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UDP Is a Competitive Antagonist at the Human P2Y₁₄ Receptor

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Abstract

G protein-coupled P2Y receptors (P2Y-R) are activated by adenine and uracil nucleotides. The P2Y₁₄ receptor (P2Y₁₄-R) is activated by at least four naturally occurring UDP sugars, with UDP-glucose (UDP-Glc) being the most potent agonist. With the goal of identifying a competitive antagonist for the P2Y₁₄-R, UDP was examined for antagonist activity in COS-7 cells transiently expressing the human P2Y₁₄-R and a chimeric Gα protein that couples Gi-coupled receptors to stimulation of phosphoinositide hydrolysis. UDP antagonized the agonist action of UDP-Glc, and Schild analysis confirmed that the antagonism was competitive ($pK_B = 7.28$). Uridine 5'-O-thiodiphosphate also antagonized the human P2Y₁₄-R (hP2Y₁₄-R) with an apparent affinity similar to that of UDP. In contrast, no antagonist activity was observed with ADP, CDP, or GDP, and other uracil analogs also failed to exhibit antagonist activity. The antagonist activity of UDP was not observed at other hP2Y-R. In contrast to its antagonist action at the hP2Y₁₄-R, UDP was a potent agonist ($EC_{50} = 0.35 \mu M$) at the rat P2Y₁₄-R. These results identify the first competitive antagonist of the P2Y₁₄-R and demonstrate pharmacological differences between receptor orthologs.

P2Y receptors (P2Y-R) are members of the superfamily of G protein-coupled receptors (GPCRs) and are activated by adenine and uridine nucleotides and nucleotide sugars. At least eight receptors comprise the P2Y-R family. P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptors are coupled to Gq and activate phospholipase C, whereas P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors couple to Gi, leading to the inhibition of adenylyl cyclase and activation of ion channels (Burnstock, 2006). The P2Y₁₁-R uniquely couples both to Gq to activate phospholipase C and to Gs to stimulate adenylyl cyclase (Communi et al., 1997; Qi et al., 2001).

The human P2Y₁₄-R (hP2Y₁₄-R) was identified as the eighth legitimate member of the P2Y receptor family (Chambers et al., 2000). UDP-glucose (UDP-Glc) was proposed to be the endogenous agonist for the P2Y₁₄ receptor (P2Y₁₄-R), with UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine acting as less potent P2Y₁₄-R agonists. P2Y₁₄-R mRNA was detected in a broad range of human tissues including placenta, stomach, intestine, adipose, brain, lung, spleen, and heart, and it was also found in specialized cells such as circulating neutrophils (Chambers et al., 2000; Scrivens and Dickenson, 2006). The rat and mouse P2Y₁₄-R exhibit 80 and 83% amino acid identity to the human receptor, and both rodent orthologs are activated by known agonists with a similar profile to that of the hP2Y₁₄-R (Freeman et al., 2001).

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Cellular UDP-Glc is released in a constitutive manner into the medium of a broad range of cell types. Although most extracellular nucleotides are metabolized quickly, UDP-Glc accumulates in the medium of several cell lines (Lazarowski et al., 2003). The mechanisms of UDP-Glc release and extracellular metabolism remain unclear. Observations of UDP-Glc-promoted signaling were reported in multiple types of immune cells (Fumagalli et al., 2003; Skelton et al., 2003; Müller et al., 2005; Scrivens and Dickenson, 2005), suggesting that the P2Y₁₄-R may have a yet-to-be-defined role in the regulation of immune system homeostasis.

Characterization of the P2Y₁₄-R has been slowed by the lack of a selective competitive antagonist. We have identified and developed novel, selective ligands for several P2Y receptors that have proven useful for pharmacological resolution of molecularly defined P2Y-R in cells and tissues (Boyer et al., 1996; Houston et al., 2006, 2008; Jacobson et al., 2006). Accordingly, we are interested in identifying a selective antagonist for the P2Y₁₄-R.

Ault and Broach (2006) recently used a yeast model system in which various nucleotides and nucleotide sugars were examined for their ability to stimulate growth of mutant P2Y₁₄-R-expressing yeast cells in studies focused on the identification of mutant P2Y₁₄ receptors with differential agonist sensitivities. Studies performed using one of these mutant receptors revealed that UDP antagonized UDP-Glc-promoted receptor activation in a concentration-dependent manner (Ault and Broach, 2006). We hypothesized that UDP acts as a competitive antagonist at the wild-type P2Y₁₄-R and, therefore, used a transfected COS-7 cell system to investigate UDP activity at the human and rat P2Y₁₄-R. In this study, we show that UDP is a selective and competitive antagonist of the hP2Y₁₄-R. Thus, signals emanating from extracellular UDP apparently occur as a consequence of activation of the P2Y₆-R as well as through antagonism of the P2Y₁₄-R. We were surprised to find that UDP is a potent full agonist at the rat P2Y₁₄-R.

Materials and Methods

Materials

UDP-Glc, UDP, ADP, CDP, GDP, and 2-methyl-thio-ADP were purchased from Sigma-Aldrich (St. Louis, MO). Diuridine 5',5'-triphosphate (UP₃U) was synthesized according to the methods detailed in Pendergast et al. (2001). The source of diuridine 5',5'-tetraphosphate (UP₄U) was as previously reported (Ivanov et al., 2007). ATP and UTP were purchased from GE Healthcare (Piscataway, NJ). [³²P]PPi was synthesized as described previously (Lazarowski et al., 2003). Uridine 5'-O-thiodiphosphate (UDPβS) as well as a mammalian expression vector for ecto-nucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) were generous gifts from Dr. José Boyer of Inspire Pharmaceuticals (Durham, NC).

Cell Culture and Transfection

COS-7 cells were grown on 12-well culture dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 4 mM L-glutamine at 37°C in a 10% CO₂ environment. FuGENE 6 (Roche Applied Science, Indianapolis, IN) was used as the transfection reagent following the manufacturer's protocol. Cells were transfected 48 h before assay with pcDNA3.1 expression vectors encoding either the human or rat P2Y₁₄ receptor with an N-terminal hemagglutinin epitope tag. The expression vector for the hP2Y₁₄-R was obtained as reported previously (Lazarowski et al., 2003). Transfections also included pcDNA3.1-*Gα_{q/i}*, a vector that directs expression of a chimera of *Gα_q* containing the last five amino acids of *Gα_i*. This chimeric G protein promotes activation of phospholipase C through *Gα_i*-coupled receptors (Coward et al., 1999). The levels of basal inositol phosphates increased markedly in COS-7 cells upon

expression of human or rat P2Y₁₄-R and G $\alpha_{q/i}$. Because we previously illustrated that UDP-sugars are basally released by various cell types (Lazarowski et al., 2003), in some experiments pcDNA3.1-expressing ENPP1 was cotransfected with the goal of hydrolyzing extracellular nucleotide sugars and potentially decreasing inositol phosphate accumulation in the absence of added P2Y₁₄-R agonists.

Inositol Phosphate Accumulation Assay

Cells were labeled 8 to 18 h before assay with 0.5 to 3 μ Ci/well *myo*-[³H]inositol (American Radiolabeled Chemicals, St. Louis, MO) in inositol-free and serum-free DMEM. Assays were initiated with the addition of 10 mM LiCl with or without drugs, and incubations continued for 45 min at 37°C. Reactions were stopped by aspiration of medium and addition of ice-cold 50 mM formic acid. After neutralization with 150 mM ammonium hydroxide, [³H]inositol phosphates were isolated by Dowex column chromatography as described previously (Nakahata and Harden, 1987). Stable cell lines for P2Y₁, P2Y₂, P2Y₄, P2Y₆, or P2Y₁₁ receptors were generated in 1321N1 human astrocytoma cells as described previously (Nicholas et al., 1996; Kennedy et al., 2000). Experiments testing the potential activity of UDP at the P2Y₂-R and P2Y₄-R included hexokinase to eliminate contamination of UTP as previously described in Nicholas et al. (1996). In brief, UDP was treated with 10 U/ml hexokinase in the presence of 22 mM glucose for 1 h at 37°C, and 1 U/ml hexokinase was included in the assay buffer for the duration of the incubation. Variability in cpm of [³H]-inositol phosphate accumulation across experiments occurred due to differences in the amount of [³H]inositol used for labeling and/or the duration of the prelabeling period.

Measurement of UDP-Glc in the Cell Medium

Quantification of UDP-Glc was performed as described previously (Lazarowski et al., 2003). In brief, incubations were in a final volume of 150 μ l containing known or unknown amounts of UDP-Glc, 25 mM HEPES, pH 7.4, 0.5 U/ml UDP-glucose pyrophosphorylase from baker's yeast (Sigma-Aldrich), and 100 nM [³²P]PPi (200,000 cpm). Incubations were terminated by addition of 0.3 mM PPi and immediate heating of samples for 2 min at 95°C, and formation of [³²P]UTP was quantified by high-performance liquid chromatography as described previously (Lazarowski et al., 2003).

Quantification of P2Y₁₄-R Expression

Cells were seeded in 12-well plates at 5×10^4 cells/well 3 days before assay and transfected with mammalian expression vectors as described above. Cells were fixed with 0.4 ml of 4% paraformaldehyde for 30 min at room temperature, washed twice with 1 ml of Hanks' balanced salt solution plus Ca²⁺/Mg²⁺, and incubated for 30 min at room temperature with 0.4 ml of DMEM plus 50 mM HEPES, pH 7.1, and 10% fetal bovine serum. Cells were incubated with mouse HA.11 monoclonal antibody at a 1:1000 dilution in 0.4 ml of medium for 1 h at room temperature. After two washes with 1 ml of Hanks' balanced salt solution plus Ca²⁺/Mg²⁺, cells were incubated with [¹²⁵I]rabbit anti-mouse IgG antibody diluted to 1:500 in 0.4 ml of medium for 2 h at room temperature. After another series of washing steps, cells were solubilized in 0.4 ml of 1 M NaOH overnight and transferred to glass tubes for quantification of radioactivity in a gamma counter.

Rat P2Y₁₄-R Subcloning

The rat homolog of the P2Y₁₄-R (rP2Y₁₄-R) was amplified from rat genomic DNA using *Pfu* polymerase (Stratagene, Carlsbad, CA) with the following primers: (5'-GAGACGCGTCCGACAACACAACACAGAAC-3') and (5'-AGACTCGAGTTACAAAGTATCTGTGCTTTCC-3'). The primers contained either a MluI (upstream primer) or a XhoI (downstream primer) restriction site, respectively (sites

are underlined), to facilitate cloning. The amplification conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s; and a final extension for 4 min at 72°C. The amplified rP2Y₁₄-R fragment was digested with MluI and XhoI, purified, and ligated into a similarly digested, modified pcDNA3 expression vector, which fuses a hemagglutinin epitope to Asp-2 at the N terminus of the receptor. An individual clone encoding the receptor was sequenced and found to be identical to the published sequence (Freeman et al., 2001).

Data Analyses

The results from each experiment are expressed as the mean $\pm$ S.E. from triplicate samples and were analyzed using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). All experiments were repeated at least three times with similar results. Schild analysis was performed using EC₅₀ values from the concentration-effect curves for UDP-Glc generated in the absence and presence of increasing concentrations of UDP. The pK_B was calculated using the equation: $\log ([A']/[A] - 1) = \log [B] - \log pK_B$ (Arunlakshana and Schild, 1959), where [A'] is the concentration of UDP-Glc necessary to produce 50% of the maximal effect in the presence of antagonist [B], and [A] is the concentration of agonist necessary to produce 50% of the maximal effect in the absence of antagonist. Drug response data presented in Figs. 3A, 5, A–D, and 7B are normalized as a percentage of the response observed with a maximally effective concentration (usually 10 μ M) of UDP-Glc. Statistics were carried out using the Student's *t* test.

Results

Agonist Activity of UDP-Glc at the hP2Y₁₄-R

To assay the functional activity of the hP2Y₁₄-R, we used COS-7 cells transiently coexpressing the hP2Y₁₄-R and a G $\alpha_{q/i}$ chimera. The G $\alpha_{q/i}$ chimera is a G α_q protein in which the last five amino acids at the C terminus have been substituted with those of G α_i . This chimeric G α subunit is activated by GPCRs that couple to the G α_i family of G proteins and signal through downstream G α_q effectors such as phospholipase C (Coward et al., 1999). Expression of the hP2Y₁₄-R or the G $\alpha_{q/i}$ chimera alone in COS-7 cells resulted in levels of [³H]inositol phosphate accumulation similar to that observed in cells expressing empty vector alone. [³H]Inositol phosphate accumulation was not changed by the addition of 100 μ M UDP-Glc to cells expressing empty vector, the hP2Y₁₄-R, or G $\alpha_{q/i}$ (Fig. 1). Coexpression of the hP2Y₁₄-R and G $\alpha_{q/i}$ resulted in increased [³H]inositol phosphate accumulation in the presence of buffer alone, as we previously reported (Lazarowski et al., 2003). Addition of 100 μ M UDP-Glc to cells coexpressing the hP2Y₁₄-R and G $\alpha_{q/i}$ resulted in a 2-fold increase in inositol phosphate accumulation, consistent with agonist-promoted activation of phospholipase C (Fig. 1).

To investigate the nature of the hP2Y₁₄-R-dependent [³H]inositol phosphate accumulation in the absence of added agonist, we coexpressed ENPP1 with the hP2Y₁₄-R and G $\alpha_{q/i}$ in COS-7 cells with the goal of removing any released nucleotide/nucleotide sugar potentially present in the medium. UDP-Glc levels (3.6 ± 1.9 nM) in the bulk medium from COS-7 cells expressing hP2Y₁₄-R and G $\alpha_{q/i}$ were similar to that of control cells (3.3 ± 0.3 nM). In contrast, coexpression of ENPP1 in cells also expressing hP2Y₁₄-R and G $\alpha_{q/i}$ resulted in an approximately 78% reduction in UDP-Glc levels (0.8 ± 0.5 nM) compared with control cells. Although expression of ENPP1 alone had no effect on basal [³H]inositol phosphate accumulation, expression of ENPP1 with hP2Y₁₄-R and G $\alpha_{q/i}$ resulted in an approximately 40% decrease ($p < 0.01$) in basal [³H]inositol phosphate levels compared with cells expressing receptor and G protein alone (Fig. 2A). Expression of ENPP1 had no effect on surface expression of the hP2Y₁₄-R as quantified with an immunoassay (data not shown; see

Materials and Methods), and it did not notably change the concentration-effect curve for added UDP-Glc (Fig. 2B). Thus, we conclude that the hP2Y₁₄-R-dependent elevation of [³H]inositol phosphate levels in the absence of added agonist occurs largely because of autocrine/paracrine release of the P2Y₁₄-R agonist. However, these results do not entirely rule out the possibility that the overexpressed P2Y₁₄-R exhibits constitutive activity in this test system.

Antagonist Effect of UDP at the hP2Y₁₄-R

Four UDP sugars were identified as agonists at the hP2Y₁₄-R, and neither UTP nor UDP exhibited agonist activity (Chambers et al., 2000). To determine whether UDP is an antagonist at the wild-type hP2Y₁₄-R, we generated a series of concentration-effect curves for UDP-Glc-promoted stimulation of phospholipase C in the presence of increasing concentrations of UDP (Fig. 3A). UDP caused a parallel rightward shift of the UDP-Glc concentration-effect curve, and Schild analysis (Fig. 3B) confirmed that the antagonism produced by UDP was competitive (slope = 1.15 ± 0.06, *n* = 3). The p*K*_B of UDP for antagonism of the hP2Y₁₄-R was 7.28 ± 0.04.

We also assessed whether UDP exhibited antagonist activity at other P2Y-R stably expressed in 1321N1 human astrocytoma cells. P2Y₁-R was maximally activated by 1 μM 2MeSADP (Fig. 4A), P2Y₂-R and P2Y₄-R were each activated by 3 μM UTP (Fig. 4, B and C), and P2Y₁₁-R was activated by 100 μM ATP (Fig. 4D). Although 10 μM UDP completely blocked a near-maximal concentration of UDP-Glc at the hP2Y₁₄-R (Fig. 3), UDP had no effect at any other P2Y receptors tested. Thus, we conclude that UDP is a selective antagonist at the hP2Y₁₄-R.

To determine whether the antagonist effect of UDP at the hP2Y₁₄-R is specific to the uracil structure, we also tested other nucleotides and nucleotide derivatives as antagonists at the hP2Y₁₄-R. In contrast to the action of UDP, other nucleoside diphosphates including ADP, CDP, and GDP, at concentrations of 10 or 100 μM, did not inhibit UDP-Glc (3 μM)-promoted [³H]inositol phosphate formation (Fig. 5). We also tested whether other uridine-based molecules would antagonize activation of the hP2Y₁₄-R by UDP-Glc. Neither UTP, UP₃U, nor UP₄U inhibited UDP-Glc activation of the receptor, although each of these nucleotides, when tested alone, produced a stimulatory effect in untransfected COS-7 cells (data not shown). The discovery of antagonist activity of UDP at the hP2Y₁₄-R is also supported by the observation that the UDP analog, UDPβS, inhibited activation of this receptor by UDP-Glc (Fig. 6). The IC₅₀ observed for UDPβS was similar to that determined for UDP under the same assay conditions. From these results, we conclude that UDP seems to be unique among naturally occurring nucleotides in its capacity to inhibit UDP-Glc-dependent activation of the hP2Y₁₄-R.

Effects of UDP at the rP2Y₁₄-R

Because pharmacological studies are often carried out with rat or mouse tissues, it is important to assess whether receptor orthologs exhibit pharmacological selectivities similar to those of human P2Y-R. Based on the precedent that ATP acts as an antagonist at the human P2Y₄-R but is an agonist at the rat P2Y₄-R (Bogdanov et al., 1998; Kennedy et al., 2000), we compared the action of UDP at the rP2Y₁₄-R with its action at the hP2Y₁₄-R. The rat P2Y₁₄-R, which exhibits approximately 80% amino acid sequence identity to the hP2Y₁₄-R and almost 90% identity in the transmembrane regions alone, was reported to display a similar UDP-sugar selectivity to that of the hP2Y₁₄-R (Freeman et al., 2001), but the actions of other uridine nucleotides on the rP2Y₁₄-R have not been reported.

Expression of either the rP2Y₁₄-R or Gα_{q/i} in COS-7 cells had no effect on [³H]inositol phosphate accumulation compared with untransfected cells, but coexpression of receptor and Gα_{q/i} resulted in markedly increased basal accumulation (Fig. 7A). Consistent with other reports (Freeman et al., 2001), UDP-Glc was a potent agonist at the rP2Y₁₄-R (Fig. 7B). Whereas UDP had no effect on inositol phosphate accumulation in wild-type COS-7 cells, in cells expressing Gα_{q/i} alone, or in cells expressing the rP2Y₁₄-R alone (data not shown), concentration of UDP-dependent increases in formation of [³H]inositol phosphates occurred in COS-7 cells coexpressing the rat P2Y₁₄-R with Gα_{q/i} (Fig. 7B). The maximal stimulatory effect observed with UDP was similar to that observed with UDP-Glc, as were the EC₅₀ values of UDP (0.35 ± 0.17 μM) and UDP-Glc (EC₅₀ = 0.28 ± μM). No additivity was observed between UDP and UDP-Glc on rP2Y₁₄-R-promoted [³H]inositol phosphate formation (Fig. 7C).

Discussion

In this study, we show that UDP is a competitive antagonist at the human P2Y₁₄-R, and this action is receptor-selective because UDP does not inhibit agonist-promoted activation of other hP2Y receptors. Moreover, the activity of UDP at the P2Y₁₄-R is species-dependent because we observed that UDP is a potent, and apparently full, agonist at the rat P2Y₁₄-R.

Chambers et al. (2000) reported in their initial study of the hP2Y₁₄-R that UDP has no agonist activity, and we observed similar results in the studies reported here. Using a reporter system in yeast, Ault and Broach (2006) generated a mutant hP2Y₁₄-R displaying a mutation in intracellular loop 1 and various mutations in several of the transmembrane regions. This mutant, selected for its ability to support growth of yeast at lower concentrations of UDP-Glc than the wild-type receptor, exhibited an enhanced UDP-Glc-stimulated response that was inhibited by UDP, and a K_B in the micromolar range was reported. The >20-fold higher potency of UDP observed in our studies probably reflects large differences in the assay systems used. For example, whereas incubations with nucleotide were continued for minutes in the current study, they were continued for hours in assays measuring P2Y₁₄-R-mediated regulation of growth of yeast. Our results illustrate that UDP is a potent, competitive antagonist of the wild-type hP2Y₁₄-R.

Demonstration of antagonist action at the hP2Y₁₄-R suggests that UDP may have broader physiological importance as an extracellular signaling molecule than has been previously appreciated. Both UDP and UDP-glucose are known to be released from cells, although the mechanisms of their release remain unclear. UDP is the most potent and selective agonist of the hP2Y₆-R (Lazarowski and Harden, 1994; Communi et al., 1996), and physiological responses attributed to UDP-initiated P2Y₆-R-promoted signaling include modulation of interleukin-8 production in monocytes (Warny et al., 2001) and human mature dendritic cells (Idzko et al., 2004). In addition, UDP was observed to induce a positive inotropic effect in mouse cardiomyocytes (Wihlborg et al., 2006) and to promote ion transport in human placental cytotrophoblast cells (Roberts et al., 2006). Our data indicate that potential contributions of the P2Y₁₄-R to responses associated with UDP must be considered. Whereas reported distribution of P2Y₆-R mRNA overlaps with that of P2Y₁₄-R mRNA in many cells and tissues, such as lung, heart, placenta, and neutrophils (Communi et al., 1996; Moore et al., 2001), it remains unclear whether the two receptor types are coexpressed in the same cells or in different cells that share the same extracellular space.

The actions of extracellular neurotransmitters and hormones are highly regulated by their release, metabolism, and reuptake. The possibility that direct antagonism of GPCR activation occurs by extracellular signaling molecules has been suggested by observations that ATP is a competitive antagonist of the hP2Y₁₂-R (Cusack and Hourani, 1982; Bodor et

al., 2003) and of the hP2Y₄-R (Bogdanov et al., 1998; Kennedy et al., 2000). The physiological relevance of UDP antagonism at the hP2Y₁₄-R will be important to investigate, as will the idea that UDP simultaneously activates the hP2Y₆-R while inhibiting the hP2Y₁₄-R.

Our finding that UDP has agonist activity at the rP2Y₁₄-R was surprising. Study of the rat and human receptors under identical conditions rules out trivial explanations for this observation. These data do not unambiguously rule out the possibility that UDP is a partial agonist at the human receptor under some conditions. However, the agonist versus antagonist action of the nucleotide at the rat versus human P2Y₁₄-R has been observed over a broad range of expression levels of these two receptors. Our activity data suggest that the binding affinity of UDP for the human and rat receptors are in fact quite similar, but development of a radioligand binding assay will be necessary to fully assess this assertion. The fact that the maximal agonist activity of UDP was similar to that of UDP-Glc over a broad range of P2Y₁₄-R expression levels (data not shown) suggests that the intrinsic efficacy of UDP at the rat P2Y₁₄-R is similar to that of UDP-Glc.

The differential activity of UDP observed between the rat and human orthologs of the P2Y₁₄-R shares similarities to the actions of ATP at the P2Y₄-R. Kennedy et al. (2000) compared the ligand selectivities of the rat and human P2Y₄ receptors under conditions that minimize the effects of released nucleotides and of extracellular bioconversion of nucleotides, and they observed that ATP is an agonist at the rat P2Y₄-R and a competitive antagonist at the human P2Y₄-R. Residues in the second extracellular loop of the P2Y₄ receptor are the primary determinants for the agonist versus antagonist activity of ATP between the two species orthologs (Herold et al., 2004). Like the P2Y₄-R, the P2Y₁₄-R shares approximately 80% amino acid sequence identity between the rat and human orthologs, with 90% identity when analysis is restricted to transmembrane regions only. Recent work by Ko et al. (2007) defines the human P2Y₁₄ receptor through structure-activity studies in conjunction with molecular modeling studies. Residues in the second extracellular loop of the hP2Y₁₄-R are predicted to interact with the diphosphate moiety and hydroxyl groups on the hexose ring of UDP-Glc. Comparative modeling of the rat P2Y₁₄-R and receptor mutagenesis directed from these predictions may identify key domains responsible for agonist efficacy at the hP2Y₁₄-R.

The mouse P2Y₁₄-R has been cloned and is reported to be activated by the same UDP-sugar agonists as the human and rat receptors (Freeman et al., 2001). The rat and mouse P2Y₁₄-R share 89% overall amino acid sequence identity and are essentially identical in their transmembrane-spanning domains and the second extracellular loop. Thus, we anticipate that the agonist action of UDP observed with the rat receptor will be similarly observed at the mouse P2Y₁₄-R.

The finding that UDP acts as a competitive antagonist at the hP2Y₁₄-R provides an excellent template for rational synthesis of antagonist analogs that exhibit high affinity at the hP2Y₁₄-R. Our structure-activity studies of UDP analogs at the hP2Y₆-R (Besada et al., 2006) also provide potential avenues for development of P2Y₁₄-R antagonists that do not act as ligands for the P2Y₆-R. Synthesis of a hydrolysis-resistant competitive antagonist for the P2Y₁₄-R is an obvious goal, as is a high-affinity radiolabeled antagonist.

UDP-glucose, UDP-galactose, UDP-glucuronic acid, and UDP-*N*-acetylglucosamine were previously identified as full or partial agonists at the human and rodent P2Y₁₄-R (Chambers et al., 2000; Freeman et al., 2001). The pharmacological selectivity for the P2Y₁₄-R is now broadened with the finding that UDP also acts at this receptor. The identification of UDP as a competitive antagonist for the hP2Y₁₄-R provides new insight into the physiological

regulation of this receptor, and it should be of pharmacological importance in delineating the functional roles subserved by this signaling protein.

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ABBREVIATIONS

P2Y-R	P2Y receptor
GPCR	G protein-coupled receptor
hP2Y ₁₄ -R	human P2Y ₁₄ -R
UDP-Glc	UDP glucose
P2Y ₁₄ -R	P2Y ₁₄ receptor
UP ₃ U	diuridine 5',5'-triphosphate
UP ₄ U	diuridine 5',5'-tetrathosphate
UDPβS	uridine 5'-O-thiodiphosphate
ENPP1	ecto-nucleotide pyrophosphatase/phosphodiesterase-1
DMEM	Dulbecco's modified Eagle's medium
rP2Y ₁₄ -R	rat homolog of the P2Y ₁₄ -R

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Fig. 1. hP2Y₁₄-R- and $G\alpha_{q/i}$ -dependent increases in [³H]inositol phosphate accumulation. COS-7 cells were transfected with empty vector or expression vectors for hP2Y₁₄-R and/or $G\alpha_{q/i}$ as described under *Materials and Methods*. Cells were labeled with [³H]inositol for 18 h before assay. LiCl (10 mM) buffer was added to the cells to inhibit inositol monophosphatase, and the cells were simultaneously incubated in the absence or presence of 10 μM UDP-Glc for 45 min. [³H]Inositol phosphates were isolated as described under *Materials and Methods*. The data shown are the means ± S.E. calculated from triplicate samples and are representative of the results obtained in three independent experiments.

Fig. 2.

Coexpression of ENPP1 reduces the basal activation of the hP2Y₁₄-R. COS-7 cells were cotransfected with expression vectors for hP2Y₁₄-R and *Ga_{q/i}* with (+) or without (–) an expression vector for ENPP1. A, [³H]inositol-labeled cells were incubated with 10 mM LiCl in the absence or presence of UDP-Glc. *, *p* < 0.01. B, [³H]inositol-labeled cells were incubated with 10 mM LiCl in the absence or presence of UDP-Glc at the indicated concentrations. The data shown are the means $\pm$ S.E. calculated from triplicate determinations.

Fig. 3.

UDP is a competitive antagonist at the hP2Y₁₄-R. A, [^3H]inositol-labeled COS-7 cells coexpressing hP2Y₁₄-R and $G\alpha_{q/i}$ were incubated with LiCl (10 mM) and increasing concentrations of UDP-glucose in the absence or presence of the indicated concentrations of UDP: ■, buffer; ▲, 0.1 μM ; ▼, 0.3 μM ; ◆, 1 μM ; ●, 3 μM ; □, 30 μM ; and ▽, 100 μM . Data shown are the means $\pm$ S.E. calculated from triplicate samples and are representative of the results obtained in three independent experiments. B, EC₅₀ values from the concentration-effect curves in A were used for Schild regression analysis. The data shown are results from a representative experiment repeated three times to yield a mean pK_B of 7.28 ± 0.04 and a slope of 1.15 ± 0.06 .

Fig. 4.

UDP is a selective antagonist at the hP2Y₁₄-R. [³H]Inositol-labeled 1321N1 human astrocytoma cells stably expressing either the human P2Y₁-R (A), human P2Y₂-R (B), human P2Y₄-R (C), or human P2Y₁₁-R (D) were incubated for 30 min with 10 mM LiCl with the cognate agonist (indicated), or 10 μ M UDP, or both agonist and UDP, and inositol phosphate accumulation was quantified as described under *Materials and Methods*. The data shown are the means $\pm$ S.E. calculated from triplicate samples and are representative of the results obtained in three or more independent experiments.

Fig. 5.

UDP is unique among nucleoside diphosphates for its antagonist effect at the hP2Y₁₄-R. CDP (A), ADP (B), GDP (C), or UDP (D) (10 or 100 μM) and LiCl (10 mM) were applied simultaneously with 3 μM UDP-Glc to [³H]inositol-labeled COS-7 cells transiently expressing hP2Y₁₄-R and Gα_{q/i}. Data were normalized to values from maximal activation of hP2Y₁₄-R by UDP-Glc alone, and the LiCl alone value was subtracted from each data point. The data shown in A, C, and D are results from a representative experiment repeated three times. The data shown in B are the average of results from four experiments.

Fig. 6.

Antagonist effect of UDPβS at the hP2Y₁₄-R. [³H]Inositol-labeled COS-7 cells transiently expressing hP2Y₁₄-R and Gα_{q/i} were incubated with 10 mM LiCl in the absence (○) or presence (●) of 1 μM UDP-Glc in the presence of the indicated concentrations of UDPβS for 30 min. The data shown are the means ± S.E. of triplicate determinations, and the results are representative of those obtained in three experiments.

Fig. 7.

Agonist effect of UDP-Glc and UDP at the rP2Y₁₄-R. A, COS-7 cells transfected with an expression vector for Gα_{q/i} and with empty vector or rP2Y₁₄-R were [³H]inositol-labeled and incubated with 10 mM LiCl in the absence or presence of 10 μM UDP-Glc. B, [³H]inositol-labeled COS-7 cells transiently expressing the rP2Y₁₄-R and Gα_{q/i} were incubated with 10 mM LiCl and UDP-Glc or UDP at the indicated concentrations, and [³H]inositol phosphate accumulation was quantified as described under *Materials and Methods*. The data shown are the means ± S.E. calculated from triplicate samples and are results from a representative experiment repeated three times, yielding an EC₅₀ of 0.28 ± 0.05 μM for UDP-Glc and 0.35 ± 0.17 μM for UDP. Data were normalized to the maximal activation of rP2Y₁₄-R by UDP-Glc. C, cells incubated with UDP-Glc (10 μM) + UDP (100 μM) exhibited no difference in [³H]inositol phosphate accumulation compared to accumulation in the presence of either agonist alone. The data shown are the means ± S.E. calculated from triplicate samples and are representative of results obtained in three independent experiments.