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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

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SUMMARY

Observation that the G protein-coupled P_{2U} receptor ($P2Y_2$ receptor) is activated by UTP as well as ATP provided the first indication that a class of uridine nucleotide-responsive receptors might exist. This hypothesis was confirmed by our identification of a uridine nucleotide-specific receptor on C6–2B rat glioma cells and by the recent cloning of two uridine nucleotide-responsive receptors, the $P2Y_6$ receptor [*J. Biol. Chem.* **270**:26152–26158 (1995)] and the $P2Y_4$ receptor [*J. Biol. Chem.* **270**:30849–30852 (1995) and *J. Biol. Chem.* **270**:30845–30848 (1995)]. The relative nucleotide selectivities of these uridine nucleotide-activated receptors have not been established. Therefore, we cloned and expressed the $P2Y_6$ and $P2Y_4$ receptors in 1321N1 human astrocytoma cells and compared their relative selectivities for UDP, UTP, and other uridine and adenine nucleotides with that of the $P2Y_2$ receptor expressed in the same cells. These comparisons were made by measuring inositol phosphate accumulation under conditions in which the initial purity and stability of agonists were rigidly ensured and quantitatively assessed. The data indicate that the $P2Y_2$ receptor is activated with similar potencies by ATP and UTP but not by ADP or UDP; the $P2Y_6$ receptor is activated most potently by UDP but weakly by UTP, ATP, and ADP; and the $P2Y_4$ receptor is activated most potently by UTP, less potently by ATP, and not at all by nucleotide diphosphates. Furthermore, the $P2Y_6$ receptor, which displays a uridine nucleotide selectivity essentially identical to that of the uridine nucleotide-specific receptor in C6–2B cells, was shown to be natively expressed in C6–2B cells and to account for the uridine nucleotide responses originally identified in these cells. These results define the uridine nucleotide selectivity of three phospholipase C-linked receptors: a receptor that is selectively activated by UDP ($P2Y_6$ receptor), selectively activated by UTP ($P2Y_4$ receptor), and activated by UTP and ATP but not by diphosphate nucleotides ($P2Y_2$ receptor).

Adenine nucleotides are released in a regulated manner from neurons and other tissues and interact with P_2 receptors to produce a wide range of physiological responses (1–3). Direct evidence of the regulated release of uridine nucleotides is limited (4–6), but occurrence of a broad range of cellular responses to UTP has led to the suggestion that extracellular uridine nucleotides also are important signaling molecules (2, 7, 8). This idea has received strong support by the widespread expression of a so-called P_{2U} receptor that is activated by both ATP and UTP. This receptor was originally cloned by Lustig *et al.* (9) and was later shown to promote both ATP- and UTP-dependent increases in inositol phosphates when expressed in 1321N1 human astrocytoma cells (10, 11).

We recently reported the existence of a uridine nucleotide-specific receptor on C6–2B rat glioma cells (12). In contrast to previously studied P_2 receptors, this phospholipase C-stimulating receptor is preferentially activated by UDP over UTP and is not activated by ATP, ADP, or analogues of these adenine nucleotides. Two additional G protein-coupled receptors that are activated by uridine nucleotides were cloned recently. We refer to these receptors as the $P2Y_6$ receptor (13)

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ABBREVIATIONS: PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; 2MeSATP, 2-methylthioadenosine-5′-triphosphate; UTPγS, uridine-5′-triphosphate-γ-thiol; 5BrUTP, 5-bromouridine-5′-triphosphate; 5BrUDP, 5-bromouridine-5′-diphosphate.

and $P2Y_4$ receptor (14, 15).[1] The relationship of these receptors to the previously studied uridine nucleotide-specific receptor of C6–2B glioma cells is not known. Furthermore, the pharmacological relationship of these receptors to the previously cloned $P2Y_2$ receptor (P_{2U} receptor) and their relative selectivities for UDP and UTP have not been clearly established. Therefore, we cloned the coding sequences of the $P2Y_6$ and $P2Y_4$ receptors and stably expressed these signaling proteins in 1321N1 human astrocytoma cells, which lack endogenous phospholipase C-activating P_2 receptors. Pharmacological analyses were carried out under conditions in which the initial purity of nucleotides and their interconversion were carefully controlled. Our data reveal that the $P2Y_6$ receptor originally cloned by Chang *et al.* (13) is analogous to the uridine nucleotide receptor of C6–2B glioma cells, exhibits high selectivity for UDP over UTP, and is not activated by ATP. In contrast, the $P2Y_4$ receptor is selective for UTP over ATP but is not activated by UDP. The previously studied $P2Y_2$ receptor, which is activated with similar potencies by ATP and UTP, was found to not be activated by nucleotide diphosphates.

Materials and Methods

PCR amplification of the coding sequences of the $P2Y_6$ and $P2Y_4$ receptors. PCR primers complementary to the published sequences of $P2Y_6$ (13) and $P2Y_4$ (14, 15) receptors were used to amplify the coding sequences from 0.2 μg of rat or human genomic DNA, respectively. The PCR primers for each receptor sequence contained at their 5′-ends either an *Eco*RI restriction site (upstream primers) or an *Xho*I restriction site (downstream primers) and were designed to include ~15 bp of 5′-untranslated sequence in the amplified fragments. The amplification conditions were 94° for 3 min; 30 cycles of 94° for 45 sec, 56° for 30 sec, 72° for 1.5 min; and 10 min at 72°. *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used to avoid fidelity errors during amplification. The amplification products were digested with *Eco*RI and *Xho*I and ligated into similarly digested pLXSN, and an individual clone encoding each receptor was sequenced on both strands using the Amplicycle Sequencing Kit (Perkin-Elmer Cetus, Norwalk, CT). The amplified rat $P2Y_6$ receptor sequence was identical to that reported by Chang *et al.* (13). The two published nucleotide sequences of the human $P2Y_4$ receptor (14, 15) differ in five positions, which translates into discrepancies in three amino acids. The sequence of the amplified $P2Y_4$ receptor gene was identical to that of Communi *et al.* (14).

Expression of $P2Y_6$ and $P2Y_4$ receptors in 1321N1 human astrocytoma cells. Recombinant retroviral particles were produced in PA317 cells after transfection with pLXSN containing the appropriate receptor sequence (16). 1321N1 human astrocytoma cells were infected with retrovirus harboring the $P2Y_6$ or $P2Y_4$ receptor coding sequence or with control retrovirus, and neomycin-resistant cells were selected with 600 μg/ml G-418 as described previously (10). The generation of 1321N1 cells expressing the $P2Y_2$ receptor using the same retroviral vector has been described previously (10, 11).

Inositol phosphate formation. Confluent cultures in 24-well dishes were incubated for 18 hr in 0.5 ml of inositol-free Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 1 μCi of *myo*-[^{3}H]inositol. The cells were preincubated with 10 mM LiCl for 10 min and then challenged with agonists in typical experiments for an additional 15 min at 37°. It should be noted that no changes of medium were made subsequent to the addition of [^{3}H]inositol (11, 17). Drug challenges were terminated by the addition of 5% trichloroacetic acid, and [^{3}H]inositol phosphates were resolved on Dowex AG1-X8 columns as described previously (11, 17).

Assessment of nucleotide purity and stability. Nucleotides obtained from commercial sources are only ≤99% pure, and their purity decreases considerably during storage in aqueous solution. Furthermore, nucleotide diphosphates contaminate commercial preparations of nucleotide triphosphates, and vice versa. The nucleotides used in this study were purified as indicated in the text using HPLC protocols that we have reported previously (11, 18). However, chemical stability of nucleotides presents a recurring problem. For example, UTP freshly purified by HPLC contains significant amounts (≤0.4%) of UDP on a second elution from the same column. The study of P_2 receptors also is complicated by the release of adenine nucleotides from most cells under a variety of conditions. In particular, 1321N1 cells release ATP during physical movement and release large amounts of ATP during changes of medium (11). Therefore, all of the experiments in this study were carried out with minimal movement of culture dishes and with no change of incubation medium.

Metabolism of nucleotides by enzymes on the extracellular face of cells also presents potential problems in delineating the pharmacological selectivity of P_2 receptors. Ectonucleotidases that are present on 1321N1 cells hydrolyze ATP, UTP, ADP, and UDP. The rate of hydrolysis of adenine nucleotides is somewhat faster than the rate of hydrolysis of UTP and UDP.[2] In studies of the pharmacological activity of ATP or UTP, care must be taken to ensure that diphosphates generated from hydrolysis of the triphosphates do not contribute to the response observed. Finally, we recently observed nucleoside diphosphokinase activity associated with the extracellular face of 1321N1 cells.[2] Thus, ~3% of added UDP (100 nM) in a volume of 0.5 ml in a 24-well dish is converted to UTP in a 10-min incubation. The percent conversion is increased to 16% and 17%, respectively, if the medium is changed before the addition of UDP or if 100 nM ATP is added concomitantly with the UDP.[2] Thus, the presence of nucleoside diphosphokinase activity in combination with release of endogenous ATP presents problems in the analysis of the effects of UDP (or ADP) alone. To circumvent this problem, we established conditions using hexokinase and glucose that quantitatively convert UTP to UDP. Thus, stock solutions of UDP (1 mM) were preincubated with 10 units/ml hexokinase and 22 mM glucose for 1 hr, which resulted in the complete conversion of UTP to UDP (data not shown). In addition, concentration-effect curves to UDP were generated in the presence of hexokinase (1 unit/ml) and glucose to ensure that any observed effects were not due to conversion of UDP to UTP during incubation with cells. This was confirmed by HPLC analysis.

Materials. UTP and ATP were from Pharmacia (Piscataway, NJ); 5BrUTP was from Sigma Chemical (St. Louis, MO); UDP, ADP, and hexokinase were from Boehringer-Mannheim Biochemicals (Indianapolis, IN); 2MeSATP and 2-methylthioadenosine-5′-diphosphate were from Research Biochemicals (Natick, MA); [^{3}H]UTP (20 Ci mmol^{-1}) and [2,8-^{3}H]ATP (15–30 Ci mmol^{-1}) were from Amersham (Arlington Heights, IL); and *myo*-[2-^{3}H]inositol (20 Ci mmol^{-1}) was from American Radiolabeled Chemicals (St. Louis, MO). 5BrUDP and [^{3}H]UDP were synthesized by incubating 5BrUTP or [^{3}H]UTP, respectively, with hexokinase and glucose as described previously (18).

[1] The nomenclature of purinergic/pyrimidinergic receptors is provisional and will be revised; thus, the terms used here are provisional. The IUPHAR Nomenclature Committee has provided general recommendations that G protein-coupled nucleotide receptors be designated as P2Y receptors (28) and that receptors in a subfamily be denoted by numbers that reflect the chronological order in which the sequences of functional receptors have become available to the public domain. However, the receptor cloned by Chang *et al.* (13) has been referred to as the $P2Y_6$ receptor (see Website http://mgddk1.niddk.nih.gov:8000/nomenclature.html) even though it was the third functional P2Y receptor for which a sequence was published. To avoid confusion, in the current study, we refer to this receptor as the $P2Y_6$ receptor.

[2] E. R. Lazarowski, W. C. Watt, R. C. Boucher, and T. K. Harden, unpublished observations.

Results

The nucleotide sequences encoding the rat $P2Y_6$ receptor and the human $P2Y_4$ receptor were amplified from genomic DNA by PCR, sequenced, and stably expressed in 1321N1 human astrocytoma cells using recombinant retroviruses. No inositol phosphate response to uridine nucleotides was observed in native 1321N1 cells or in 1321N1 cells infected with control virus. In contrast, UTP (100 μM) promoted marked inositol lipid hydrolysis in 1321N1 cells selected after infection with recombinant retroviruses harboring the coding sequence of either the $P2Y_6$ or the $P2Y_4$ receptor (Table 1). Uridine nucleotide-stimulated inositol phosphate accumulation was similar to ($P2Y_4$ receptor) or 2-fold greater ($P2Y_6$ receptor) than that observed with 1 mM carbachol.

As was previously reported by Chang *et al.* (13), UTP apparently was a potent agonist at the $P2Y_6$ receptor (Fig. 1A). However, assessment of the activity of UDP revealed that this diphosphate nucleotide was 100-fold more potent than UTP. The potent effects of UDP ($K_{0.5}$ = 0.19 ± 0.04 μM) potentially could be explained by the presence of UTP in the preparation of UDP or by conversion of UDP to UTP by nucleoside diphosphokinase or other enzymatic activities associated with the cells. However, incubation of UDP stock solutions with hexokinase and glucose and coincubation of UDP with hexokinase and glucose during incubations with 1321N1 cells had no effect on the concentration-effect curve of UDP (Fig. 1A). These incubation conditions were shown by HPLC analysis to result in essentially complete absence of UTP, and therefore the presence of UTP does not account for the effects of UDP. Conversely, the effect of UTP on the $P2Y_6$ receptor could be explained by the presence of UDP as a contaminant in the UTP stocks or by conversion of UTP to UDP during incubation with cells. To circumvent these problems, we purified UTP through HPLC immediately before use and reduced the incubation time with cells to 5 min to minimize any possible contribution of UDP generated from UTP by cell surface nucleotidases. Under these conditions, UTP was essentially inactive at the $P2Y_6$ receptor (Fig. 1A, *inset*). These data indicate that the $P2Y_6$ receptor is highly selective for UDP over UTP.

The uridine nucleotide selectivity of the $P2Y_4$ receptor also was determined after stable expression in 1321N1 cells. As reported recently by Communi *et al.* (14) and Nguyen *et al.* (15), UTP was the most potent agonist (Fig. 1B; $K_{0.5}$ = 0.8 ± 0.2 μM). Although the addition of UDP alone resulted in a concentration-dependent increase in inositol phosphate accumulation, UDP had little or no effect on inositol phosphate accumulation under conditions in which hexokinase and glucose were included in the assay medium to ensure that any UTP that was present initially in the stock solution of UDP or that was formed during incubation with 1321N1 cells was converted to UDP (Fig. 1B). Thus, although the $P2Y_4$ receptor is activated by UTP, in marked contrast to the $P2Y_6$ receptor, it is not activated by UDP.

We previously reported that although UDP was ~100-fold less potent than UTP for activation of the $P2Y_2$ receptor, it nevertheless was a full agonist (11). The relative selectivities of UDP versus UTP for $P2Y_6$ and $P2Y_4$ receptors led us to reexamine the effects of UDP on the $P2Y_2$ receptor under conditions in which hexokinase and glucose were included to ensure that no contribution of UTP could occur. As illustrated in Fig. 1C, our previous conclusion of full agonist effects of UDP on the $P2Y_2$ receptor was incorrect. Although marked increases in inositol phosphate accumulation were observed with increasing concentrations of UDP in the ab-

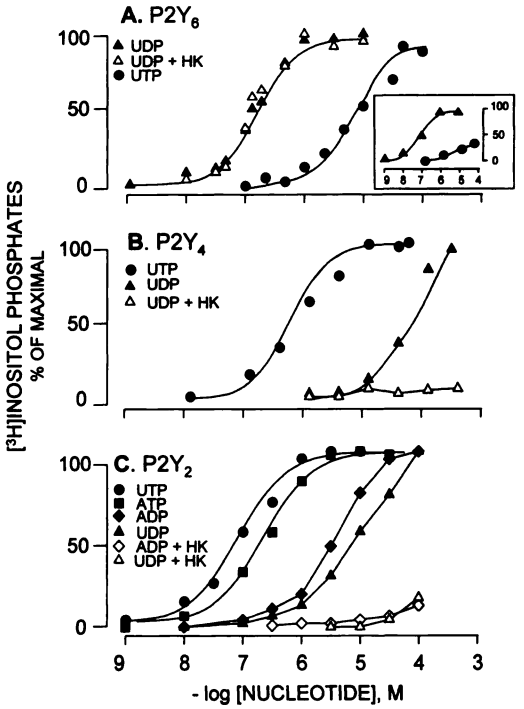

Fig. 1. Effect of hexokinase and glucose on the relative potencies of UDP and ADP in 1321N1 cells expressing the $P2Y_2$, $P2Y_6$, or $P2Y_4$ receptor. A, 1321N1 cells heterologously expressing the $P2Y_6$ receptor were labeled with [^{3}H]inositol and challenged for 15 min with uridine nucleotides in the presence of 10 mM LiCl, and the resulting [^{3}H]inositol phosphates were quantified. The nucleotides were either untreated UTP ●, untreated UDP (▲), or UDP preincubated for 1 hr with 10 units/ml hexokinase and 25 mM glucose (△) to remove contaminating UTP. Assays with pretreated nucleotides contained an additional 1 unit/ml hexokinase in the incubation medium. *Inset*, [^{3}H]inositol phosphates were measured in the same cells after a 5-min incubation with either UDP or HPLC-purified UTP. B, 1321N1 cells expressing the $P2Y_4$ receptor were challenged with either UTP or UDP. Treatment of UDP with hexokinase and glucose (△) was carried out as in A. C, 1321N1 cells expressing the $P2Y_2$ receptor were challenged with either ATP, UTP, ADP, or UDP. Treatment of ADP and UDP with hexokinase and glucose (open symbols) was carried out as in A. The data were normalized to the maximal effect of UDP ($P2Y_6$ receptor) or UTP ($P2Y_2$ and $P2Y_4$ receptors) in each experiment and represent a combination of at least two to eight different experiments. The standard error of each value was always <10% of the mean value.

TABLE 1

Effects of UTP and carbachol on [^{3}H]inositol phosphate formation in 1321N1 cells infected with empty retrovirus or with retrovirus containing the $P2Y_6$ or $P2Y_4$ receptor coding sequences

Confluent cells were labeled with *myo*-[^{3}H]inositol, preincubated with 10 mM LiCl, and challenged with the indicated agonist for 15 min as described in Materials and Methods. The data are the mean ± standard deviation from one experiment performed with triplicate samples that is representative of 5 to 10 independent experiments.

Condition	Vector	$P2Y_6$	$P2Y_4$
		cpm × 10^{-3}	
Control	0.8 ± 0.05	1.1 ± 0.1	1.2 ± 0.2
Carbachol (1 mM)	9.2 ± 0.7	7.7 ± 0.6	7.3 ± 0.4
UTP (100 μM)	0.7 ± 0.06	13.9 ± 1.3	7.5 ± 0.2

sence of hexokinase and glucose, the inclusion of hexokinase and glucose to prevent any contribution of UTP completely eliminated the apparent effects of UDP. UDP could be quantitatively recovered from the incubation medium in assays carried out in the presence of hexokinase and glucose. Likewise, although ADP initially seemed to be a full agonist at the $P2Y_2$ receptor, ADP had no effect when assays were carried out in the presence of hexokinase and glucose to eliminate any contribution of ATP (Fig. 1C). Thus, the $P2Y_2$ receptor is activated by ATP and UTP but not by ADP or UDP; i.e., it is nucleoside triphosphate specific. In summary, there are three known receptors that are activated by uridine nucleotides; two of these (the $P2Y_2$ and $P2Y_4$ receptors) are activated by UTP but not by UDP, and one (the $P2Y_6$ receptor) is highly selective for UDP over UTP.

The effects of other uridine nucleotides on the three receptors also were studied. We previously synthesized UTPγS and reported that this hydrolysis-resistant nucleotide analog is essentially equipotent with UTP for activation of the $P2Y_2$ receptor (18). As predicted from the results with UTP, UTPγS also was a potent activator of the $P2Y_4$ receptor (Fig. 2B; $K_{0.5}$ = 1.6 ± 0.2 μM) but was a poor agonist at the $P2Y_6$ receptor (data not shown). Conversely, although 5BrUDP was the most potent agonist studied for activation of the $P2Y_6$ receptor ($K_{0.5}$ = 0.13 ± 0.02 μM), it was essentially

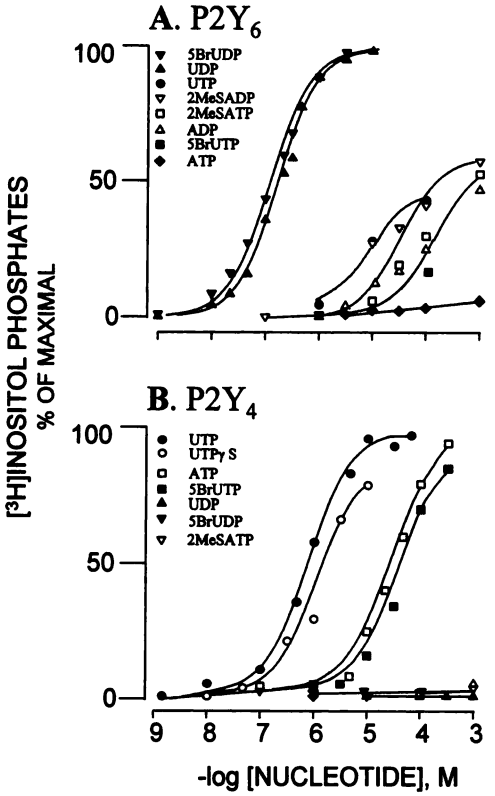

Fig. 2. Pharmacological selectivities of $P2Y_6$ and $P2Y_4$ receptors for various adenine and uridine nucleotides. Inositol phosphate accumulation was measured in 1321N1 cells expressing either the $P2Y_6$ (A) or $P2Y_4$ (B) receptor after a 15-min incubation with the indicated adenine and uridine nucleotides. UDP and ADP were pretreated with hexokinase and glucose as described in the legend to Fig. 1. The curve for UTP in A is from a 5-min incubation with HPLC-purified UTP. The data were normalized as in Fig. 1 and represent a combination of two to eight independent experiments. The standard error of each value was always <10% of the mean value.

inactive at the $P2Y_4$ receptor (Fig. 2B). The selectivity of the three uridine nucleotide-activated receptors for 5BrUDP also was consistent with their selectivity for UDP (Fig. 2 and data not shown). Although it was 30-fold less potent than UTP, 5BrUTP was a full agonist at the $P2Y_4$ receptor (Fig. 2B; $K_{0.5}$ = 49 ± 0.6 μM). Initially, it seemed that 5BrUTP was a potent full agonist at the $P2Y_6$ receptor. However, commercial sources of 5BrUTP contain ≤4% 5BrUDP, and no effect on $P2Y_6$ receptors was observed with 100 μM 5BrUTP that was purified by HPLC (Fig. 2A).

The relative selectivities of $P2Y_2$, $P2Y_6$, and $P2Y_4$ receptors for ATP and other adenine nucleotides also were examined. ATP was a full agonist at the $P2Y_4$ receptor, although the potency of ATP ($K_{0.5}$ = 3.9 ± 0.7 μM) was 50-fold less than UTP for activation of this receptor (Fig. 2). These results contrast in part with those of Communi *et al.* (14), who found that the maximal effect observed with a saturating concentration of ATP was only 50% of that observed with UTP. Furthermore, Nguyen *et al.* (15) reported that ATP was inactive at the $P2Y_4$ receptor, and although data were not presented, the authors commented that ATP was an antagonist of this receptor. The effects of ATP at the $P2Y_4$ receptor were neither additive with nor blocked the effects of UTP (data not shown), indicating that ATP and UTP are both full agonists at the $P2Y_4$ receptor under our assay conditions. In contrast to the results of Chang *et al.* (13), no effect of ATP was observed on the $P2Y_6$ receptor stably expressed in 1321N1 cells (Fig. 2). These results are not explained by hydrolysis of ATP during the assay because ATP could be recovered from the medium after incubation with 1321N1 cells under these assay conditions (data not shown).

We and others have reported that although ATP is essentially equipotent to UTP for activation of the $P2Y_2$ receptor, 2MeSATP, which is a very potent agonist for the $P2Y_1$ receptor, is inactive at the $P2Y_2$ receptor. 2MeSATP also had little effect on inositol phosphate accumulation in 1321N1 cells expressing either the $P2Y_6$ or $P2Y_4$ receptors (Fig. 2). The lack of full agonist effect of 2MeSATP at the $P2Y_6$ receptor contrasts with the full agonist effect reported by Chang *et al.* (13).

We previously reported the existence on C6–2B glioma cells of a phospholipase C-activating receptor that exhibited strict selectivity for activation by uridine nucleotides and selectivity of UDP over UTP for activation (12). The relative order of potency of four uridine nucleotides for promotion of inositol lipid hydrolysis in C6–2B glioma cells was essentially identical to that observed for the $P2Y_6$ receptor expressed in 1321N1 cells when the nucleotides were used without prior purification and in the absence of hexokinase and glucose, as was the case in our original study of the receptor on C6–2B glioma cells. Furthermore, multiple independent $P2Y_6$ receptor cDNA clones were isolated from a C6–2B cell cDNA library probed with the rat $P2Y_6$ receptor sequence. Thus, the $P2Y_6$ receptor is natively expressed in C6–2B rat glioma cells and apparently accounts for the uridine nucleotide-selective receptor activity previously observed in these cells.

Discussion

The existence of pyrimidinergic receptors was hypothesized almost a decade ago from the work of Urquilla (8),

Seifert and Schultz (7), von Kügelgen *et al.* (19), Dubyak (20), and others. The most compelling support for this hypothesis followed from the demonstration of activation of phospholipase C by a so-called P_{2U} receptor in a broad range of tissues. Observation of equipotent activation by UTP and ATP relegated this receptor to membership in the "purinergic" receptor family. However, a uridine nucleotide-specific receptor (i.e., a "pyrimidinergic" receptor) was subsequently identified on C6–2B rat glioma cells (12), and nucleotide sequences encoding two different G protein-coupled receptors that are selectively activated by uridine nucleotides have been reported very recently (13–15). Current convention considers these receptors to be members of a superfamily of "P_{2Y}" receptors,[1] although this designation necessarily requires inclusion of pyrimidinergic receptors that are not activated by adenine nucleotides and that do not fit the original "purinergic" moniker.

The availability of DNA clones encoding three different receptors that are activated by uridine nucleotides has allowed us to assess the relative selectivity of activation of these signaling proteins by naturally occurring uridine nucleotides. We found that precise characterization of relative selectivities requires attention to the initial purity of nucleotides and their stability during incubation with cells. Commercial preparations of purine and pyrimidine nucleotides are predictably impure, and the stability of these molecules can be low in the presence of a myriad of extracellular enzymes that promote hydrolysis or interconversion. Thus, we routinely analyzed the purity of the nucleotides that were used and maintained assay conditions that reduced the likelihood of interconversion of nucleotide molecules. Furthermore, we quantified nucleotides after incubation in the presence of cells to ensure that significant breakdown or interconversion had not occurred. Although the duration of agonist incubations with cells was routinely 15 min, much shorter times of exposure resulted in concentration-effect curves that were indistinguishable from those obtained after longer exposure. Thus, the occurrence of agonist-induced desensitization did not contribute substantively to the relative potencies that are reported. Finally, by measuring inositol phosphate accumulation rather than Ca^{2+} mobilization, we quantified a response that is more proximal to the initial agonist-receptor interaction and eliminated uncertainties that might arise from elevation of Ca^{2+} levels by more than one molecular mechanism. Thus, the relative potencies reported here are likely an accurate reflection of the relative affinities of uridine and adenine nucleotide molecules for the $P2Y_2$, $P2Y_6$, and $P2Y_4$ receptors.

Our results indicate that these three receptors can be clearly delineated into a UDP-preferring, uridine nucleotide-specific receptor (the $P2Y_6$ receptor), a UTP-preferring receptor (the $P2Y_4$ receptor), and a receptor that is activated by both uridine and adenine triphosphates (the $P2Y_2$ receptor). The two receptors that preferentially recognize uridine nucleotides exhibit a markedly different UTP/UDP selectivity. The $P2Y_6$ receptor is highly selective for UDP over UTP, whereas the $P2Y_4$ receptor is potently activated by UTP and is not activated by UDP.

The observed uridine nucleotide selectivities were notably different from those previously described for each receptor. We have reported that UDP is a full agonist at the $P2Y_2$ receptor (11). However, this effect of UDP was apparently artifactual because we illustrate here that incubation of UDP-containing medium with concentrations of hexokinase and glucose that quantitatively convert UTP to UDP resulted in loss of the observed effects of UDP on $P2Y_2$ receptor-promoted inositol phosphate formation. Similarly, although Communi *et al.* (14) reported that UDP and UTP were equipotent at the $P2Y_4$ receptor, generation of concentration-effect curves under conditions that prevented potential contributions of UTP to the response observed with UDP revealed that UDP is essentially inactive at the $P2Y_4$ receptor. Notably, although Chang *et al.* (13) reported that the $P2Y_6$ receptor was activated selectively by UTP, we demonstrate here that UDP-free UTP produced only negligible responses in 1321N1 cells expressing the $P2Y_6$ receptor. These results with $P2Y_2$, $P2Y_6$, and $P2Y_4$ receptors emphasize the need to take into account cross-contamination of stock solutions of UDP and UTP and their potential biological interconversion by nucleoside diphosphokinases and ectonucleotidases.

The selectivities of receptors for adenine nucleotides observed here also were different from those previously reported for the $P2Y_6$ and $P2Y_4$ receptors. ATP was a full agonist at the $P2Y_4$ receptor, and 2MeSATP and ADP were weak partial agonists at the $P2Y_6$ receptor. The reasons for the discrepancies between the current results and those previously reported for adenine nucleotides and adenine nucleotide analogs are unclear, although one or more of the aforementioned concerns involving nucleotide stability or purity may be involved.

In contrast to the physiological importance of adenine nucleotides released from neurons, platelets, and various other tissues, regulated release of uridine nucleotides is less well established (4–6). Perhaps the strongest evidence for physiological relevance of extracellular UDP and UTP emanates from the identification of at least three different phospholipase C-activating receptors that are stimulated by uridine nucleotides. Furthermore, the fact that two of these receptors are activated either selectively or specifically by uridine nucleotides provides compelling support for the existence of important responses that are regulated by extracellular uridine nucleotides. Unambiguous evidence for regulated release of uridine nucleotides is needed to confirm the physiological importance of pyrimidinergic receptor-signaling responses. It also will be important to establish whether release of UDP rather than UTP can be an important initial signaling step associated with $P2Y_6$ receptor-regulated physiological responses.

There are almost certainly additional ATP- and UTP-activated receptors that have not been cloned. For example, we and others have studied an adenylyl cyclase-inhibiting receptor that is preferentially activated by adenine nucleotides and whose pharmacological and second messenger signaling selectivities are not accounted for by the $P2Y_1$ receptor (21, 22). It is perhaps ironic that of the four nucleotide-activated G protein-coupled receptors that have been cloned, three are potently or selectively activated by uridine nucleotides. Conversely, at least four ligand-gated ion channel receptors have been cloned that are activated by ATP (23–27), but to our knowledge none of these are activated by UTP. This suggests the speculation that extracellular adenine nucleotides are prominently involved in fast neuronal and muscle contractile responses, whereas extracellular uridine nucleotides play an

important, albeit unproved, role in responses that need not occur on millisecond time scales.

In summary, three receptors that are activated by uridine nucleotides have been identified. These receptors exhibit remarkably different uridine-versus-adenine and triphosphate-versus-diphosphate nucleotide selectivities. The existence of distinct agonist selectivities heightens the possibility of selective drug development for each of these receptors, and an important next step in their evolution as potential drug targets will be to clearly associate these proteins of known structure with specific members of the exceptionally broad class of physiological responses that are known to be regulated by extracellular nucleotides.

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