0026-895X/00/050926-06$3.00/0
MOLECULAR PHARMACOLOGY

MOL 57:926–931, 2000 /12988/817849

ATP, an Agonist at the Rat $P2Y_4$ Receptor, Is an Antagonist at the Human $P2Y_4$ Receptor

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Received September 20, 1999; accepted January 18, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The nucleotide selectivities of the human $P2Y_4$ ($hP2Y_4$) and rat $P2Y_4$ ($rP2Y_4$) receptor stably expressed in 1321N1 human astrocytoma cells were determined by measuring increases in intracellular [Ca^{2+}] under conditions that minimized metabolism, bioconversion, and endogenous nucleotide release. In cells expressing the $hP2Y_4$ receptor, UTP, GTP, and ITP all increased intracellular [Ca^{2+}] with a rank order of potency of UTP (0.55) > GTP (6.59) = ITP (7.38), (EC_{50}, μM). ATP, CTP, xanthine 5′-triphosphate (XTP), and diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A), all at 100 μM, were inactive at the $hP2Y_4$ receptor. In cells expressing the $rP2Y_4$ receptor, all seven nucleotides increased intracellular [Ca^{2+}] with similar maximal effects and a rank order of potency of UTP (0.20) > ATP (0.51) > Ap_4A (1.24) ≈ ITP (1.82) ≈ GTP (2.28) > CTP (7.24) > XTP (22.9). Because ATP is inactive at the $hP2Y_4$ receptor, we assessed whether ATP displayed antagonist activity. When coapplied, ATP shifted the concentration-response curve to UTP rightward in a concentration-dependent manner, with no change in the maximal response. A Schild plot derived from these data gave a pA_2 value of 6.15 (K_B = 708 nM) and a slope near unity. Additionally, CTP and Ap_4A (each at 100 μM) inhibited the response to an EC_{50} concentration of UTP by ~40 and ~50%, respectively, whereas XTP had no effect. The inhibitory effects of ATP, CTP, and Ap_4A were reversible on washout. Thus, ATP is a potent agonist at the $rP2Y_4$ receptor but is a competitive antagonist with moderate potency at the $hP2Y_4$ receptor.

P2Y receptors are G protein-coupled receptors activated by extracellular nucleotides. Molecular cloning and characterization studies have identified five functional human P2Y (hP2Y) receptor subtypes ($hP2Y_{1,2,4,6,11}$). All five P2Y receptors are linked to activation of phospholipase C, generation of inositol phosphates, and release of intracellular Ca^{2+} stores (North and Barnard, 1997; Harden, 1998; King et al., 1998). In addition, the $hP2Y_{11}$ receptor also promotes activation of adenylyl cyclase and accumulation of cAMP (Communi et al., 1997). Because there are few subtype-selective antagonists, the pharmacologic characterization of P2Y receptors has relied on the rank order of potency of the natural agonists ATP, ADP, UTP, UDP, and some structural analogs.

Several factors can confound the pharmacologic characterization of P2Y receptors. These include the purity of nucleotides, the presence of ecto-nucleotidase activity, the release of ATP and UTP from cells after mechanical stimulation or change in medium, and the bioconversion of nucleotides by ecto-nucleoside diphosphokinase (NDPK) activity (Kennedy and Leff, 1995; Lazarowski et al., 1995; Nicholas et al., 1996; Zimmermann, 1996; Harden et al., 1997; Lazarowski et al., 1997a,b,c; Leon et al., 1997). Furthermore, the level of receptor reserve can influence agonist activity. Notably, ATP is a partial agonist at the $hP2Y_1$ receptor with a maximal response of 80% that of the full agonist ADP when expressed at high levels in 1321N1 cells (Palmer et al., 1998). Desensitization of the $hP2Y_1$ receptor abolished responses to ATP, as predicted for a partial agonist, but shifted rightward the concentration-response curve to ADP with little decrease in the maximum response, as predicted for a full agonist. Furthermore, ATP inhibited the response to ADP in desensitized cells. Thus, under conditions of low receptor reserve, the partial agonist ATP has no agonist activity, but instead acts as an antagonist. This may explain reports that ATP inhibits responses to ADP at $hP2Y_1$ receptors when expressed in Jurkat cells (Leon et al., 1997; Hechler et al., 1998).

The confounding factors mentioned above also are evident with $P2Y_4$ receptors, especially when assaying cells at high density in static bathing solutions. For example, UDP was at first reported to be a full agonist at the $hP2Y_4$ receptor (Communi et al., 1995). Subsequently, it was found to be virtually inactive when UDP solutions were made UTP-free

This work was supported by United States Public Health Service Grant GM38213 (T.K.H., R.A.N.) and grants from The Wellcome Trust and The Caledonian Research Foundation (C.K.). During this study, R.A.N. was an Established Investigator of the American Heart Association.

ABBREVIATIONS: $hP2Y_4$, human $P2Y_4$ receptor; Ap_4A, diadenosine 5′, 5‴-P^1,P^4-tetraphosphate; $rP2Y_4$, rat $P2Y_4$ receptor; XTP, xanthine 5′-triphosphate; NDPK, nucleoside diphosphokinase.

and when accumulation of UTP via NDPK activity was prevented (Nicholas et al., 1996). Such factors may also underlie the discrepancies in the literature regarding the effects of ATP at the $P2Y_4$ receptor. When inositol phosphate accumulation was measured, UTP was a potent, full agonist at the $hP2Y_4$ receptor, whereas ATP was reported to be either a full agonist with low potency (Nicholas et al., 1996) or a partial agonist (Communi et al., 1995, 1996; Harper et al., 1998). However, ATP was inactive when the release of intracellular $[Ca^{2+}]$ stores was monitored (Nguyen et al., 1995; Lazarowski et al., 1997a,b). The most likely explanation for this discrepancy is that in static medium, ATP serves as a γ-phosphate donor for ecto-NDPK to convert UDP accumulated in the medium to UTP.

Recently, two separate reports detailed the cloning and nucleotide selectivity of the rat $P2Y_4$ ($rP2Y_4$) receptor (Bogdanov et al., 1998; Webb et al., 1998). The rat receptor has 83% sequence identity with its human homologue (90% in transmembrane regions and extracellular loops) and is expressed in a wider range of tissues than is the $hP2Y_4$ receptor. In contrast to the $hP2Y_4$ receptor, both UTP and ATP were reported to be full agonists at the $rP2Y_4$ receptor when intracellular Ca^{2+} mobilization was measured, either directly (Webb et al., 1998) or indirectly (Bogdanov et al., 1998). Furthermore, ITP and Ap_4A were shown to activate the $rP2Y_4$ receptor when expressed in *Xenopus* oocytes (Bogdanov et al., 1998).

The aim of this study was to compare and contrast the nucleotide selectivities of $hP2Y_4$ and $rP2Y_4$ receptors expressed in the same cell line under conditions in which the complicating factors described above were minimized or eliminated. Throughout this study, we have used pure nucleotides and have minimized the influence of nucleotide breakdown and bioconversion of agonists by measuring intracellular $[Ca^{2+}]$ in a field of continuously and rapidly superfused single cells expressing either the $hP2Y_4$ or the $rP2Y_4$ receptor. Our data indicate that $rP2Y_4$ and $hP2Y_4$ receptors display markedly different selectivities for a wide range of nucleotides. Furthermore, we show that ATP, although an agonist at the $rP2Y_4$ receptor, acts as an antagonist at the $hP2Y_4$ receptor. Preliminary accounts of these results have been published (Kennedy et al., 1999; Qi et al., 1999).

Experimental Procedures

Materials. AmpliTaq DNA polymerase and the Amplicycle sequencing kit were obtained from Perkin-Elmer Cetus, (Norfolk, CT). All tissue culture reagents and Hanks' balanced salt solution were supplied by the Lineberger Comprehensive Cancer Center tissue culture facility (University of North Carolina, Chapel Hill, NC). Fura-2/AM ester was obtained from Molecular Probes (Eugene, OR). Sodium salts ATP, UTP, CTP, and GTP (Pharmacia, Piscataway, NJ) and sodium salts ITP and XTP (Sigma, St. Louis, MO) contained no other contaminating nucleotides. Ap_4A (Sigma) was treated with apyrase before use. ADP and UDP were from Boehringer Mannheim Biochemicals (now Roche Molecular Biochemicals, Indianapolis, IN). Stock solutions of ADP and UDP in Dulbecco's modified Eagle's medium high glucose were treated for 2 h immediately before use with 50 and 250 U/ml hexokinase, respectively.

Polymerase Chain Reaction (PCR) Amplification of the Coding Sequence of the $rP2Y_4$ Receptor. PCR primers complementary to the published sequence of the $rP2Y_4$ receptor (Bogdanov et al., 1998) were used to amplify the coding sequence from 0.18 μg of rat genomic DNA using AmpliTaq DNA polymerase. The PCR primers contained at their 5′ ends either an *Eco*RI restriction site (5′-GAGAGAATTCTACTTGTAGGGGGCCATGA-3′; upstream primer) or an *Xho*I restriction site (5′-GAGACTCGAGTCATATC-CAGCAGCAGGGTT-3′; downstream primer) and were designed to include 15 and 18 base pairs, respectively, of untranslated sequence at the 5′- and 3′-ends of the amplified fragment. The amplification conditions were 94°C for 3 min; 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 70 s; and a final extension for 7 min at 72°C. The amplified product was purified, digested with *Eco*RI and *Xho*I, and ligated into the similarly digested retroviral expression vector pLXSN. An individual clone encoding the receptor was sequenced using the Amplicycle sequencing kit and found to be identical with that reported by Bogdanov et al. (1998). Amplification of the $hP2Y_4$ receptor from genomic human DNA and its ligation into the pLXSN vector has been described previously (Nicholas et al., 1996).

Expression of $hP2Y_4$ and $rP2Y_4$ Receptors in 1321N1 Cells. Recombinant retrovirus particles were produced by calcium phosphate-mediated transfection of PA317 cells with the pLXSN vector containing the appropriate receptor sequence (Nicholas et al., 1996; Comstock et al., 1997). 1321N1 human astrocytoma cells, which show no functional responses to P2Y receptor agonists, were grown in monolayer culture at 37°C in 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. The cells were infected with retrovirus harboring the $hP2Y_4$ or $rP2Y_4$ coding sequence or with control retrovirus. Geneticin-resistant cells were selected after 2 weeks with 1 mg/ml G-418 and then were maintained in medium containing 0.4 mg/ml G-418.

Intracellular $[Ca^{2+}]$ Measurements. Intracellular $[Ca^{2+}]$ was quantified as described previously (Palmer et al., 1998). 1321N1 cells stably expressing the $hP2Y_4$ or $rP2Y_4$ receptor were grown on glass coverslips for 1 to 3 days to a density approximately 20% of confluence. Coverslips containing 750 nM Fura-2/AM-loaded cells were encased in an acrylic chamber (200 μl volume) and superfused at 1.4 ml/min with Hanks' buffered saline solution (+ Ca^{2+}, Mg^{2+}). The flow-through chamber was secured to the stage of a Nikon TMS inverted fluorescence microscope retrofitted for use with epifluorescence. Cells were exposed to alternating excitation wavelengths of 340 and 380 nm from a 300 W Xenon lamp, and fluorescence emission at 510 nm was monitored by an integrating CCD camera. The 340/380 nm fluorescence emission ratio was determined and converted to intracellular $[Ca^{2+}]$ concentration by comparing ratios to a standard curve. Agonists were applied for 30 s in the superfusate via a valve attached to a six-well reservoir, and the change in intracellular $[Ca^{2+}]$ was measured in 9 to 16 individual cells per coverslip and averaged. To generate concentration-effect curves, each concentration of nucleotide was applied only once to each individual coverslip (to avoid receptor desensitization), and the average response from 9 to 16 cells/coverslip was measured from 4 to 6 coverslips. Data were recorded and processed using an InCyt IM2 (Intracellular Imaging Inc., Cincinnati, OH) digital imaging system.

Statistics. Data in the text are expressed as the mean ± S.E. or geometric mean with 95% confidence limits for EC_{50} values. Vertical lines in graphs indicate S.D. of the mean and have been omitted in some cases for clarity. Concentration-response curves were fitted to the data by logistic (Hill equation), nonlinear regression analysis (GraphPad Prism, San Diego, CA). Data were compared using one-way ANOVA and Tukey's comparison or by Student's paired t test, with $P < .05$ considered to be statistically significant.

Results

Agonist Activity of Nucleoside Triphosphates and Ap_4A at $hP2Y_4$ and $rP2Y_4$ Receptors. When applied in the superfusate for 30 s, UTP, GTP and ITP (100 μM) all evoked

a rapid, reversible rise in intracellular [Ca^{2+}] with a similar time course (Fig. 1A). In general, reproducible responses could be obtained if agonists were applied at 5-min intervals, although at higher concentrations of agonist some desensitization was observed. When UTP was applied for 5 min, the intracellular [Ca^{2+}] returned to baseline in the continued presence of UTP and did not oscillate (data not shown).

To generate concentration-response curves, intracellular Ca^{2+} levels evoked by agonists were averaged from 9 to 16 cells/coverslip, and the values from 4 to 6 coverslips were averaged for each concentration of nucleotide. UTP, GTP, and ITP each increased intracellular [Ca^{2+}] in a concentration-dependent manner, with a rank order of potency of UTP > GTP ≈ ITP (Fig. 1B; Table 1). Each agonist also evoked a similar maximum increase in intracellular [Ca^{2+}] (90–105 nM). In contrast, ATP, CTP, XTP, and Ap_4A were all without effect at 100 μM (Fig. 1A). Cells infected with control pLXSN virus showed no response to any of the nucleotides (data not shown).

In contrast to cells expressing the $hP2Y_4$ receptor, all seven nucleotides tested evoked a rapid, reversible rise in intracellular [Ca^{2+}] with a similar time course in cells expressing the $rP2Y_4$ receptor (Fig. 2A). Each nucleotide acted in a concentration-dependent manner with a rank order of potency of UTP > ATP > Ap_4A ≈ ITP ≈ GTP > CTP > XTP (Fig. 2B; Table 1). The maximum increase in intracellular [Ca^{2+}] evoked by each nucleotide was similar (102–118 nM).

Agonist Effects of Nucleoside Diphosphates at the $hP2Y_4$ and $rP2Y_4$ Receptors. We also tested the agonist effects of the nucleoside diphosphates ADP and UDP. We have reported previously that when measuring inositol phosphate accumulation, UDP is inactive at the $hP2Y_4$ receptor if care is taken to remove contaminating UTP by treating UDP stock solutions with hexokinase and glucose (Nicholas et al., 1996). Consistent with these data, UDP at both 10 and 100 μM was essentially inactive at the $hP2Y_4$ receptor when

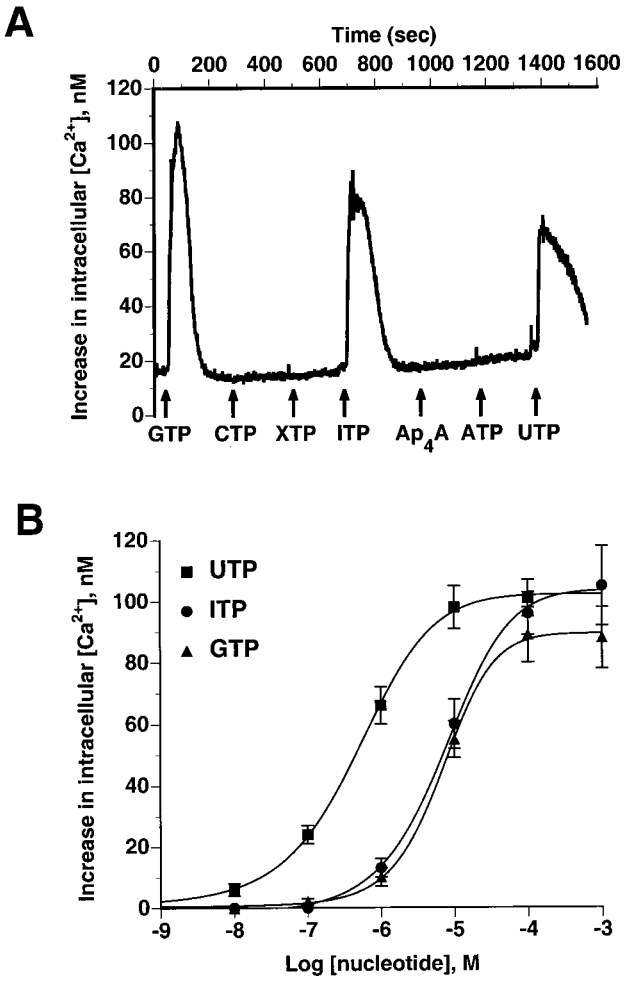

Fig. 1. The capacity of nucleotides to increase intracellular [Ca^{2+}] in 1321N1 cells expressing the $hP2Y_4$ receptor. A, averaged increases in intracellular [Ca^{2+}] from 9 to 16 cells on one coverslip superfused for 30 s with 100 μM concentrations of the indicated nucleotides. B, concentration-response relationship of the average peak increase in intracellular [Ca^{2+}] evoked by UTP, ITP, and GTP. Each point was determined from a minimum of four coverslips, with 9 to 16 cells monitored per coverslip, as described in *Experimental Procedures*. Vertical lines indicate S.D. of the mean and have been omitted in some cases for clarity.

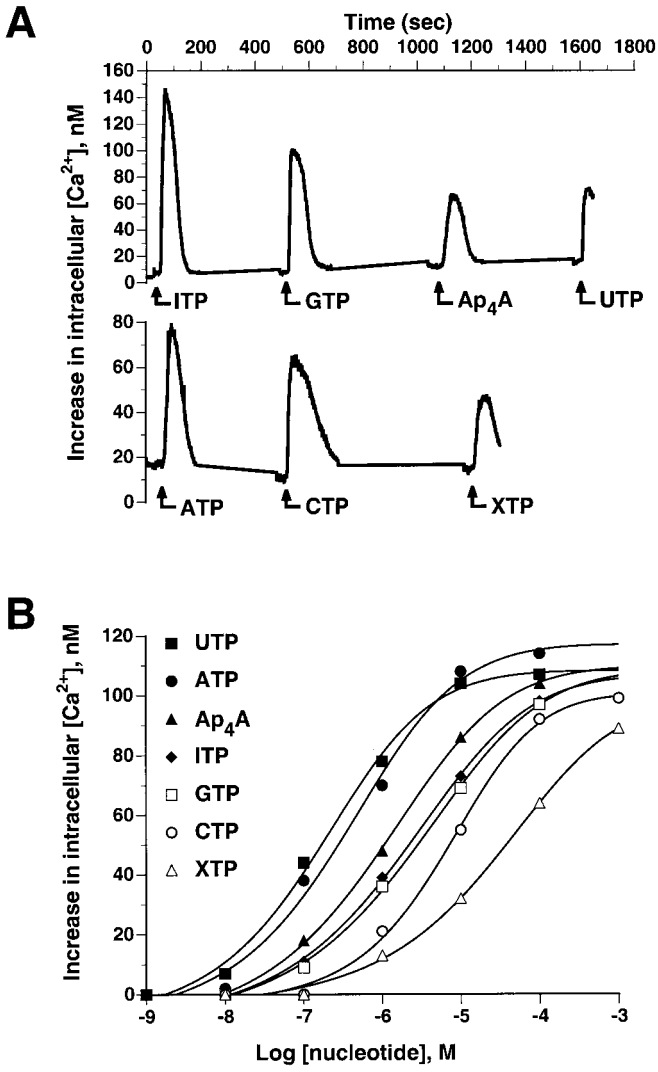

Fig. 2. The capacity of nucleotides to increase intracellular [Ca^{2+}] in 1321N1 cells expressing the $rP2Y_4$ receptor. A, averaged increases in intracellular [Ca^{2+}] from 9 to 16 cells on one coverslip superfused for 30 s with 100 μM concentrations of the indicated nucleotides. B, concentration-response relationship of the average peak increase in intracellular [Ca^{2+}] evoked by the indicated nucleotides. Each point was determined from a minimum of four coverslips, with 9 to 16 cells monitored per coverslip, as described in *Experimental Procedures*.

assessed by measuring increases in intracellular [Ca^{2+}], and this lack of activity also was observed in cells expressing the $rP2Y_4$ receptor (Fig. 3). In contrast, ADP at 10 and 100 μM showed statistically significant partial agonist activity at both the $hP2Y_4$ (15% of maximum) and the $rP2Y_4$ (34% of maximum) receptors (Fig. 3). Thus, ADP (but not UDP) weakly activates both species homologues of the $P2Y_4$ receptor.

Antagonist Effects of Nucleoside Triphosphates at the $hP2Y_4$ and $rP2Y_4$ Receptors. Because ATP, Ap_4A, CTP, and XTP were full agonists at the $rP2Y_4$ receptor but failed to activate the $hP2Y_4$ receptor, we investigated whether these nucleotides could act as antagonists at the $hP2Y_4$ receptor. Cells expressing the $hP2Y_4$ receptor were superfused with one of the four nucleotides at either 10 or 100 μM for 1 min, followed by coadministration of 100 nM UTP, a concentration close to its EC_{50} value at the $hP2Y_4$ receptor. Figure 4 shows that at these concentrations, only ATP substantially inhibited the response to UTP. CTP and Ap_4A were much less effective and, at 100 μM, inhibited the response to UTP by ~40 and ~50%, respectively, whereas XTP had no effect. The inhibitory effects of ATP, CTP, and Ap_4A were reversible on washout.

The inhibitory action of ATP was then studied in greater detail. Coapplication of increasing concentrations of ATP (3–100 μM) to cells expressing the $hP2Y_4$ receptor produced a progressive rightward shift of the concentration-response curve to UTP, with no change in the maximum response (Fig. 5A). A Schild plot derived from these data (Fig. 5B) could be fit by a straight line with a slope near unity (0.94) and yielded a pA_2 value of 6.15 (K_B = 708 nM). Thus, ATP appears to act as a competitive antagonist with moderate potency at the $hP2Y_4$ receptor.

Discussion

The results of this study show that the nucleotide selectivities of the $hP2Y_4$ and $rP2Y_4$ receptors are markedly different. Whereas only UTP, GTP, and ITP are full agonists at both receptors, the $rP2Y_4$ receptor is also activated by ATP, Ap_4A, CTP, and XTP. Thus, the $rP2Y_4$ receptor is activated by virtually all common nucleoside triphosphates, but the $hP2Y_4$ receptor has a restricted nucleotide selectivity. In addition, whereas ATP is a reasonably potent agonist at the $rP2Y_4$ receptor, it acts as a competitive antagonist at the $hP2Y_4$ receptor. Our data are consistent with previous reports that UTP and ATP are full, equipotent agonists at the $rP2Y_4$ receptor (Bogdanov et al., 1998; Webb et al., 1998) and that UTP is a full agonist at the $hP2Y_4$ receptor (Communi et al., 1995, 1996; Nguyen et al., 1995; Nicholas et al., 1996; Harper et al., 1998). However, this is the first demonstration that ATP can act as a competitive antagonist at the $hP2Y_4$ receptor.

In this study, the release of intracellular Ca^{2+} stores evoked by extracellular nucleotides was monitored under conditions in which potential complicating factors, such as release of endogenous ATP and UTP and the extracellular metabolism and bioconversion of nucleotides, were minimized. Previous studies using inositol phosphate production as a measure of activity at the $hP2Y_4$ receptor have reached the very different conclusion that ATP is either a full agonist

TABLE 1

Potency of nucleotides at $hP2Y_4$ and $rP2Y_4$ receptors for increasing intracellular [Ca^{2+}]

Values shown are EC_{50} (95% confidence limits) in μM units for each nucleotide.

Nucleotide	$hP2Y_4$	$rP2Y_4$
UTP	0.55 (0.27–1.15)	0.20 (0.02–1.77)
ATP	N.E.	0.51 (0.03–7.54)
Ap_4A	N.E.	1.24 (0.24–6.40)
GTP	6.59 (4.15–10.5)	2.28 (0.25–20.0)
ITP	7.38 (6.09–8.93)	1.82 (0.26–12.7)
CTP	N.E.	7.24 (3.20–16.4)
XTP	N.E.	22.9 (5.64–93.0)

N.E., no effect at 100 μM.

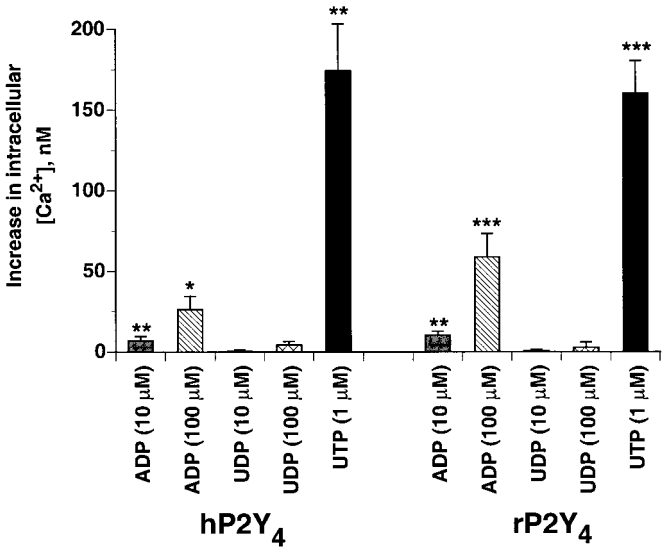

Fig. 3. Agonist activities of ADP and UDP. Increases in intracellular [Ca^{2+}] were monitored in 1321N1 cells expressing either the $hP2Y_4$ or $rP2Y_4$ receptor after superfusion with hexokinase-treated ADP and UDP at 10 or 100 μM. Each bar represents the mean response from three separate measurements, with 9 to 16 cells monitored per measurement. *$P \leqq .05$, **$P \leqq .01$, and ***$P \leqq .001$, relative to control responses.

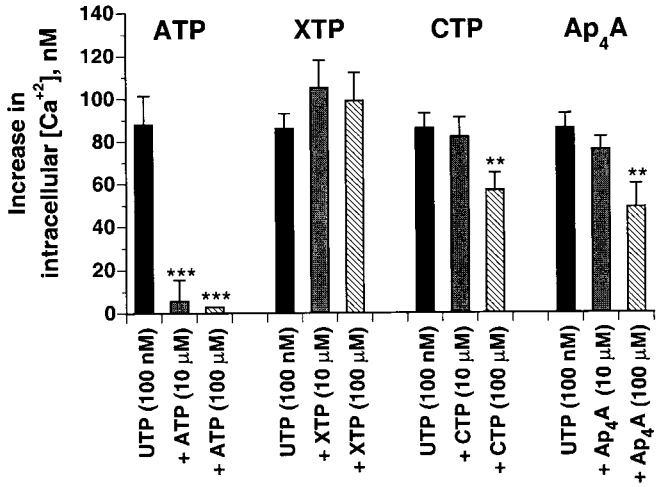

Fig. 4. Antagonist activities of ATP, CTP, XTP, and Ap_4A at the $hP2Y_4$ receptor. The capacity of 10 and 100 μM of the indicated nucleotides to antagonize the increase in intracellular [Ca^{2+}] evoked by UTP (100 nM) in 1321N1 cells expressing the $hP2Y_4$ receptor was measured as described in *Experimental Procedures*. Each bar represents the mean response from three separate measurements, with 9 to 16 cells monitored per measurement. *$P \leqq .05$, **$P \leqq .01$, and ***$P \leqq .001$, relative to control responses.

with low potency (Nicholas et al., 1996) or a partial agonist (Communi et al., 1995,1996; Harper et al., 1998). However, these and other data suggest that ATP-promoted increases in inositol phosphates are actually contingent on the production of UTP, which then activates the $hP2Y_4$ receptor.

Inositol phosphate production is measured by applying agonists for 10 to 20 min to cells cultured to a high density in a static bathing solution, which is an ideal condition for the transphosphorylation of ATP to UTP by ecto-NDPK. We have previously demonstrated in cells expressing the $hP2Y_4$ receptor that UTP produces a rapid accumulation of inositol phosphates, whereas the response to ATP is preceded by an ~10-min delay (Lazarowski et al., 1997b). It was demonstrated further that under these conditions, NDPK catalyzes the transfer of the terminal phosphate group of exogenous ATP to endogenous UDP, producing UTP. This suggests that the delay in the response to ATP was attributable to the production of UTP from UDP that had accumulated in the medium. Communi et al. (1996) also saw a lag in the production of inositol phosphates by ATP, but not by UTP, and they have interpreted this discrepancy in terms of two distinct activation states of the $hP2Y_4$ receptor, one with a strong selectivity for UTP and the other with a wider agonist selectivity. Although we cannot discount such a mechanism, the demonstration of NDPK activity (and UTP production) in medium on addition of ATP is compelling evidence consistent with bioconversion of nucleotides and subsequent receptor activation.

In this study, ATP did not increase intracellular $[Ca^{2+}]$ in cells expressing the $hP2Y_4$ receptor. In contrast to the studies in which the production of inositol phosphates was measured, in this study cells were grown to low density and perfused constantly, and agonists were applied for 30 s. Such conditions minimize the influence of released nucleotides and their extracellular metabolism, because any compound released or metabolite produced is immediately washed away. For example, we have shown previously that if $[Ca^{2+}]$ is measured in a static bathing solution, ATP can produce a small rise with a several-minute delay (Lazarowski et al., 1997a). Additionally, UDP alone is inactive, but coadministration of ATP and UDP evokes a rapid rise in $[Ca^{2+}]$. Again, these data are consistent with the suggestion that the apparent agonist effect of ATP at the $hP2Y_4$ receptor requires transphosphorylation to UTP via NDPK.

An alternative possibility is that ATP is truly a partial agonist at the $hP2Y_4$ receptor and that the cells used to study intracellular $[Ca^{2+}]$ expressed the receptor at low levels, but those used when inositol phosphate accumulation was measured expressed the receptor at higher levels. ATP would act as an antagonist in the former assay, but as a partial or full agonist in the latter. For example, ATP is a partial agonist at the $hP2Y_1$ receptor, and its action is dependent on the level of receptor expression (Palmer et al., 1998). However, several factors make this possibility unlikely for the $hP2Y_4$ receptor. In this study, the potency and efficacy of UTP were virtually identical at both the $hP2Y_4$ and $rP2Y_4$ receptors, suggesting that the levels of receptor expression were similar. Furthermore, we have shown previously that the same high concentrations of ATP that evoke inositol phosphate accumulation through the $hP2Y_4$ receptor do not induce release of intracellular $[Ca^{2+}]$ in the same cells (Lazarowski et al., 1997b). These factors, together with the slow onset of increase in inositol phosphate accumulation in response to ATP, strongly suggest that ATP has no agonist activity at the $hP2Y_4$ receptor.

We demonstrate here that ATP is a competitive antagonist at the $hP2Y_4$ receptor. Nguyen et al. (1995) reported previously that 100 μM ATP inhibited the rise in intracellular $[Ca^{2+}]$ evoked by UTP, although no data were shown. Communi et al. (1996) and Harper et al. (1998) also showed that ATP inhibited the accumulation of inositol phosphates evoked by UTP, but the potency of ATP was much lower than reported here. This may reflect the concomitant transphosphorylation of ATP to UTP, which would decrease the effective concentration of ATP and increase that of UTP. In our study, ATP was moderately potent as an antagonist at the $hP2Y_4$ receptor, with a K_B = 708 nM. Although endogenous inhibitory modulators of ligand-gated ion channel activity

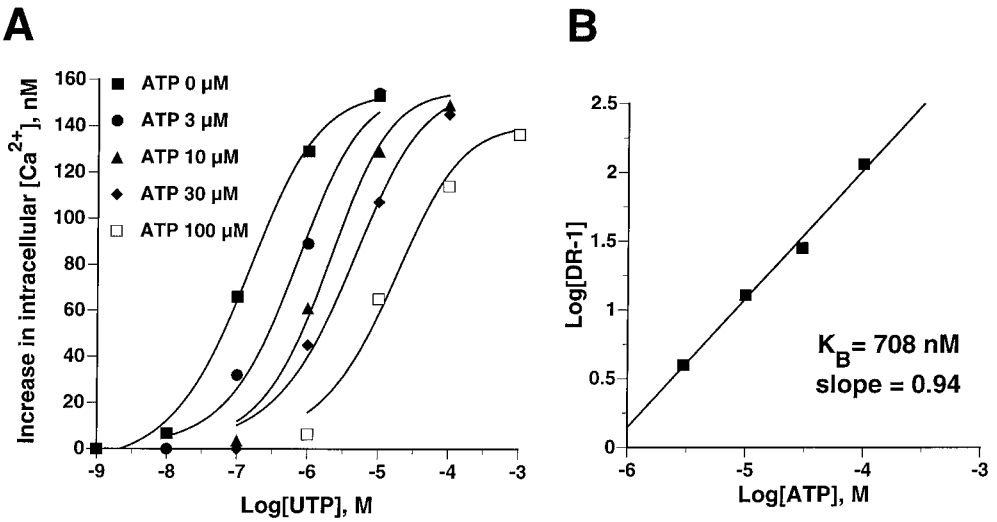

Fig. 5. ATP is a competitive antagonist at the $hP2Y_4$ receptor. A, the concentration-response curves for UTP-evoked increases in intracellular $[Ca^{2+}]$ in 1321N1 cells expressing the $hP2Y_4$ receptor were determined in the presence of increasing concentrations of ATP. Each point is the mean response from three experiments, with 9 to 16 cells monitored per experiment. B, a Schild plot derived from the data was fit by a straight line (R^2 = 0.993) with a slope of 0.94 and an x-axis intercept of −6.15.

have been identified, to our knowledge this is the first demonstration of an endogenous antagonist for a cloned G protein-coupled receptor, although ATP has been shown previously to be a partial agonist at the $hP2Y_1$ receptor (Palmer et al., 1998).

In our experiments, CTP and Ap_4A were weak antagonists at the $hP2Y_4$ receptor and XTP was inactive. However, all were agonists at the $rP2Y_4$ receptor. Indeed, all compounds tested were full agonists at the $rP2Y_4$ receptor. Of the four other cloned mammalian P2Y receptors, only the $P2Y_2$ receptor shows any similarity to this profile. GTP (Chen et al., 1996) and ITP (Fillipov et al., 1997) are agonists at the $rP2Y_2$ receptor, whereas Ap_4A activates the $hP2Y_2$ receptor (Lazarowski et al., 1995). Additional studies are required for a comprehensive comparison of these subtypes.

The pharmacological profile of the $rP2Y_4$ receptor is in fact closest to two nonmammalian p2y receptors. At the recently cloned turkey p2y receptor, which shows greatest sequence identity (~55%) with $hP2Y_4$ and $rP2Y_4$ receptors (Boyer et al., 1997), UTP, ATP, ITP, GTP, XTP, CTP, and Ap_4A are all full agonists (Boyer et al., 2000). Bogdanov et al. (1997) cloned a p2y receptor from *Xenopus laevis* (sometimes referred to as the p2y8 receptor) that again shows greatest sequence identity (~62%) with mammalian $P2Y_4$ receptors. ATP, CTP, GTP, ITP, and UTP (100 μM) were all active at this receptor. At present, the mammalian orthologues of these two receptors have yet to be identified, but it is conceivable that these receptors represent species homologues of the mammalian $P2Y_4$ receptor. This hypothesis warrants additional investigation.

In conclusion, $hP2Y_4$ and $rP2Y_4$ receptors have very different pharmacologic properties. In particular, ATP is a competitive antagonist at the former and a potent, full agonist at the latter. This is surprising, because they share 83% amino acid sequence identity across their whole sequence and 90% identity in the transmembrane spanning regions and extracellular loops. Such a difference is unusual, but not unique, because human and rodent $5\text{-}HT_{1B}$ receptors share 93% amino acid sequence identity, but a single amino acid difference confers distinct pharmacologic properties to synthetic ligands (Martin, 1998). Additionally, there also are differences in the pharmacologic selectivities of the human and rat $5\text{-}HT_2$ receptor, although these are not as profound as those for the $hP2Y_4$ and $rP2Y_4$ receptors described here (Martin, 1998). Because ATP binds to both species homologues of the $P2Y_4$ receptor but only activates the $rP2Y_4$ receptor, this suggests that amino acid changes have occurred in the human receptor that alter its ability to achieve the activated state on binding of ATP. The residue(s) involved in the differences between the species homologues of the $P2Y_4$ receptor is under active investigation.

Acknowledgments

We thank José Boyer for many helpful discussions and Ian Thorpe for help in carrying out some of the initial Ca^{2+} measurements.

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