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Agonist Action of Adenosine Triphosphates at the Human $P2Y_1$ Receptor

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ABSTRACT

The agonist selectivity for adenosine di- and triphosphates was determined for the human $P2Y_1$ receptor stably expressed in human 1321N1 astrocytoma cells and was studied under conditions in which nucleotide metabolism was both minimized and assessed. Cells were grown at low density on glass coverslips, encased in a flow-through chamber, and continuously superfused with medium, and Ca^{2+} responses to nucleotides were quantified. Superfusion with high performance liquid chromatographically purified ADP, ATP, 2-methylthio-ADP, and 2-methylthio-ATP resulted in rapid Ca^{2+} responses, with EC_{50} values of 10 ± 5, 304 ± 51, 2 ± 1, and 116 ± 50 nM, respectively. Similar peak responses were observed with maximal concentrations of these four agonists and with the hydrolysis-resistant adenine nucleoside triphosphate adenosine-5′-*O*-(3-thiotriphosphate). No conversion of [^{3}H]ATP to [^{3}H]ADP occurred under these conditions. Similar full agonist activities of ATP, 2-methylthio-ATP, and ADP were observed in human embryonic kidney 293 cells, which natively express the $P2Y_1$ receptor. In contrast to these results, Leon *et al*. [*FEBS Lett* **403:**26–30 (1997)] and Hechler *et al.* [*Mol Pharmacol* **53:**727–733 (1998)] recently reported that, whereas ADP and 2-methylthio-ADP were agonists, ATP and 2-methylthio-ATP were weak antagonists in studies of the human $P2Y_1$ receptor expressed in human Jurkat cells. To assess whether differences in the degree of receptor reserve might explain this discrepancy of results, $P2Y_1$ receptor-expressing 1321N1 cells were incubated for 24 hr with adenosine-5′-*O*-(2-thiodiphosphate), with the goal of down-regulating the level of functional receptors. Pretreatment with adenosine-5′-*O*-(2-thiodiphosphate) resulted in a 10-fold rightward shift in the concentration-effect curve for ADP; in contrast, the agonist activity of ATP was completely abolished. Taken together, our results indicate that adenosine di- and triphosphates are agonists at the human $P2Y_1$ receptor. However, the intrinsic efficacy of ATP is less than that of ADP, and the capacity of ATP to activate second messenger responses through this receptor apparently depends on the degree of $P2Y_1$ receptor reserve.

P2Y receptors are G protein-coupled receptors that are activated by extracellular nucleotides (Fredholm *et al.*, 1994). Molecular cloning and heterologous protein expression have led to unambiguous identification of at least five mammalian P2Y receptor subtypes (Communi *et al.*, 1997; Fredholm *et al.*, 1997). Antagonists capable of discriminating among the subtypes of P2Y receptors are not generally available, and pharmacological characterization of these receptors has been limited to descriptions of the rank order of potencies for activation by nucleotide agonists.

Metabolism of extracellular nucleotides complicates delineation of the agonist selectivity of P2Y receptors (Harden *et al.*, 1997). For example, 1321N1 human astrocytoma cells (a cell line commonly used for heterologous expression of P2Y receptors) express both ectonucleotidase and extrafacial nucleoside diphosphokinase activities, which modify exogenously applied nucleotides (Lazarowski *et al.*, 1997a, 1997b). Endogenous nucleotides also are released from these cells (Lazarowski *et al.*, 1995, 1997a) and other cells (Osipchuk and Cahalan, 1992; Grierson and Meldolesi, 1995; Schlosser *et al.*, 1996; Grygorczyk and Hanrahan, 1997), constitutively or as a result of mechanical stimulation, and promote elevation of basal levels of second messengers and receptor desensitization (Harden *et al.*, 1997). The purity of nucleotides obtained from commercial sources also is important (Nicholas *et al.*, 1996; Harden *et al.*, 1997; Leon *et al.*, 1997), and marked effects on the apparent agonist selectivity of $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors were demonstrated when precautions were taken to ensure nucleotide purity and stability (Nicholas *et al.*, 1996).

The $P2Y_1$ receptor, which promotes phospholipase C-catalyzed generation of inositol phosphates and subsequent mobilization of intracellular calcium, has been characterized by an agonist potency order of 2MeSADP > 2MeSATP > ADP > ATP (Webb *et al.*, 1993; Filtz *et al.*, 1994; Henderson *et al.*,

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ABBREVIATIONS: 2MeSADP, 2-methylthio-ADP; 2MeSATP, 2-methylthio-ATP; ADPβS, adenosine-5′-*O*-(2-thiodiphosphate); ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); HEK, human embryonic kidney; HPLC, high performance liquid chromatography.

1995; Schachter *et al.*, 1996; Boyer *et al.*, 1996). However, the previously described agonist activity of ATP and ATP analogues at the $P2Y_1$ receptor recently has been questioned (Leon *et al.*, 1997; Hechler *et al.*, 1998). Whereas ADP and 2MeSADP were potent full agonists, ATP and 2MeSATP purified from contaminating nucleoside diphosphates by HPLC did not exhibit agonist activity and acted as weak antagonists of the human $P2Y_1$ receptor-promoted calcium response in suspensions of Jurkat T cells heterologously expressing this receptor. This observed adenine nucleoside diphosphate specificity of the $P2Y_1$ receptor is strikingly similar to the pharmacological selectivity of a receptor on platelets that is referred to as the P2T receptor (Hourani and Hall, 1994), which led to the suggestion that the two receptors may be the same signaling protein (Leon *et al.*, 1997).

Unambiguous description of the pharmacological selectivity of P2Y receptors requires the use of purified nucleotides in a rapid assay of agonist action that is performed under conditions that minimize the confounding influences of ectoenzyme activities and endogenous nucleotide release. To this end, we have adapted a digital imaging system to record intracellular calcium responses in human $P2Y_1$ receptor-expressing 1321N1 cells grown at low density and continuously superfused with buffer. This method provides a test system that both minimizes and allows accurate quantification of the metabolism of superfused molecules. Both ATP and 2MeSATP were active as $P2Y_1$ receptor agonists in this assay system, using cells that either heterologously or natively expressed the $P2Y_1$ receptor. Our results suggest that the extent of the agonist activity of adenosine triphosphates at the human $P2Y_1$ receptor is dependent on the extent of receptor reserve.

Experimental Procedures

Materials. 2MeSADP and 2MeSATP were obtained from Research Biochemicals International (Natick, MA), ADP, ADPβS, and ATPγS were from Boehringer Manheim Biochemicals (Indianapolis, IN), ATP and UTP were from Pharmacia (Piscataway, NJ), [^{3}H]ATP (15–30 Ci/mmol) was from Amersham (Arlington Heights, IL), grade I apyrase was from Sigma Chemical Co. (St. Louis, MO), and fura-2/acetoxymethyl ester was from Molecular Probes (Eugene, OR). The sources of all other reagents have been reported (Schachter *et al.*, 1996).

HPLC. Separation of nucleotides was accomplished by HPLC with a Zorbax strong anion exchange column (4.6 mm × 25 cm). Samples were eluted with a linear gradient of NaCl (0–1 M) in a buffer of 10 mM NH_4HPO_4, pH 6.5. Nucleotides were detected by absorbance at 260 nm and were identified from the elution profiles of authentic standards. Assays were routinely carried out with freshly purified nucleotides.

Intracellular calcium measurement. 1321N1 human astrocytoma cells stably expressing the human $P2Y_1$ receptor (hP2Y1–1321N1 cells) (Schachter *et al.*, 1996) and human HEK293 cells (Schachter *et al.*, 1997) were grown on glass coverslips for 2–3 days to a cell density of approximately 20% of confluence. Intracellular calcium was quantified essentially as described (Palmer *et al.*, 1994). Coverslips containing fura-2 (0.5–1.0 μM)-loaded cells were encased in a flow-through chamber (0.2-ml volume) and superfused continuously (1.4 ml/min) with Hanks' buffered saline solution, with or without agonist. Solution changes were accomplished by means of a valve attached to a gravity-driven six-well reservoir. The flow-through chamber was secured to the stage of a Nikon Diaphot inverted fluorescence microscope. The cells were exposed to alternating excitation wavelengths of 340 and 380 nm, and fluorescence emission at 510 nm was monitored by a silicone-intensified tube camera. The 340/380-nm fluorescence emission ratio was determined and converted to the intracellular Ca^{2+} concentration using the equation of Grynkiewicz *et al.* (1985). Data were recorded and processed using an Image 1/FL digital imaging system (Universal Imaging Corp., West Chester, PA).

Results

We previously observed in assays of inositol lipid hydrolysis that ATP and 2MeSATP, although somewhat less potent than ADP and 2MeSADP, respectively, were full or nearly full agonists at the $P2Y_1$ receptor (Filtz *et al.*, 1994; Schachter *et al.*, 1996). Considering the contrasting results of Gachet and co-workers (Leon *et al.*, 1997; Hechler *et al.*, 1998), we compared the activities of adenine nucleoside di- and triphosphates at the human $P2Y_1$ receptor under conditions designed to circumvent the confounding influences of secreted endogenous nucleotides and nucleotide metabolism (see Experimental Procedures). Rapid calcium responses to nucleotide agonists were recorded from small numbers of continuously superfused cells. The commercially obtained preparations of ATP and 2MeSATP used in this study were contaminated by approximately 1% and 5%, respectively, with the corresponding nucleoside diphosphate. Therefore, all nucleotides were purified by strong anion exchange HPLC just before testing of the hP2Y1–1321N1 cells.

Superfusion of hP2Y1–1321N1 cells with HPLC-purified ATP, ADP, 2MeSATP, or 2MeSADP resulted in rapid increases in intracellular Ca^{2+} concentrations (Fig. 1). The superfusion time required to elicit a response and the rate of elevation of intracellular Ca^{2+} concentrations were identical for all four agonists. Moreover, maximally effective concentrations of 2MeSATP and ATP elicited responses that were comparable in magnitude to those produced by ADP, although in some experiments the maximal effect of ATP was somewhat less than that of ADP. Assuming that ADP exhibited a relative efficacy of 1.00 for eliciting a maximal response, the averaged relative efficacies of HPLC-purified ATP and 2MeSATP were 0.80 ± 0.08 (mean ± standard error of 18 experiments) and 1.00 ± 0.05 (mean ± standard error of 12 experiments), respectively. Although the response tracings of ADP versus ATP were often superimposable (Fig. 1), in some experiments differences in the descending portion of response tracings were noted (data not shown).

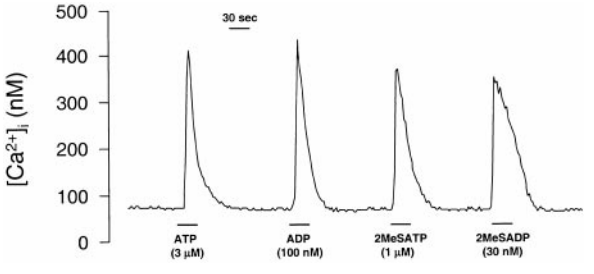

Fig. 1. Calcium responses of hP2Y1–1321N1 cells to HPLC-purified adenine nucleoside di- and triphosphates. Cells grown on glass coverslips to a density of approximately 20% of confluence were continuously superfused with Hanks' buffered saline solution, with or without nucleotides. The presence of nucleotides and the times of superfusion are indicated by *bars below and above the trace*, respectively. The trace shown is that of a single cell from a field of eight cells, all of which responded to the four nucleotides. Similar results were obtained from four identical experiments. $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

Complete concentration-effect curves were generated for the adenine nucleoside di- and triphosphates (Fig. 2). The nucleoside diphosphates were more potent than their triphosphate counterparts, and a rank order of potency for stimulation of Ca^{2+} mobilization of 2MeSADP > ADP > 2MeSATP > ATP was observed (Fig. 2, Table 1) The position of ADP in this order of potency differs from that we previously reported for the human $P2Y_1$ receptor in studies of agonist-promoted inositol phosphate formation (Schachter *et al.*, 1996).

Although nucleotides were exposed only briefly to a few cells under continuous flow in these experiments, sufficient conversion of nucleoside triphosphate to diphosphate might occur to account for the agonist activity of ATP and 2MeSATP. To examine this possibility, the conversion of [^{3}H]ATP to [^{3}H]ADP was directly measured under the same conditions in which intracellular calcium responses were recorded. A 10 μM solution of ATP containing HPLC-purified [^{3}H]ATP was superfused over coverslips, with or without hP2Y1–1321N1 cells, for 30 sec. The effluent was collected and ^{3}H-labeled nucleotides were resolved by HPLC. [^{3}H]ADP was not present in amounts that were statistically significantly above the background levels in control superfusate. Moreover, no increase in [^{3}H]ADP levels was detected in the superfusate from chambers containing cells (Table 2). Thus, conversion of the nucleoside triphosphates to their diphosphate forms cannot account for the observed responses to ATP and 2MeSATP. This conclusion is supported by the observation that the hydrolysis-resistant analogue ATPγS (made diphosphate-free by the treatment described in the legend to Fig. 3) elicited Ca^{2+} responses in hP2Y1–1321N1 cells (Fig. 3).

Disparate conclusions regarding the relative activities of diphosphates and triphosphates at the human $P2Y_1$ receptor could be explained by differences in the levels of functional receptors in various $P2Y_1$ receptor test systems. Agonists with low intrinsic efficacy might be equally effective, compared with full agonists, for stimulation of a maximal response in cells exhibiting considerable receptor reserve. In contrast, responses to a low-efficacy agonist would be submaximal or absent in cells with no receptor reserve. Thus, the possibility that ATP is a low-efficacy agonist at the human $P2Y_1$ receptor was examined by subjecting hP2Y1–1321N1 cells to agonist-induced desensitization. Cells were incubated for 24 hr with 100 μM ADPβS (ADPβS is a full agonist with an EC_{50} of 66 nM) and then rechallenged with a range of concentrations of ADP or ATP. The resulting concentration-effect curve for ADP was shifted approximately 10-fold to the right and slightly downward in ADPβS-pretreated cells. In contrast, prolonged preincubation with ADPβS essentially eliminated the capacity of ATP to elicit a calcium response in hP2Y1–1321N1 cells (Fig. 4). Although 30 μM ATP exhibited no agonist activity in ADPβS-desensi-

TABLE 1

Potencies of HPLC-purified adenine nucleoside di- and triphosphates in human P2Y1-1321N1 cells

Concentration-effect curves were generated for elevation of intracellular Ca^{2+} concentrations, as described in the legend to Fig. 2. EC_{50} values were calculated by nonlinear regression (GraphPAD Prism; GraphPAD Software). Values in parentheses are 95% confidence intervals.

Nucleotide	EC_{50}
	nM
2MeSADP	2 (0.5–6)
ADP	10 (4–25)
2MeSATP	116 (18–719)
ATP	304 (191–483)

TABLE 2

Lack of detectable conversion of ATP to ADP during superfusion of 1321N1 human astrocytoma cells

[^{3}H]ATP was purified by HPLC, added to a 10 μM solution of ATP in Hanks' buffered saline solution (1 μCi/ml), and then superfused for 30 sec (1.4 ml/min) over coverslips with or without cells (three experiments for each condition). ^{3}H-labeled nucleotides in the effluent were resolved by HPLC, and radioactivity in the fractions corresponding to authentic standards was quantitated. Data are given as averages ± standard errors.

	Radioactivity	
	[^{3}H]ATP	[^{3}H]ADP
	cpm	
No cells	117,343 ± 5,724	63 ± 35
Cells	112,445 ± 6,456	54 ± 51

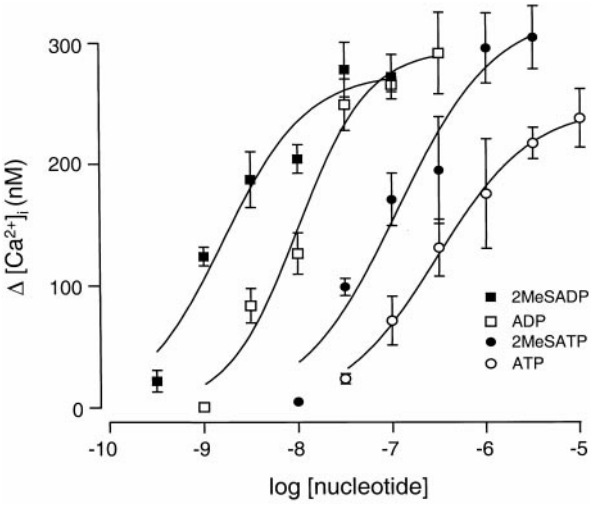

Fig. 2. Concentration dependence of adenine nucleotide-promoted Ca^{2+} mobilization in hP2Y1–1321N1 cells. Single coverslips were exposed to one concentration of nucleotide in Hanks' buffered saline solution for 30 sec. Traces of intracellular Ca^{2+} concentrations were recorded from 10–20 individual cells/coverslip and averaged. Therefore, the value of the peak intracellular Ca^{2+} concentration obtained from each coverslip reflects both the response magnitude of individual cells and the number of responding cells. *Data points*, mean ± standard error of the averaged peak calcium responses from four to seven coverslips. $\Delta[Ca^{2+}]_i$, change in intracellular Ca^{2+} concentration.

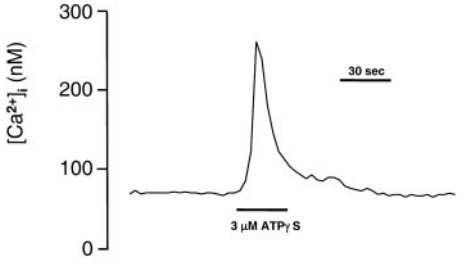

Fig. 3. ATPγS-promoted calcium responses in hP2Y1–1321N1 cells. ATPγS was treated with 2.5 units/ml apyrase (grade I) for 30 min at 37° and then purified by strong anion exchange HPLC before being tested as an agonist with hP2Y1–1321N1 cells. The trace shown is from a single cell in a field of 10 responding cells. Similar responses to 3 μM ATPγS were obtained from cells on seven additional coverslips. The presence of ATPγS in Hanks' buffered saline solution and the time are indicated by the *bars below and above the trace*, respectively. $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

tized cells, this concentration of ATP partially blocked the calcium response to 300 nM ADP in these pretreated cells (Fig. 5). Thus, the agonist action of ATP, but not ADP, was largely lost under conditions that likely substantially reduced the number of functional $P2Y_1$ receptors. These results suggest that ATP is a low-efficacy agonist at the human $P2Y_1$ receptor and that the capacity of ATP to elicit a calcium response is dependent on the levels of functional $P2Y_1$ receptors.

Because most studies examining the relative agonist activities of ADP and ATP at the human $P2Y_1$ receptor were carried out with heterologously expressed receptors, it also was important to test these activities at natively expressed $P2Y_1$ receptors. Thus, agonist activities of 2MeSATP and ATP were examined by measuring Ca^{2+} responses from a small number of superfused HEK293 cells, which natively express the $P2Y_1$ receptor (Schachter *et al.*, 1997). These cells also natively express a phospholipase C-coupled $P2Y_2$ receptor that we have shown mediates a minor component of the inositol phosphate response to ATP. Consistent with our earlier findings, a maximal concentration of UTP stimulated calcium responses from approximately one third of the cells tested (Fig. 6, A and B). In contrast, HPLC-purified 2MeSATP, which is inactive at the $P2Y_2$ receptor (Lazarowski *et al.*, 1995), and ATP elicited rapid calcium responses from essentially all HEK293 cells (Fig. 6, A and C).

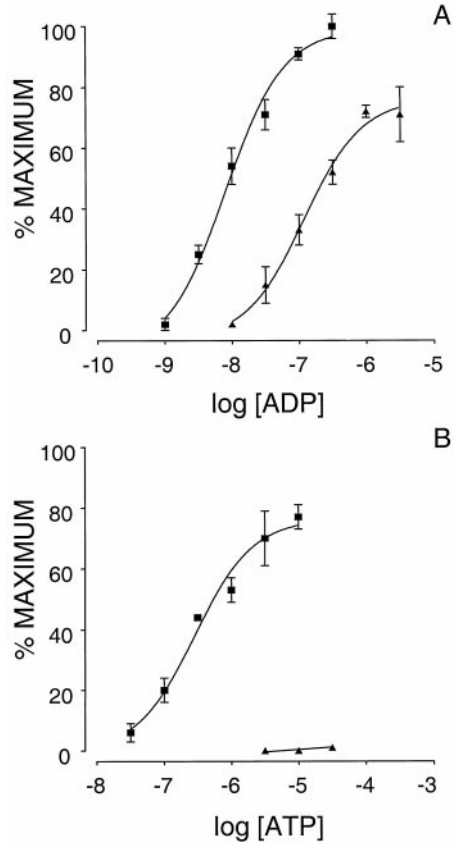

Fig. 4. $P2Y_1$ receptor agonist activity in ADPβS-pretreated hP2Y1–1321N1 cells. Concentration-effect curves were generated for mobilization of Ca^{2+} (as described in the legend to Fig. 2) for ADP (A) and ATP (B) in hP2Y1–1321N1 cells after a 24-hr preincubation with (▲) or without (■) 100 μM ADPβS. Values are presented as percentages (mean ± standard error) of the maximal response to 300 nM ADP in untreated control cells.

These results are consistent with the idea that the response of HEK293 cells to ATP is predominantly attributable to the $P2Y_1$ receptor and that ATP and 2MeSATP are both agonists at the human $P2Y_1$ receptor present at natively expressed levels.

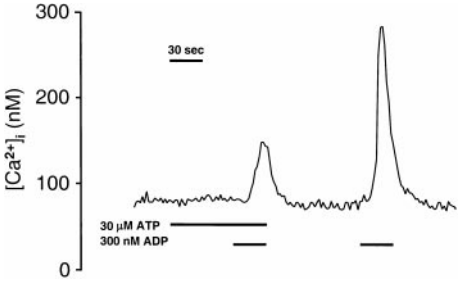

Fig. 5. Antagonism by ATP of the calcium response to ADP in ADPβS-pretreated hP2Y1–1321N1 cells. Cells pretreated for 24 hr with 100 μM ADPβS (as described in the legend to Fig. 4) were continuously superfused with Hanks' buffered saline solution, with or without nucleotides. The presence of ATP and ADP and time are indicated by the *bars below and above the trace*, respectively. The averaged trace from a field of eight cells is shown. Similar results were obtained from two additional coverslips. $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

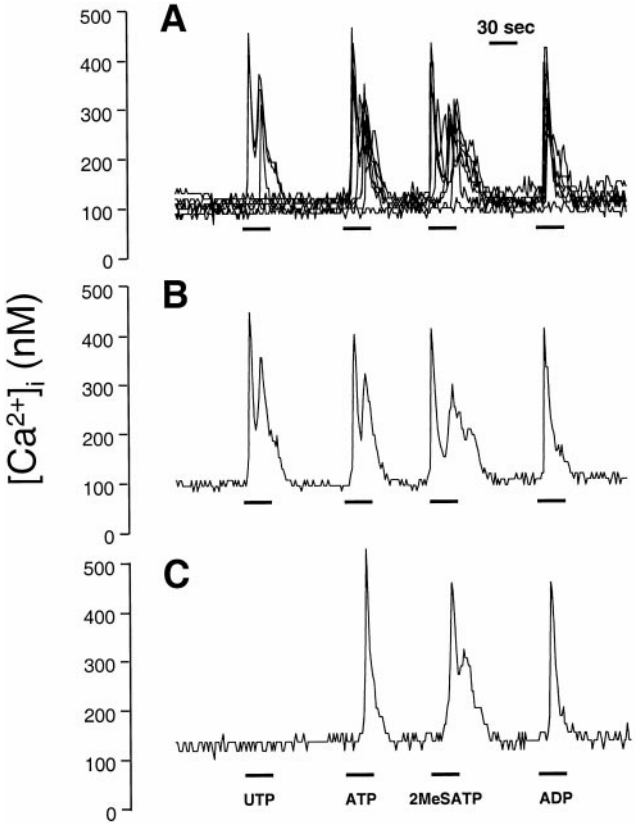

Fig. 6. ATP- and 2MeSATP-promoted calcium responses through natively expressed $P2Y_1$ receptors. HEK293 cells were continuously superfused with Hanks' buffered saline solution. The presence of nucleotides and time are indicated by the *bars below and above (in A) the traces*, respectively. A, Composite of intracellular calcium responses of eight individual cells from a single microscopic field. Three of eight cells responded to a maximally effective concentration of UTP (3 μM) through the $P2Y_2$ receptor. Seven of eight cells responded to maximal concentrations of HPLC-purified ATP (3 μM), 2MeSATP (1 μM), and ADP (300 nM). B, Trace from one of the three cells in A that responded to all nucleotides, demonstrating both $P2Y_2$ and $P2Y_1$ receptor-mediated promotion of calcium signaling. C, Trace from one of the four cells in A that responded only to the $P2Y_1$ receptor agonists. These results are representative of six similar experiments. $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

Discussion

A $P2Y_1$ receptor-expressing cell preparation was used under conditions that circumvent potential contributions of nucleotide metabolism or release to the rapid responses observed with superfused agonists. HPLC purification of test molecules immediately before assay also ensured that the effects measured were not a combination of responses to both nucleoside di- and triphosphates. Under these conditions, adenosine diphosphates were clearly the most potent agonists at the human $P2Y_1$ receptor, as was recently emphasized by Gachet and co-workers (Leon *et al.*, 1997; Hechler *et al.*, 1998). However, in contrast to those results, we observed that ATP, 2MeSATP, and ATPγS also were $P2Y_1$ receptor agonists.

The absence of a reliable binding assay for $P2Y_1$ receptors has relegated this (and other P2Y receptors) to a group of signaling proteins for which ligand binding affinities cannot be directly determined. Therefore, assays of the activation of phospholipase C or of downstream signaling responses have evolved as the primary means to assess ligand interactions at P2Y receptors. These assays provide accurate monitoring of second messenger responses but only indirectly reflect ligand-receptor interactions, as well as introducing limitations associated with most steady-state biochemical analyses. Occurrence of agonist-induced receptor desensitization is a potential problem. The existence of a complex set of ectoenzymes further complicates study of extracellular nucleotides, because triphosphates can be converted to diphosphates by nucleotidases (Zimmermann, 1996) and triphosphates can be formed from diphosphates by nucleoside diphosphokinases (Lazarowski *et al.*, 1997a). Thus, simple elimination of contaminating nucleotides in stock solutions of a given nucleotide by HPLC purification does not preclude the contribution of rapid formation of similar molecules as metabolites during measurements of signaling responses.

We have emphasized the differences in apparent agonist selectivity that the aforementioned factors can produce in studies of the uridine nucleotide-activated $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors (Nicholas *et al.*, 1996). The results of Leon *et al.* (1997) with $P2Y_1$ receptors stably expressed in Jurkat cells established that similar considerations apply in delineating agonist selectivities at the $P2Y_1$ receptor. Whereas previous studies from our laboratory and other laboratories showed that both 2MeSATP and ATP were potent full agonists at the $P2Y_1$ receptor (Webb *et al.*, 1993; Filtz *et al.*, 1994; Henderson *et al.*, 1995; Schachter *et al.*, 1996), this was not the case with the Jurkat cell-expressed $P2Y_1$ receptor when nucleotides were purified by HPLC before agonist activity testing and rapid measurements of Ca^{2+} accumulation were carried out to minimize metabolism or interconversion of agonists during drug testing (Leon *et al.*, 1997). Although the results of Leon *et al.* (1997) prompted us to adopt a $P2Y_1$ receptor test system that alleviated problems of nucleotide metabolism, our initial results were not entirely consistent with those reported in their well-controlled studies. That is, as we previously observed in the less definitive studies of inositol phosphate accumulation, ATP activated the $P2Y_1$ receptor to promote Ca^{2+} accumulation, and the maximal effects of ATP were very similar to those of ADP. Thus, in our hands ATP is not simply an antagonist at the human $P2Y_1$ receptor. The same conclusions can be drawn from our data with 2MeSATP and, perhaps more importantly, ATPγS, which is a nonhydrolyzable triphosphate analogue that is a full or nearly full agonist at the $P2Y_1$ receptor.

One possible explanation for differences between our results and those of Leon *et al.* (1997) and Hechler *et al.* (1998) is that $P2Y_1$ receptors might be expressed at much higher levels in the 1321N1 cells used in the current study, compared with the Jurkat cell-expressed $P2Y_1$ receptors. Because the studies of Leon *et al.* (1997) and Hechler *et al.* (1998) were carried out with suspended cells at relatively high cell densities, release of endogenous ATP and basal activation of the expressed $P2Y_1$ receptors also were more likely to occur than in the current study, which was carried out with relatively few cells in a monolayer under constant superfusion. Thus, lowering of functional receptor levels by agonist-induced desensitization before drug treatment is a possibility with the Jurkat cell-expressed receptors. Conversely, relatively high levels of $P2Y_1$ receptor expression in 1321N1 cells may generate a large receptor reserve that, in turn, permits detection of agonist activity of nucleotides that exhibit much lower intrinsic efficacy than does ADP. The 20-fold greater potencies of ADP and 2MeSADP determined at $P2Y_1$ receptors expressed in 1321N1 cells, compared with the values reported for the receptors expressed in Jurkat cells, suggests greater functional $P2Y_1$ receptor expression in our studies, compared with those of Leon *et al.* (1997) and Hechler *et al.* (1998). Moreover, experiments with agonist-preincubated 1321N1 cells directly confirm this possibility. That is, preincubation of $P2Y_1$ receptor-expressing 1321N1 cells with ADPβS resulted in a complete loss of response to ATP but only a shift to the right of the concentration-effect curve for ADP, with no substantial decrease in the maximal effect. Indeed, some antagonist activity of ATP was apparent in experiments with the desensitized cells. Thus, we conclude from these studies that both ADP and ATP are agonists at the human $P2Y_1$ receptor and that the intrinsic efficacy of ADP is greater than that of ATP.

The physiological significance of our observations is not completely clear. The agonist activity of ATP is not restricted to the unnatural situation of heterologous overexpression of $P2Y_1$ receptors in 1321N1 cells, because ATP was also an agonist at human $P2Y_1$ receptors natively expressed in HEK293 cells and is likely to be a physiologically important activator of $P2Y_1$ receptors in various target tissues. Our results emphasize that it will be important in future studies to assess the accuracy with which *in vitro* tests of receptor activity reflect the extent of functional reserve of $P2Y_1$ receptors *in vivo*. ADP clearly is more potent than ATP and, under conditions of little $P2Y_1$ receptor reserve, would be a more efficacious agonist. Therefore, the importance of ATP as an agonist at the $P2Y_1$ receptor would depend on the receptor level in target tissues and on the extracellular concentration of ATP, relative to that of ADP. Clearly, the 30-fold greater potency of ADP, relative to ATP, at the $P2Y_1$ receptor ensures that lower concentrations of ADP are detected. The studies of both Leon *et al.* (1997) and Kunapuli and co-workers (Jin *et al.*, 1998; Daniel *et al.*, 1998) strongly suggest that the $P2Y_1$ receptor is prominently involved in the platelet aggregation response to ADP and that, at least in *in vitro* studies, the agonist action of ADP is antagonized by ATP.

We doubt that the antagonist action of ATP that may exist under conditions of low $P2Y_1$ receptor reserve has physiolog-

ical importance, because this antagonist activity is observed only at concentrations of ATP much higher than those necessary to observe full agonist actions of ADP. However, the data of Leon *et al.* (1997) and Hechler *et al.* (1998) and those presented here indicate that the rate of metabolism of extracellular ATP to ADP may prove to be an important temporal determinant of the agonist action of released adenine nucleotides at the $P2Y_1$ receptor. Clearly, multiple factors exist that can serve to regulate the extracellular action of adenine nucleotides.

References

Boyer JL, Schachter JB, Sromek SM, Palmer RK, Jacobson KA, Nicholas RA, and Harden TK (1996) Avian and human homologues of the $P2Y_1$ receptor: pharmacological, signaling, and molecular properties. *Drug Dev Res* **39:**253–261.

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

Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith B, and Kunapuli SP (1998) Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* **273:**2024–2029.

Filtz TM, Li Q, Boyer JL, Nicholas RA, and Harden TK (1994) Expression of a cloned P_{2Y}-purinergic receptor that couples to phospholipase C. *Mol Pharmacol* **46:**8–14.

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, and Williams M (1994) Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46:**143–156.

Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson KA, Schwabe U, and Williams M (1997) Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol Sci* **18:**79–82.

Grierson JP and Meldolesi J (1995) Shear stress-induced $[Ca^{2+}]_i$ transients and oscillations in mouse fibroblasts are mediated by endogenously released ATP. *J Biol Chem* **270:**4451–4456.

Grygorczyk R and Hanrahan JW (1997) CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli. *Am J Physiol* **272:**C1058–C1066.

Grynkiewicz G, Poenie M, and Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:**3440–3450.

Harden TK, Lazarowski ER, and Boucher RC (1997) Release, metabolism and interconversion of adenine and uridine nucleotides: implications for G protein-coupled P2 receptor agonist selectivity. *Trends Pharmacol Sci* **18:**43–46.

Hechler B, Vigne P, Leon C, Breittmayer JP, and Gachet C (1998) ATP derivatives are antagonists of the P2Y1 receptor: similarities to the platelet ADP receptor. *Mol Pharmacol* **53:**727–733.

Henderson DJ, Elliot DG, Smith GM, Webb TE, and Dainty IA (1995) Cloning and characterization of a bovine P_{2Y} receptor. *Biochem Biophys Res Commun* **212:**648–656.

Hourani SMO and Hall DA (1994) Receptors for ADP on human blood platelets. *Trends Pharmacol Sci* **15:**103–108.

Jin J, Daniel JL, and Kunapuli SP (1998) Molecular basis for ADP-induced platelet activation. II. The $P2Y_1$ receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* **273:**2030–2034.

Lazarowski ER, Homolya L, Boucher RC, and Harden TK (1997a) Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *J Biol Chem* **272:**24348–24354.

Lazarowski ER, Homolya L, Boucher RC, and Harden TK (1997b) Identification of an ecto-nucleoside diphosphokinase and its contribution to interconversion of P2 receptor agonists. *J Biol Chem* **272:**20402–20407.

Lazarowski ER, Watt WC, Stutts MJ, Boucher RC, and Harden TK (1995) Pharmacological selectivity of the cloned human phospholipase C-linked P_{2U}-purinergic receptor: potent activation by diadenosine tetraphosphate. *Br J Pharmacol* **116:**1619–1627.

Leon C, Hechler B, Vial C, Leray C, Cazenave J-P, and Gachet C (1997) The $P2Y_1$ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett* **403:**26–30.

Nicholas RA, Watt WC, Lazarowski ER, Li Q, and Harden TK (1996) Uridine nucleotide selectivity of three phospholipase C-activating P_2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol Pharmacol* **50:**224–229.

Osipchuk Y and Cahalan M (1992) Cell-to-cell spread of calcium signals mediated by ATP receptors in mast cells. *Nature (Lond)* **359:**241–244.

Palmer RK, Yule DI, McEwen EL, Williams JA, and Fisher SK (1994) Agonist-specific calcium signaling and phosphoinositide hydrolysis in human SK-N-MCIXC neuroepithelioma cells. *J Neurochem* **63:**2099–2107.

Schachter JB, Li Q, Boyer JL, Nicholas RA, and Harden TK (1996) Second messenger cascade specificity and pharmacological selectivity of the human P_{2Y1} receptor. *Br J Pharmacol* **118:**167–173.

Schachter JB, Sromek SM, Nicholas RA, and Harden TK (1997) HEK293 human embryonic kidney cells endogenously express the $P2Y_1$ and $P2Y_2$ receptors. *Neuropharmacology* **36:**1181–1187.

Schlosser SF, Burgstahler AD, and Nathanson MH (1996) Isolated rat hepatocytes can signal to other hepatocytes and bile duct cells by release of nucleotides. *Proc Natl Acad Sci USA* **93:**9948–9953.

Webb TE, Simon J, Krishek BJ, Bateson AN, Smart TG, King BF, Burnstock G, and Barnard EA (1993) Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett* **324:**219–225.

Zimmermann H (1996) Extracellular purine metabolism. *Drug Dev Res* **39:**337–352.

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