversible antagonist would allow more unequivocal delineation of the reasons for the high apparent potency of agonists at the expressed human P_{2U}-purinoceptor.

The shift to the left of agonist concentration effect curves with the expressed P_{2U}-purinoceptor allowed a more detailed study of agonists of relative low potency than was previously possible. Thus, we were able to demonstrate that, even in this P_{2U}-purinoceptor test system exhibiting a 30 fold increase in 'sensitivity', 2MeSATP, 2MeSADP, and α,β-MeATP, which are agonists for P_{2Y}-, P_{2T}-, and P_{2X}-purinoceptors, respectively, had essentially no effect. In contrast, several compounds, most notably 2ClATP, 5BrUTP, 8BrATP, and GTP, were full agonists under the conditions of this test system.

A surprising aspect of the pharmacological analysis of the cloned human P_{2U}-purinoceptor is the remarkable potency observed with Ap_4A. This compound is one of a group of diadenosine polyphosphates that are synthesized and released from a variety of tissues including neurones, chromaffin cells, and platelets (Foldgaard & Klenow, 1982; del Castillo *et al.*, 1988; Pintor & Miras-Portugal, 1993; Pintor *et al.*, 1995). Neither the mode of regulation of synthesis and release nor the

physiological significance of the extracellular presence of these molecules has been established. However, Miras-Portugal and coworkers have made a strong case for the existence of a specific population of binding sites for the diadenosine polyphosphates and have termed the idea of a putative P_{2D}-purinoceptor that subserves a signalling role involving these molecules (Pintor & Miras-Portugal, 1993; 1995; Miras-Portugal *et al.*, 1994). No second messenger- or ion-channel regulating activity has been unambiguously demonstrated for these molecules, although high concentrations of several diadenosine polyphosphates have been shown to mobilize Ca^{2+} in chromaffin and endothelial cells (Castro *et al.*, 1992; 1994; Pintor & Miras-Portugal, 1993). Ap_4A activates human P_{2U}-purinoceptors with a potency in the range of that of ATP and UTP, suggesting that Ap_4A may be an endogenous regulator of P_{2U}-purinoceptors. Indeed, Ap_4A might stand as a more likely regulator in tissues where its release is well-documented relative to the dearth of evidence suggesting any physiological release of UTP. Whether interaction of Ap_4A with P_{2U}-purinoceptors explains most or even all of its actions in tissues where a putative P_{2D}-purinoceptor has been proposed to be the involved entity will need to be defined. Although we cannot totally rule out the possibility that an endogenous P_{2D}-like receptor was selected during sub-cloning the HP2U-1321N1 cells, our data showing lack of additivity between Ap_4A and UTP, and cross-desensitization data strongly suggest that Ap_4A is a P_{2U}-purinoceptor agonist. Since Ap_4A is not subject to the high rate of nucleotidase-catalyzed hydrolysis that is the case for ATP and UTP, the diadenosine polyphosphates or analogues of these compounds could find considerable therapeutic value.

In summary, the availability of cloned human P_{2U}-purinoceptors stably expressed in a human tumour cell line has allowed a pharmacological analysis of a potentially therapeutically important signalling protein of defined function. The high sensitivity of the cloned cell line to P_{2U}-receptor agonists as well as the identification of a diadenosine polyphosphate as a potent P_{2U}-purinoceptor agonist should serve as springboards to identification of stable, receptor-selective, high potency P_{2U}-purinoceptor agonists. This system also provides a sensitivity that should permit clearer identification of partial agonist structures that may provide lead compounds for synthesis of heretofore unavailable P_{2U}-purinoceptor antagonists.

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