G<sub>i</sub>-Dependent Cell Signaling Responses of the Human P2Y<sub>14</sub> Receptor in Model Cell Systems

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ABSTRACT

Eight G protein-coupled receptors comprise the P2Y receptor family of cell signaling proteins. The goal of the current study was to define native cell signaling pathways regulated by the uridine nucleotide sugar-activated P2Y<sub>14</sub> receptor (P2Y<sub>14</sub>-R). The P2Y<sub>14</sub>-R was stably expressed in human embryonic kidney (HEK) 293 and C6 rat glioma cells by retroviral infection. Nucleotide sugar-dependent P2Y<sub>14</sub>-R activation was examined by measuring inhibition of forskolin-stimulated cAMP accumulation. The effect of P2Y<sub>14</sub>-R activation on mitogen-activated protein kinase signaling also was studied in P2Y<sub>14</sub>-HEK293 cells and in differentiated HL-60 human myeloid leukemia cells. UDP-Glc, UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine promoted inhibition of forskolin-stimulated cAMP accumulation in P2Y<sub>14</sub>-HEK293 and P2Y<sub>14</sub>-C6 cells, and this signaling effect was abolished by pretreatment of cells with pertussis toxin. Inhibition of cAMP formation by nucleotide sugars also was observed in direct assays of adenyl cyclase activity in membranes prepared from P2Y<sub>14</sub>-C6 cells. UDP-Glc promoted concentration-dependent and pertussis toxin-sensitive extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in P2Y<sub>14</sub>-HEK293 cells. P2Y<sub>14</sub>-R mRNA was not observed in wild-type HL-60 cells but was readily detected in dimethyl sulfoxide-differentiated cells. Consistent with this observation, no effect of UDP-Glc was observed in wild-type HL-60 cells, but UDP-Glc-promoted pertussis toxin-sensitive activation of ERK1/2 occurred after differentiation. These results illustrate that the human P2Y<sub>14</sub>-R signals through G<sub>i</sub> to inhibit adenyl cyclase, and P2Y<sub>14</sub>-R activation also leads to active extracellular signal-regulated kinase (ERK) 1/2 phosphorylation. This work also identifies two stable P2Y<sub>14</sub>-R-expressing cell lines and differentiated HL-60 cells as model systems for the study of P2Y<sub>14</sub>-R-dependent signal transduction.

The P2Y<sub>14</sub>-R is a seven-transmembrane-spanning G protein-coupled receptor that is activated by UDP-Glc and other UDP sugars (Chambers et al., 2000). P2Y<sub>14</sub>-R mRNA is expressed in stomach, intestine, placental and adipose tissues, lung, heart, and throughout the brain, and in many types of immune cells (Lee et al., 2003; Moore et al., 2003; Skelton et al., 2003; Scrivens and Dickenson, 2006). Consistent with its prominent immune cell expression, the P2Y<sub>14</sub>-R has been implicated in several immune cell functions. Moore et al. (2003) reported that P2Y<sub>14</sub>-R mRNA is up-regulated in several brain regions after immunological challenge of mice with lipopolysaccharide. In addition, UDP-Glc was reported to promote chemotaxis of bone marrow-derived hematopoietic stem cells (Lee et al., 2003).

The P2Y<sub>14</sub>-R is a member of a subgroup of P2Y receptors, which includes the P2Y<sub>12</sub> and P2Y<sub>13</sub> receptors thought primarily to activate heterotrimeric G proteins of the G<sub>i</sub> family, but a comprehensive understanding of the signal transduction pathways activated by the P2Y<sub>14</sub>-R is not available. Nonetheless, several reports are consistent with the idea that the P2Y<sub>14</sub>-R couples to G<sub>i</sub>. For example, Chambers and his colleagues illustrated in their initial study of the cloned human P2Y<sub>14</sub>-R that UDP-Glc promotes pertussis toxin-sensitive binding of radiolabeled GTP<sub>S</sub> to membranes prepared from HEK293 cells expressing this receptor. Modest UDP-Glc-promoted inhibition of cAMP accumulation also has been reported for murine T-lymphocytes (Scrivens and Dickinson, 2005), human neutrophils (Scrivens and Dickinson, 2006), and C6 glia cells (Krzeminski et al., 2008), although the effects were not shown unambiguously to involve the P2Y<sub>14</sub>-R. Other pharmacological studies of the P2Y<sub>14</sub>-R have

ABBREVIATIONS: P2Y<sub>14</sub>-R, P2Y<sub>14</sub> receptor; HEK, human embryonic kidney; MAP, mitogen-activated protein kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; IBMX, 3-isobutyl-1-methylxanthine; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; RT, reverse transcriptase; PCR, polymerase chain reaction; fMLP, formyl-Met-Leu-Phe; PAR, protease-activated receptor; FPR, formyl peptide receptor.
relied on coexpression with the promiscuous G protein, Ga\textsubscript{16} (Chambers et al., 2000), or with a chimeric G\textsubscript{i} (Moore et al., 2003; Fricks et al., 2008) engineered to couple G\textsubscript{i}-activating receptors to activation of phosphoinositide hydrolysis and Ca\textsuperscript{2+} mobilization (Coward et al., 1999).

Studies of the P2Y\textsubscript{14}-R have been limited by inability to detect and quantify receptor expression directly through the use of antibodies or radioligand binding assays and also by the absence of high-affinity, nonhydrolyzable, selective agonists, and competitive antagonists to verify receptor-specific signal transduction. It is critical that we understand the cell signaling processes engaged in response to P2Y\textsubscript{14}-R activation, and with this goal in mind, we generated two different cell lines that stably express the human P2Y\textsubscript{14}-R. Robust P2Y\textsubscript{14}-R-dependent inhibition of adenylyl cyclase was observed in both cell lines, and P2Y\textsubscript{14}-R-dependent MAP kinase signaling was studied in P2Y\textsubscript{14}-HEK293 cells. We also discovered that expression of native P2Y\textsubscript{14}-R is induced during differentiation of HL-60 human myeloid leukemia cells and that UDP-Glc promotes activation of MAP kinase signaling in these cells. The model cell systems reported here should provide useful platforms for investigation of the P2Y\textsubscript{14}-R at the cellular and biochemical level.

Materials and Methods

Cell Culture. HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 10% CO\textsubscript{2} environment. C6 rat glioma cells were cultured in DMEM supplemented with 5% FBS in a 5% CO\textsubscript{2} environment. HL-60 cells were maintained in Iscove’s medium supplemented with 10% FBS. Differentiation of HL-60 cells was achieved by inclusion of 1.3% DMSO in the culture medium for 5 days (Servant et al., 2000).

P2Y\textsubscript{14}-R Expression. Human P2Y\textsubscript{14}-R cDNA was amplified and ligated into the retroviral expression vector pLXSN as described previously (Wolff et al., 2005). Retrovirus encoding the P2Y\textsubscript{14}-R was produced in PA317 cells according to the method of Johnson et al. (1998) and was used to infect HEK293 cells or C6 glioma cells. Geneticin-resistant cells were selected for 2 weeks in medium containing 0.4 mg/ml G418. Clonal HEK293 cells stably expressing the hP2Y\textsubscript{14} were obtained by performing serial dilutions of cells in 96-well plates and growing clonal populations from a single cell under selection medium.

Membrane Preparation. Membranes were prepared as described previously (Smith and Harden, 1985). In brief, P2Y\textsubscript{14}-C6 rat glioma cells were grown on 150-mm dishes until confluent. Cells were washed gently with phosphate-buffered saline and then lysed with ice-cold 1 mM Tris, pH 7.4. Cells were harvested by scraping off the dishes and homogenized with a glass homogenizer for 10 strokes. Lysates were centrifuged at 40,000 \texttimes g for 30 min. Washed membranes were resuspended in Hanks’ balanced salt solution for the assay at a density of 5 \times 10\textsuperscript{6} cells/ml, 0.2 ml/assay. Drugs were added for the indicated times, and the assay was terminated by aspiration of medium. The cells were washed once with phosphate-buffered saline, and Laemmli buffer containing 60 \mu M dithiothreitol was added to each well. The resultant cell lysates were passed through a 27-gauge needle 10 times, heated to 95°C for 5 min, and proteins were resolved by electrophoresis on a 12.5% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, blocked with 5% bovine serum albumin, washed with Tris-buffered saline/Tween 20 (20 mM Tris, pH 7.4, 120 mM NaCl, 0.1% Tween 20), and then incubated with antibody for phospho-ERK1/2, phospho-p38, or phospho-e-Jun NH\textsubscript{2}-terminal kinase (JNK), according to the manufacturer’s directions. After washing with Tris-buffered saline/Tween 20, membranes were incubated with horseradish peroxidase-conjugated goat-anti-mouse (phospho-ERK1/2, phospho-JNK) or goat-anti-rabbit (phospho-p38) antibody, membranes were washed, incubated with chemiluminescent substrate (Pico West system; Thermo Fisher Scientific, Waltham, MA), and then exposed to film. Membranes were stripped with 200 mM glycine, pH 2.6, for 1 h at 25°C, then reporbed with a primary antibody against total MAP kinase to verify equal loading of lanes. HL-60 cells were serum-starved 24 h before the assay and resuspended in Hanks’ balanced salt solution for the assay at a density of 5 \times 10\textsuperscript{6} cells/ml, 0.2 ml/assay. Drugs were added for the indicated times, and the lysates were lysed by adding 1 volume of Laemmli buffer containing 60 \mu M dithiothreitol to the cells. Lysates were analyzed as described above.

Reverse Transcriptase-PCR. HL-60 cells were harvested, washed, and lysed in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated by chloroform extraction, and isolation of mRNA was performed using the Oligotex mRNA Mini kit (QIAGEN, Valencia, CA). cDNA was generated using the reverse transcriptase (RT) III SuperMix First-Strand Synthesis kit (Invitrogen). P2Y\textsubscript{14}-R-specific primers (5’-ACTACGGCTCATCATTCAATGACATCCTTAACACTCTGGTTGGTGAGAAT-3’ and 5’-GTGTAGTGCATCTCAAACTACTTGTGGTGGAGATGC-3’) were used in the PCR reactions, and conditions were as follows: 95°C, 30 s; 55°C, 30 s; and 72°C, 1 min, for 38 cycles. PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Negative controls for each sample were performed without reverse transcriptase added to the reaction.

Data Analysis. EC\textsubscript{50} values were determined using Prism software (GraphPad Software Inc., San Diego, CA) and are presented as mean \pm S.E. Statistical significance was determined by analysis of variance, and p < 0.02 was considered statistically significant. All experiments were repeated at least three times.

Materials. IBMX, creatine phosphate, creatine phosphokinase, forskolin, formyl-Met-Leu-Phe (fMLP), and GTP were purchased from Sigma-Aldrich (St. Louis, MO). UDP-Glc, UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine were all from Fluka (Buchs, Switzerland), purchased through Sigma-Aldrich. HPLC analysis of the UDP-Glc used in the current experiments revealed >98% purity. ATP was purchased from GE Healthcare (Chalfont St. Giles, UK). Pertussis toxin was purchased from List Biological Laboratories Inc.(Campbell, CA). [\textsuperscript{3H}]Adenine and [\textsuperscript{3H}]cAMP were purchased from American Radiolabeled Chemicals (St. Louis, MO).
nine-labeled P2Y14-HEK293 cells were incubated with 200 μM IBMX alone (open bars) or IBMX with 30 μM forskolin and the indicated concentrations of UDP-Glc (filled bars). The data shown are presented as mean ± S.E. and are representative of results from three independent experiments.

Results

UDP Sugars Promote Inhibition of Forskolin-Stimulated cAMP Formation in Cells Stably Expressing the Human P2Y14-R. To examine potential regulation of adenylyl cyclase activity downstream of the human P2Y14-R, we stably expressed this receptor in HEK293 cells. UDP-Glc promoted concentration-dependent inhibition of forskolin-stimulated accumulation of cAMP in P2Y14-R-expressing HEK293 cells. An EC50 value of 82 ± 11 nM (n = 3) was observed for UDP-Glc, and the maximal inhibition of forskolin-stimulated cAMP accumulation ranged from 50 to 60% (Fig. 1). In contrast, no effect of UDP-Glc was observed on basal or forskolin-stimulated cAMP accumulation in HEK293 cells infected with vector alone. Preincubation of P2Y14-HEK293 cells with pertussis toxin resulted in complete loss of UDP-Glc-dependent inhibition of cAMP accumulation (Fig. 1), indicating that the P2Y14-R signals through Gα-subunits of the G family.

Although UDP-Glc is reported to be a full agonist at the P2Y14-R, several studies of the P2Y14-R have reported variable effects of other nucleotide sugars (Chambers et al., 2000; Scrivens and Dickenson, 2006). Therefore, we tested the capacity of UDP sugars to activate the P2Y14-R in P2Y14-HEK293 cells. UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine all promoted inhibition of cAMP accumulation, and the maximal inhibition observed was similar to that of UDP-Glc. The potencies of UDP-galactose and UDP-glucuronic acid were similar to that of UDP-Glc, whereas UDP-N-acetylglucosamine exhibited an approximately 10-fold higher EC50 value (Fig. 2; Table 1). Although marked P2Y14 receptor-dependent effects on cAMP accumulation were observed in P2Y14-HEK293 cells, incubation of these cells with UDP-Glc had no effect on inositol phosphate accumulation (data not shown).

We concluded that it was important to stably express the P2Y14-R in several cell backgrounds. Previous studies by our lab and other labs revealed robust inhibition of adenylyl cyclase in C6 rat glioma cells expressing several different G protein-coupled receptors (Schachter et al., 1997; Thomas et al., 2000; Castillo et al., 2007). Therefore, we also infected C6 cells with the recombinant pLXSN virus harboring the hP2Y14-R coding sequence. Again, although UDP-Glc had no effect on cAMP accumulation in C6 cells expressing empty vector alone, robust inhibitory effects were observed with UDP-Glc in P2Y14-R-expressing C6 cells, with 70 to 80% maximal inhibition of forskolin-stimulated accumulation of cAMP (Fig. 3). An EC50 value of 107 ± 68 nM was determined (Table 1). The action of UDP-Glc in P2Y14-C6 cells was completely blocked by pretreatment of the cells with pertussis toxin (Fig. 3). The concentration-dependent inhibition of cAMP formation with other UDP sugars also was established, and the EC50 values are reported in Table 1.

Effect of UDP-Glc on Adenylyl Cyclase Activity in Membranes from P2Y14-R-Expressing C6 Cells. Based on the robust P2Y14-R-dependent inhibition of cAMP accumulation observed in C6 glioma cells, we also isolated plasma membrane-enriched fractions to determine whether UDP-Glc and P2Y14-R-dependent inhibition of adenylyl cyclase activity could be observed in a cell-free system. Although no effect of UDP-Glc was observed in membranes from control cells, approximately 50% inhibition of forskolin-stimulated
TABLE 1

<table>
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<th>Agonist</th>
<th>EC50 ± S.E.</th>
<th>Maximal Inhibition</th>
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<th>EC50 ± S.E.</th>
<th>Maximal Inhibition</th>
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<tr>
<td>UDP-glucose</td>
<td>82 ± 11</td>
<td>51 ± 1</td>
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<td>92 ± 51</td>
<td>76 ± 0.5</td>
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<tr>
<td>UDP-galactose</td>
<td>96 ± 29</td>
<td>60 ± 2</td>
<td></td>
<td>240 ± 53</td>
<td>72 ± 8</td>
</tr>
<tr>
<td>UDP-glucuronic acid</td>
<td>60 ± 7</td>
<td>55 ± 0.3</td>
<td></td>
<td>108 ± 66</td>
<td>79 ± 1</td>
</tr>
<tr>
<td>UDP-N-acetylglucosamine</td>
<td>919 ± 205</td>
<td>58 ± 3</td>
<td></td>
<td>229 ± 68</td>
<td>74 ± 8</td>
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adenylyl cyclase activity was observed in the presence of 10 μM UDP-Glc in membranes isolated from P2Y14-C6 glioma cells (Fig. 4). This UDP-Glc-promoted response did not occur in membranes from P2Y14-C6 cells pretreated with pertussis toxin.

**MAP Kinase Activation by UDP-Glc in P2Y14-HEK293 Cells.** Many important biological processes are regulated by MAP kinase signaling pathways. To assess the capacity of the P2Y14-R to activate MAP kinase signaling cascades, P2Y14-HEK293 cells were treated with UDP-Glc, and cell lysates were analyzed for phosphorylated ERK1/2 by Western blotting. Although no effect was observed in mock-infected cells, UDP-Glc-dependent ERK1/2 phosphorylation occurred in P2Y14-HEK293 cells (Fig. 5A).

The time course for UDP-Glc activation of ERK1/2 was compared with that occurring as a consequence of activation of PAR2, which is natively expressed in HEK293 cells (Amadesi et al., 2004; Dai et al., 2007). Incubation of cells with the PAR2 agonist peptide SLIGKV (100 μM) resulted in marked phosphorylation of ERK1/2 within 5 min, but phosphorylation quickly diminished thereafter. In contrast, UDP-Glc-dependent activation of ERK1/2 was not maximal until at least 15 min of incubation and was retained for at least 30 min. The effect of UDP-Glc on ERK1/2 phosphorylation was concentration-dependent (Fig. 5C), and the EC50 (30 nM) observed was similar to that obtained in studies of inhibition of cAMP accumulation in these cells. As was observed in the studies of adenylyl cyclase activity, P2Y14-R-regulated ERK1/2 phosphorylation was not observed in cells preincubated with pertussis toxin (Fig. 5B).

The capacity of the P2Y14-R to activate p38 and JNK also was examined. Although p38 was phosphorylated with a peak response observed within 5 min after treatment of cells with sorbitol, no UDP-Glc-dependent activation of p38 was observed at any time point up to 60 min (Fig. 5D). In contrast to a robust activation observed in the presence of anisomycin, we also observed no effect of UDP-Glc on JNK phosphorylation (Fig. 5D).

**Functional P2Y14-R Are Endogenously Expressed in Differentiated HL-60 Cells.** With the goal of identifying a cell line that natively expresses the P2Y14-R, we tested by RT-PCR several candidate cell lines previously reported to express P2Y14-R mRNA and other cell lines derived from tissues reported to express this receptor (Chambers et al., 2000; Moore et al., 2003; Skelton et al., 2003). Although no P2Y14-R mRNA was detected in undifferentiated HL-60 cells, differentiation of these cells with 1.3% DMSO to a neutro-
phil-like cell resulted in a marked increase in expression of P2Y14-R mRNA (Fig. 6A).

We also investigated the cell signaling responses discussed above to determine whether functional P2Y14-R could be observed in differentiated HL-60 cells. In multiple experiments, 100 μM UDP-Glc exhibited no effect on cAMP accumulation promoted by forskolin, amthamine, or agonist for the Gs-coupled H2 histamine receptor, or forskolin + amthamine (data not shown). Although a formyl peptide receptor (FPR) is natively expressed by HL-60 cells (Boulay et al., 1990; Klinker et al., 1996), we also failed to observe effects of 1 μM fMLP on cAMP accumulation in these cells.

Potential activation of the MAP kinase signaling pathway also was studied in HL-60 cells. Although no effect of UDP-Glc was observed in wild-type cells, time-dependent activation of ERK1/2 by UDP-Glc was observed after differentiation of the cells. This effect was apparent within 5 min and peaked within 30 min (Fig. 6B). In contrast to the time course of the phosphorylation response to UDP-Glc, cells treated with 1 μM fMLP exhibited a robust ERK1/2 activation at 5 min that quickly diminished thereafter (Fig. 6C). Neither

**Fig. 5.** UDP-Glc-promoted activation of MAP kinase signaling in P2Y14-HEK293 cells. A, empty vector or P2Y14-R-expressing HEK293 cells were serum-starved for 18 h and then incubated with 100 μM UDP-Glc or 100 μM SLIGKV for the times indicated. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, the samples were transferred to nitrocellulose membranes, and the samples were probed with antibodies for phospho-ERK1/2 and total ERK1/2 as described under Materials and Methods. Results shown are representative of three individual experiments. B, cells were pretreated in the absence (−PTX) or presence (+PTX) of 100 ng/ml pertussis toxin overnight, then treated with UDP-Glc for 15 min. Results shown are representative of those obtained from three independent experiments. C, cells were incubated with varying concentrations of UDP-Glc for 15 min. Phospho-ERK1/2 and ERK1/2 were quantified using Scion Image software (Scion Corporation, Frederick, MD) and graphed using GraphPad Prism software. Each phospho-ERK1/2 lane was normalized to the corresponding ERK1/2 control and plotted as fold stimulation over control. Data shown are the average ± S.E. of four independent experiments. D, P2Y14-HEK293 cells were treated with 0.2 M sorbitol, 0.01 mg/ml anisomycin, or 100 μM UDP-Glc for the indicated times. Phospho-p38, phospho-JNK, total p38, and total JNK were determined as described under Materials and Methods.

**Fig. 6.** UDP-Glc-promoted activation of MAP kinase activation in differentiated HL-60 cells. A, RT-PCR of undifferentiated (−DMSO) or differentiated (+DMSO) HL-60 cells in the absence (−) or presence (+) of RT. PCR with hP2Y14-R-specific primers was performed as described under Materials and Methods. B, wild-type or differentiated (see Materials and Methods) HL-60 cells were serum-starved for 18 h before addition of 100 μM UDP-Glc, and incubation was continued for the indicated times. Western blots for phospho-ERK1/2 and ERK1/2 were generated as described under Materials and Methods. Phospho-ERK1/2 was quantified using Scion Image software and graphed using GraphPad Prism software. Each phospho-ERK1/2 lane was normalized to the corresponding ERK1/2 control and plotted as fold stimulation over control. Data shown are the mean ± S.E. of six independent experiments (, p < 0.02). C, serum-starved, differentiated HL-60 cells were incubated with 1 μM formyl-Met-Leu-Phe for the indicated times, and lysates were analyzed as described under Materials and Methods. D, differentiated HL-60 cells were treated in the absence (−PTX) or presence (+PTX) of 200 ng/ml pertussis toxin for 4 h before assay. Cells were incubated for the indicated times with 100 μM UDP-Glc and phospho-ERK1/2, and total ERK1/2 was quantified as described under Materials and Methods.
UDP-Glc- nor fMLP-dependent ERK1/2 activation was observed in cells preincubated with pertussis toxin (Fig. 6D), consistent with the notion that the UDP-Glc-promoted ERK1/2 activation in differentiated HL-60 cells occurs through a mechanism involving Gi in differentiated HL-60 cells.

Discussion
In this report, we demonstrate that the human P2Y_{14}-R couples to inhibition of adenylyl cyclase in a pertussis toxin-sensitive manner in HEK293 and C6 cells stably expressing this receptor. This work provides the first unequivocal demonstration of P2Y_{14}-R-dependent inhibition of adenylyl cyclase in a membrane preparation. Robust stimulation of MAP kinase signaling also occurs with activation of the P2Y_{14}-R. This was the predominant activity observed with native P2Y_{14}-R in differentiated HL-60 human myeloid leukemia cells.

UDP-Glc is released from many cell types. This phenomenon was initially demonstrated by Lazarowski et al. (2003), who illustrated both basal and mechanically induced release of UDP-Glc from multiple mammalian cell types. Constitutive release of UDP-Glc also occurs in yeast (Esther et al., 2008). Although the mechanism(s) underlying UDP-Glc release is not well established, calcium-dependent release of UDP-Glc was demonstrated in Calu-3 cells (Kreda et al., 2007), and UDP-Glc release occurred downstream of thrombin receptor-promoted signaling pathways in human astrocytoma 1321N1 cells (Kreda et al., 2008).

UDP sugars in addition to UDP-Glc are predictably present in the extracellular space. UDP-Glc, UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine all were reported previously as potent agonists of the P2Y_{14}-R in test systems that involved coexpression of the receptor with a phospholipase C-activating G protein (Chambers et al., 2005). The fMLP receptor, FPR, has been reported to couple to Gi, but the mechanism linking FPR to ERK1/2 activation is not clear (Selvatici et al., 2006; Huet et al., 2007). Therefore, it is possible that the signaling pathway(s) responsible for P2Y_{14}-R-promoted MAP kinase signaling differs from that engaged by PAR2 or FPR. We speculate that pertussis toxin-sensitive Grα-subunits are necessary but not sufficient, and additional pathways or regulatory mechanisms are involved in the MAP kinase response downstream of one or more of these receptors. Our studies do not rule out the possibility that the P2Y_{14}-R desensitizes at a slower rate than PAR2 in HEK293 cells or FPR in HL-60 cells. Additional studies will be necessary to address questions about the mechanism of P2Y_{14}-R-dependent activation of MAP kinase, but its pertussis toxin sensitivity clearly indicates that Gi is an important signaling component in the pathway.

In summary, this work unequivocally demonstrates that the human P2Y_{14}-R promotes inhibition of adenylyl cyclase and activation of MAP kinase signaling pathways. Our work also provides several model cell lines for study of P2Y_{14}-R signaling and its potential role in neutrophil biology.

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