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Quantification of G_i-Mediated Inhibition of Adenylyl Cyclase Activity Reveals That UDP Is a Potent Agonist of the Human $P2Y_{14}$ Receptor

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ABSTRACT

The $P2Y_{14}$ receptor was initially identified as a G protein-coupled receptor activated by UDP-glucose and other nucleotide sugars. We have developed several cell lines that stably express the human $P2Y_{14}$ receptor, allowing facile examination of its coupling to native G_i family G proteins and their associated downstream signaling pathways (*J Pharmacol Exp Ther* **330:** 162–168, 2009). In the current study, we examined $P2Y_{14}$ receptor-dependent inhibition of cyclic AMP accumulation in human embryonic kidney (HEK) 293, C6 glioma, and Chinese hamster ovary (CHO) cells stably expressing this receptor. Not only was the human $P2Y_{14}$ receptor activated by UDP-glucose, but it also was activated by UDP. The apparent efficacies of UDP and UDP-glucose were similar, and the EC_{50} values (74, 33, and 29 nM) for UDP-dependent activation of the $P2Y_{14}$ receptor in HEK293, CHO, and C6 glioma cells, respectively, were similar to the EC_{50} values (323, 132, and 72 nM) observed for UDP-glucose. UDP and UDP-glucose also stimulated extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in $P2Y_{14}$ receptor-expressing HEK293 cells but not in wild-type HEK293 cells. A series of analogs of UDP were potent $P2Y_{14}$ receptor agonists, but the naturally occurring nucleoside diphosphates, CDP, GDP, and ADP exhibited agonist potencies over 100-fold less than that observed with UDP. Two UDP analogs were identified that selectively activate the $P2Y_{14}$ receptor over the UDP-activated $P2Y_6$ receptor, and these molecules stimulated phosphorylation of ERK1/2 in differentiated human HL-60 promyeloleukemia cells, which natively express the $P2Y_{14}$ receptor but had no effect in wild-type HL-60 cells, which do not express the receptor. We conclude that UDP is an important cognate agonist of the human $P2Y_{14}$ receptor.

The metabotropic P2Y receptors include a subgroup of five receptors, the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors, that primarily signal through G_q-activated signaling pathways and a subgroup of three receptors, the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors, that primarily signal by activating heterotrimeric G proteins of the G_i family (Abbracchio et al., 2006; Burnstock, 2007). The human $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ receptors are activated by adenine nucleotides. The human $P2Y_4$ and $P2Y_6$ receptors are activated by uridine nucleotides, and the $P2Y_2$ receptor is activated by both ATP and UTP.

The $P2Y_{14}$-R exhibits the most unique agonist selectivity of the P2Y receptors extant; it was initially identified as an orphan G protein-coupled receptor that is activated by nucle-

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ABBREVIATIONS: $P2Y_{14}$-R, $P2Y_{14}$ receptor; $P2Y_6$-R, $P2Y_6$ receptor; $hP2Y_{14}$-R, human $P2Y_{14}$ receptor; $P2Y_{14}$-HEK293 cells, human embryonic kidney 293 cells stably expressing the human $P2Y_{14}$ receptor; $P2Y_{14}$-C6 cells, C6 rat glioma cells stably expressing the human $P2Y_{14}$ receptor; $P2Y_{14}$-CHO cells, Chinese hamster ovary cells stably expressing the human $P2Y_{14}$ receptor; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; UDPβS, uridine 5′-*O*-thiodiphosphate; MAP, mitogen-activated protein; MRS2802, α,β-difluoromethylene-UDP; MRS2907, 2-thio-UDP-β-propyl ester; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; GPCR, G protein-coupled receptor.

otide sugars, such as UDP-glucose, UDP-galactose, UDP-*N*-acetylglucosamine, and UDP-*N*-glucuronic acid, rather than by adenine and/or uridine nucleotides (Chambers et al., 2000; Freeman et al., 2001). Although the $P2Y_{14}$-R has been considered a nucleotide sugar-regulated receptor, we recently reported that UDP is a competitive antagonist/partial agonist at the human ortholog (Fricks et al., 2008). Moreover, our experiments with the rat $P2Y_{14}$-R suggested that UDP is a potent full agonist at this receptor. These studies revealing activities of UDP were carried out in COS-7 cells transiently coexpressing recombinant $P2Y_{14}$-R with a chimeric G protein, $G\alpha_{q/i}$ (Coward et al., 1999) that couples G_i-activating receptors to activation of phospholipase C-β isozymes. Thus, we questioned whether the action of UDP previously observed by measuring inositol lipid hydrolysis was a vagary of the test system used. To address this issue, agonist activities were quantified in three different stable cell lines we developed that allow study of $P2Y_{14}$-R-dependent inhibition of adenylyl cyclase activity. These experiments measuring a natural cell signaling response of the $P2Y_{14}$-R reveal that not only is UDP an agonist of the human receptor, but its potency and apparent efficacy are similar to that of UDP-glucose and other nucleotide sugars. Thus, the pervasive view that the $P2Y_{14}$-R is selectively activated by UDP-glucose and other nucleotide sugars should be reassessed with the idea that extracellular UDP is also an important cognate agonist of this receptor.

Materials and Methods

Cell Lines Stably Expressing $P2Y_{14}$-R. Human embryonic kidney 293 cells stably expressing the $hP2Y_{14}$-R ($P2Y_{14}$-HEK293 cells) and C6 rat glioma cells stably expressing the $hP2Y_{14}$-R ($P2Y_{14}$-C6 cells) were generated as described previously (Fricks et al., 2009). Chinese hamster ovary cells stably expressing the $hP2Y_{14}$-R ($P2Y_{14}$-CHO cells) were produced using similar methods. In brief, $hP2Y_{14}$-R cDNA was ligated into the retroviral expression vector pLXSN as described previously (Wolff et al., 2005), and recombinant retrovirus for $hP2Y_{14}$-R expression was produced in PA317 cells. CHO cells were incubated with $hP2Y_{14}$-R virus and 0.4 mg/ml G418 for 2 weeks to produce $hP2Y_{14}$-R-expressing cells.

Cell Culture. HEK293 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% G418 (Geneticin) at 37°C in a 5% CO_2 environment. C6 rat glioma cells were cultured in DMEM supplemented with 5% fetal bovine serum and 1% G418 in a 5% CO_2 environment. CHO cells were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum and 1% G418 at 37°C in a 5% CO_2 environment. 1321N1 Human astrocytoma cells were grown in DMEM supplemented with 5% fetal bovine serum in a 5% CO_2 environment. HL-60 promyeloleukemia cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Differentiation of HL-60 cells was achieved by inclusion of 1.3% DMSO in the culture medium for 5 days.

Cyclic AMP Accumulation. Cells were grown in 24-well plates and incubated with 1 μCi of [^{3}H]adenine/well in serum-free DMEM for 2 h before assay. Assays were initiated by the addition of HEPES-buffered, serum-free DMEM containing 200 μM 3-isobutyl-1-methylxanthine and agonists, and incubation continued for 10 min at 37°C. Incubations were terminated by aspiration of medium and addition of 500 μl of ice-cold 5% trichloroacetic acid. [^{3}H]cAMP was isolated by sequential Dowex and alumina chromatography and quantified by liquid scintillation counting as described previously (Harden et al., 1982).

Inostiol Phosphate Accumulation. $P2Y_6$-R-dependent activation of phospholipase C was measured by quantifying [^{3}H]inositol phosphate accumulation in 1321N1 human astrocytoma cells as described previously (Besada et al., 2006).

MAP Kinase Activation Assays. Phosphorylation of ERK1/2 was quantified as we have described in detail (Fricks et al., 2009). In brief, wild-type HEK293 or $P2Y_{14}$-HEK293 cells were serum-starved for 18 h before addition of either UDP or UDP-glucose for 15 min at 37°C. Undifferentiated or differentiated HL-60 cells were serum-starved for 18 h before addition of agonist for 30 min at 37°C. Cell extracts were subjected to SDS-polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, which was sequentially incubated with antibody for phospho-ERK1/2, with horseradish peroxidase-conjugated goat anti-mouse antibody, and with chemiluminescent substrate (Pierce West Pico system; Thermo Fisher Scientific, Waltham, MA) and then was exposed to film. Membranes were stripped and reprobed with a primary antibody against total ERK1/2 to verify equal loading of lanes.

Data Analysis. EC_{50} values were determined using Prism software (GraphPad, San Diego, CA) and are presented as mean ± S.E. All experiments were repeated at least three times.

Materials. 3-Isobutyl-1-methylxanthine and forskolin were purchased from SigmaAldrich (St. Louis, MO). UDP-glucose, UDP, CDP, GDP, and ADP were all from FLUKA and were purchased from SigmaAldrich. High-performance liquid chromatography analysis of UDP-glucose and all of the nucleotides revealed >95% purity. Pertussis toxin was purchased from List Biologicals (Campbell, CA). [^{3}H]Adenine was purchased from American Radiolabeled Chemicals (St. Louis, MO). G418, serum, and all cell culture medium were from Invitrogen (Carlsbad, CA). Antibodies for phosphoERK1/2 and ERK1/2 were purchased from Cell Signaling Technologies (Danvers, MA).

Results

Agonist-promoted activation of G_i and consequential inhibition of adenylyl cyclase is thought to be a primary signaling response of the $P2Y_{14}$-R and other members of the $P2Y_{12}$-R subfamily of P2Y receptors. However, most studies of $P2Y_{14}$-R-promoted signaling have relied on overexpression of either $G\alpha_{16}$ or a chimera of $G\alpha_q$ ($G\alpha_{q/i}$) that engineers coupling of this G_i-coupled receptor to activation of phospholipase C (Chambers et al., 2000; Freeman et al., 2001; Fricks et al., 2008). Using C6 rat glioma cells stably expressing the human $P2Y_{14}$-R, we recently illustrated that adenylyl cyclase activity in both intact cells and isolated membranes was inhibited by UDP-glucose in a concentration- and pertussis toxin-sensitive manner (Fricks et al., 2009). Based on these and similar results obtained with $hP2Y_{14}$-HEK293 cells, we reasoned that cells displaying $P2Y_{14}$-R-promoted inhibition of adenylyl cyclase provide a more physiologically relevant system for quantification of $P2Y_{14}$-R-dependent signaling over an engineered signaling system that depends on overexpression of a G protein that activates phospholipase C. Given our recent observation of activities of UDP at the $P2Y_{14}$-R transiently expressed with an unnatural chimeric G protein, $G\alpha_{q/i}$, in COS-7 cells, we believed it important to quantify the relative actions of UDP and UDP-glucose for promotion of a native cell signaling response in cell lines stably expressing the human $P2Y_{14}$-R.

Whereas UDP-glucose had no effect on cyclic AMP accumulation in wild-type HEK293 cells (Fig. 1A), incubation of $P2Y_{14}$-HEK293 cells with this nucleotide sugar resulted in a concentration-dependent inhibition of forskolin-stimulated cyclic AMP accumulation (Fig. 1B). Likewise, UDP had no effect in wild-type HEK293 cells (Fig. 1A) but promoted a

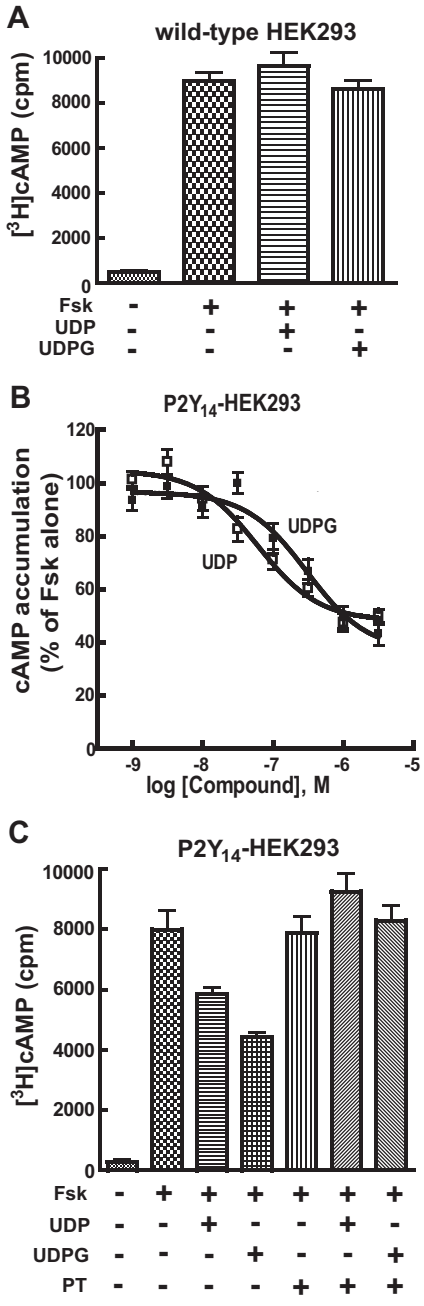

Fig. 1. UDP-glucose and UDP promote pertussis toxin-sensitive inhibition of cyclic AMP accumulation in P2Y$_{14}$-HEK293 cells. A, the capacity of 100 μM UDP or 100 μM UDP-glucose (UDPG) to inhibit forskolin (Fsk; 30 μM)-stimulated cyclic AMP accumulation was quantified in wild-type HEK293 cells as described under *Materials and Methods*. The data are mean ± S.E.M. from triplicate determinations and are representative of three separate experiments. B, concentration-dependent inhibition of forskolin-stimulated cyclic AMP accumulation by UDP-glucose and UDP. P2Y$_{14}$-HEK293 cells were incubated with 30 μM forskolin alone or 30 μM forskolin plus the indicated concentrations of UDP-glucose (■) or UDP (□). The data are presented as mean ± S.E.M. of triplicate determinations and are representative of data from six experiments. C, blockade of the P2Y$_{14}$-R-dependent effects of UDP-glucose and UDP by pertussis toxin (PT). P2Y$_{14}$-HEK293 cells were incubated overnight with vehicle or 100 ng/ml pertussis toxin, and cyclic AMP accumulation was subsequently measured in the presence of 30 μM forskolin alone or with 30 μM forskolin plus 10 μM UDP-glucose or 10 μM UDP. The data are presented as mean ± S.E.M. of results from three separate experiments.

concentration-dependent decrease in cyclic AMP accumulation in P2Y$_{14}$-HEK293 cells. The potency (Table 1) and maximal effect (Table 2) of UDP for inhibition of forskolin-stimulated cyclic AMP accumulation were very similar to the potency and maximal effect of UDP-glucose. As was previously observed with UDP-glucose (Fricks et al., 2009), UDP had no effect on cyclic AMP accumulation in P2Y$_{14}$-HEK293 cells in which GPCR-promoted activation of G_i was inhibited by preincubation of cells with pertussis toxin (Fig. 1C).

The effect of UDP on cyclic AMP accumulation was observed in several different cell lines cloned from hP2Y$_{14}$-R virus-infected HEK293 cells (data not shown). Although these results strongly suggest that UDP is a robust natural agonist of this receptor, we concluded it prudent to test possible agonist action of UDP at P2Y$_{14}$-R stably expressed in other cell backgrounds. Therefore, activity of UDP was assessed in a stable cell line (P2Y$_{14}$-C6 cells) that we recently developed (Fricks et al., 2009), as well as in a third stable cell line (P2Y$_{14}$-CHO cells) that was generated as described under *Materials and Methods*. Again, neither UDP-glucose nor UDP affected basal or forskolin-stimulated cyclic AMP accumulation in wild-type C6 (Fig. 2A) or CHO (data not shown) cells. In contrast, concentration-dependent effects of both UDP-glucose and UDP were observed in P2Y$_{14}$-C6 (Fig. 2B) and P2Y$_{14}$-CHO (Fig. 2C) cells. The maximal inhibition of forskolin-stimulated cyclic AMP accumulation observed with UDP was similar to that observed with UDP-glucose in these two cell lines (Table 2), and the EC_{50} values of UDP and UDP-glucose also were similar (Table 1). The effect of both UDP-glucose and UDP on cyclic AMP accumulation was blocked by preincubation of P2Y$_{14}$-C6 cells (Fig. 3A) or P2Y$_{14}$-CHO cells (Fig. 3B) with pertussis toxin.

TABLE 1

Comparative potencies of UDP-glucose and UDP in cell lines stably expressing the human P2Y$_{14}$-R. Concentration effect curves for UDP-glucose- and UDP-promoted inhibition of forskolin-stimulated cyclic AMP accumulation were generated in P2Y$_{14}$-HEK293, P2Y$_{14}$-C6, and P2Y$_{14}$-CHO cells as described under *Materials and Methods*. Assays were carried out in triplicate, and EC_{50} values were determined from individual experiments by applying the Prism software package. The EC_{50} values are presented as mean ± S.E.M. averaged from 6 experiments each with C6 and CHO cells and from 21 experiments with HEK293 cells.

	EC_{50}	
Cell Line	UDP-Glucose	UDP
	nM	
P2Y$_{14}$-HEK293 cells	323 ± 84	74 ± 18
P2Y$_{14}$-CHO cells	132 ± 76	33 ± 20
P2Y$_{14}$-C6 cells	72 ± 17	29 ± 9

TABLE 2

The maximal percentage inhibition of forskolin-stimulated cAMP accumulation was calculated from the curves generated as described in Table 1.

	Maximal Inhibition of Forskolin-Stimulated cAMP Accumulation	
Cell Line	UDP-Glucose	UDP
	%	
P2Y$_{14}$-HEK293 cells	60 ± 6	58 ± 2
P2Y$_{14}$-CHO cells	70 ± 10	68 ± 9
P2Y$_{14}$-C6 cells	86 ± 3	91 ± 1

The agonist activity of other naturally occurring nucleoside diphosphates was tested in $P2Y_{14}$-HEK293 cells. Both CDP and GDP exhibited agonist activities, although the potencies observed were more than 100-fold less than that observed with UDP (Fig. 4). The effect of ADP, which is the cognate agonist of the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors, was also examined. Incubation of $P2Y_{14}$-HEK293 cells with low micromolar concentrations of ADP resulted in a reproducible increase in cyclic AMP accumulation, and high micromolar concentrations of ADP reduced cyclic AMP accumulation to levels similar to that observed with forskolin alone. The stimulatory effect of ADP also was observed in wild-type HEK293 cells, but the small inhibitory effect observed at high concentrations of ADP was not (data not shown). Thus, ADP exhibited only $P2Y_{14}$-R-dependent activity at very high concentrations relative to those necessary for agonist effects of UDP. A potential explanation of the stimulatory effect of ADP in both wild-type and $P2Y_{14}$-HEK293 cells is that ec-

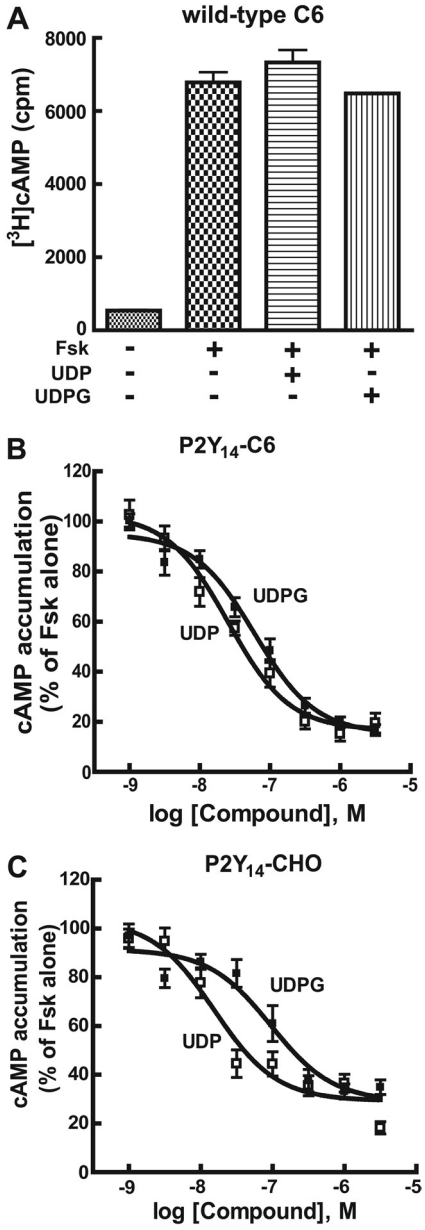

Fig. 2. $P2Y_{14}$-*R*-dependent activities of UDP-glucose and UDP in $P2Y_{14}$-C6 and $P2Y_{14}$-CHO cells. A, the capacity of 100 μM UDP or 100 μM UDP-glucose (UDPG) to inhibit forskolin (30 μM)-stimulated cyclic AMP accumulation was quantified in wild-type C6 rat glioma cells as described under *Materials and Methods*. The data are mean ± S.E.M. from triplicate determinations and are representative of results from three separate experiments. B, concentration-dependent inhibition of forskolin-stimulated cyclic AMP accumulation by UDP-glucose and UDP in $P2Y_{14}$-C6 cells. $P2Y_{14}$-C6 cells were incubated in the presence of 30 μM forskolin alone or with 30 μM forskolin plus the indicated concentrations of UDP-glucose (■) or UDP (□). The data are presented as mean ± S.E.M. of triplicate determinations and are representative of data from six separate experiments. C, concentration-dependent inhibition of forskolin-stimulated cyclic AMP accumulation by UDP-glucose and UDP in $P2Y_{14}$-CHO cells. $P2Y_{14}$-CHO cells were incubated in the presence of 30 μM forskolin alone or with 30 μM forskolin plus the indicated concentrations of UDP-glucose (■) or UDP (□). The data are presented as mean ± S.E.M. of triplicate determinations and are representative of data from six separate experiments.

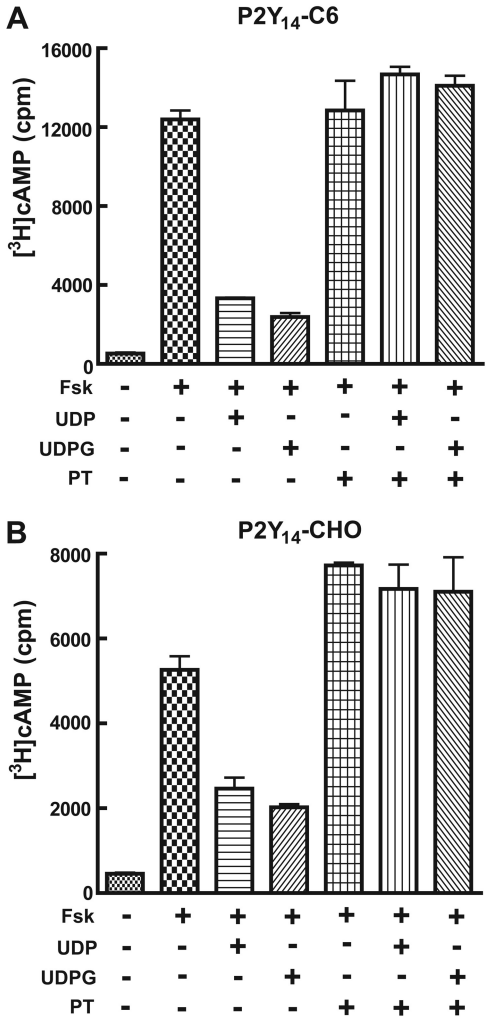

Fig. 3. Pertussis toxin sensitivity of the effects of UDP-glucose and UDP in $P2Y_{14}$-C6 cells and $P2Y_{14}$-CHO cells. A, blockade of the $P2Y_{14}$-R-dependent effects of UDP-glucose and UDP by pertussis toxin in $P2Y_{14}$-C6 cells. $P2Y_{14}$-C6 cells were incubated overnight with vehicle or 100 ng/ml pertussis toxin, and cyclic AMP accumulation was subsequently measured in the presence of 30 μM forskolin alone or with 30 μM forskolin plus 10 μM UDP-glucose or 10 μM UDP. The data are presented as mean ± S.E.M. of results from three separate experiments. B, blockade of the $P2Y_{14}$-R-dependent effects of UDP-glucose and UDP by pertussis toxin in $P2Y_{14}$-CHO cells. $P2Y_{14}$-CHO cells were incubated overnight with vehicle or 100 ng/ml pertussis toxin, and cyclic AMP accumulation was subsequently measured in the presence of 30 μM forskolin alone or with 30 μM forskolin plus 10 μM UDP-glucose or 10 μM UDP. The data are presented as mean ± S.E.M. of results from three separate experiments.

toenzymes convert ADP to adenosine, which in turn activates an endogenous A_{2B} adenosine receptor known to be present on HEK293 cells (Cooper et al., 1997; Mundell et al., 1999).

Potential agonist activities of the UDP analogs, UDPβS (Malmsjö et al., 2000) and 2-thio-UDP (Besada et al., 2006) also were examined in P2Y$_{14}$-HEK293 cells. As is illustrated in Fig. 5, both UDPβS (EC_{50} = 26 ± 11 nM, n = 3) and 2-thio-UDP (EC_{50} = 2 ± 1 nM, n = 3) were potent agonists at the hP2Y$_{14}$-R.

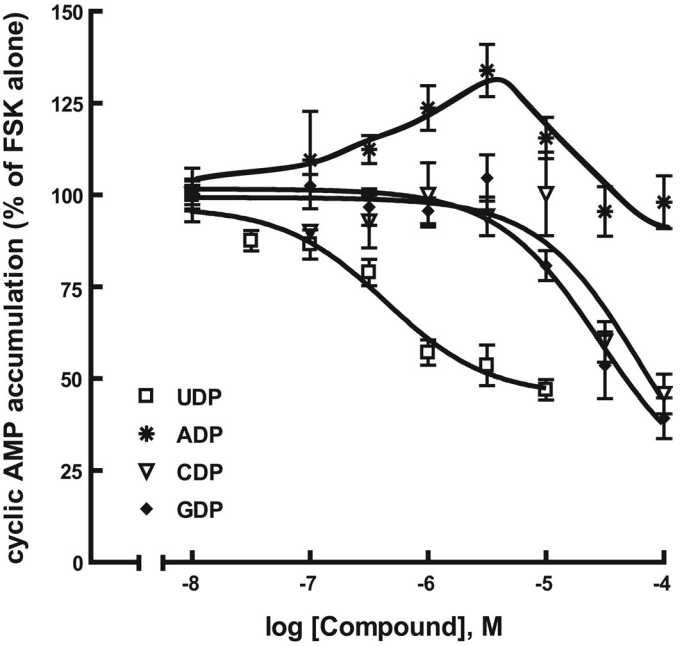

Fig. 4. Agonist activities of nucleoside diphosphate molecules in P2Y$_{14}$-HEK293 cells. P2Y$_{14}$-HEK293 cells were incubated in the presence of 30 μM forskolin alone or with 30 μM forskolin plus the indicated concentrations of UDP (□), CDP (▽), GDP (◆), or ADP (*). The data are presented as mean ± S.E.M. of results pooled from three separate experiments.

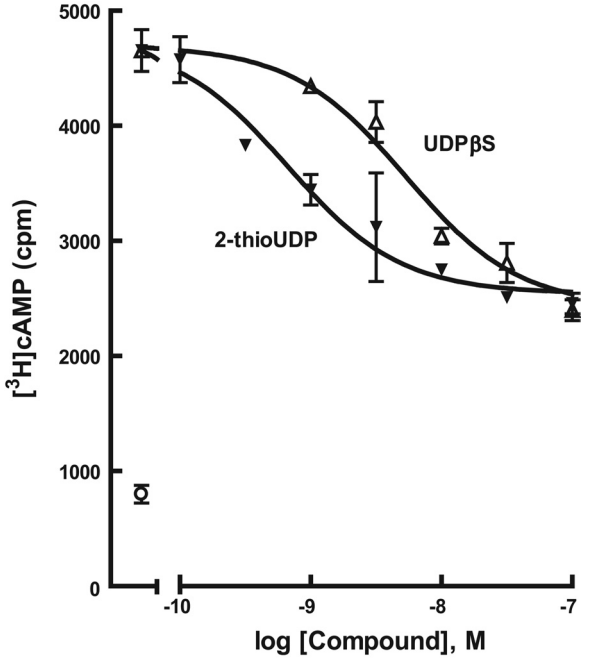

Fig. 5. Agonist activities of 2-thio-UDP and UDPβS in P2Y$_{14}$-HEK293 cells. P2Y$_{14}$-HEK293 cells were incubated in the absence (○) or presence of 30 μM forskolin alone or with 30 μM forskolin plus the indicated concentrations of 2-thio-UDP (▼) or UDPβS (△). The data are presented as mean ± S.E.M. of triplicate determinations and are representative of data from three separate experiments.

We recently reported that stable expression of the human P2Y$_{14}$ receptor in HEK293 cells confers a robust MAP kinase signaling response to UDP-glucose (Fricks et al., 2009). Therefore, the capacity of UDP to promote P2Y$_{14}$-R-dependent phosphorylation of ERK1/2 also was examined. As illustrated in Fig. 6, 10 μM UDP had no effect on the MAP kinase response in wild-type HEK293 cells but promoted marked ERK1/2 phosphorylation in P2Y$_{14}$-HEK293 cells. Thus, as was observed in measurements of cyclic AMP accumulation, quantification of MAP kinase signaling also reveals that UDP and UDP-glucose exhibit similar agonist activities at the P2Y$_{14}$ receptor.

The results presented thus far with the human P2Y$_{14}$-R stably expressed in three different cell types strongly suggest that UDP is a potent agonist at this receptor. We also recently discovered that the P2Y$_{14}$-R is natively expressed in HL-60 promyeloleukemia cells (Fricks et al., 2009). Whereas neither P2Y$_{14}$-R mRNA nor a MAP kinase signaling response to UDP-glucose was detectable in wild-type HL-60 cells, differentiation of these cells by addition of DMSO to the growth medium resulted in expression of P2Y$_{14}$-R mRNA as well as phosphorylation of ERK1/2 in response to UDP-glucose. Thus, HL-60 cells also were studied to examine whether UDP activates signaling responses downstream of a natively expressed P2Y$_{14}$-R. ERK1/2 phosphorylation was observed in response to UDP in differentiated HL60 cells (data not shown), but further experiments revealed that a robust response to UDP also was seen in wild-type HL60 cells in the absence of P2Y$_{14}$-R expression (Fig. 7A). Reverse transcription-polymerase chain reaction analyses revealed that the response observed to UDP in wild-type HL60 cells is probably due to the presence of a UDP-activated P2Y$_6$-R, because mRNA for this receptor is prominently expressed in both wild-type and differentiated HL60 cells (data not shown). Although quantification of inhibition of cyclic AMP accumulation potentially might allow resolution of the effects of UDP on the G_i-coupled P2Y$_{14}$-R versus G_q-coupled P2Y$_6$-R in these cells, we have been unable to observe inhibition of adenylyl cyclase in response to UDP-glucose or formyl-Met-Leu-Phe, which activates a G_i-linked receptor for chemotactic peptides in HL-60 cells (Fricks et al., 2009).

In the absence of availability of another cell line that

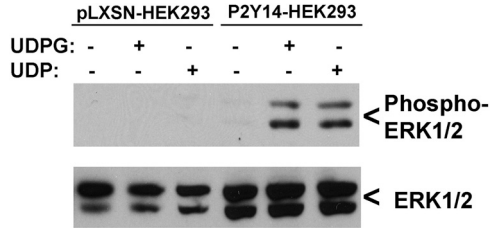

Fig. 6. P2Y$_{14}$-*R*-dependent activation of MAP kinase signaling by UDP. Empty vector or P2Y$_{14}$-HEK293 cells were serum-starved for 18 h before incubation with vehicle, 10 μM UDP, or 10 μM UDP-glucose for 15 min. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, the samples transferred to nitrocellulose membranes, and the membranes probed with antibodies for phospho-ERK1/2 and total ERK1/2 as described under *Materials and Methods*. The results shown are representative of data from three individual experiments.

natively expresses the $P2Y_{14}$-R, we considered a pharmacological approach to show that the $P2Y_{14}$-R natively expressed on differentiated HL-60 cells responds to agonists in addition to nucleotide sugars. As illustrated above (Fig. 5), two UDP analogs activated the $P2Y_{14}$-R stably expressed in HEK293 cells. Because these two molecules also are potent activators of the $P2Y_6$-R (data not shown), they are not useful for selectively activating the $P2Y_{14}$-R of HL-60 cells. However, our recent chemical syntheses (A. Das, H. Ko, M. O. Barrett, L. B. Burianek, T. K. Harden, and K. A. Jacobson, submitted) have identified analogs of UDP that activate the $P2Y_{14}$-R without activating the $P2Y_6$-R, and the relative activities of two of these, MRS2802 and MRS2907, at the $P2Y_{14}$-R versus $P2Y_6$-R are illustrated in Fig. 7. MRS2802 inhibited forskolin-stimulated cyclic AMP accumulation in $P2Y_{14}$-HEK293 cells with an EC_{50} of approximately 50 nM (Fig. 7B, left), whereas little inositol phosphate response to this molecule was observed at concentrations up to 10 μM in 1321N1 human astrocytoma cells stably expressing the human $P2Y_6$-R (Fig. 7B, right). MRS2907 also selectively activated the $P2Y_{14}$-R over the $P2Y_6$-R (Fig. 7C). Given the $P2Y_{14}$-R selectivity of these two UDP analogs, we examined their capacity to stimulate ERK1/2 phosphorylation in wild-type and differentiated HL-60 cells. Neither MRS2802 nor MRS2907 promoted ERK1/2 phosphorylation in wild-type HL-60 cells (Fig. 7D), but as was the case with UDP-glucose, a MAP kinase signaling response to both analogs was observed after induction of expression of the $P2Y_{14}$-R by differentiation of these cells by treatment with DMSO. These results are consistent with the idea that UDP analogs, and by deduction UDP itself, are potent agonists of the $P2Y_{14}$-R in a native environment.

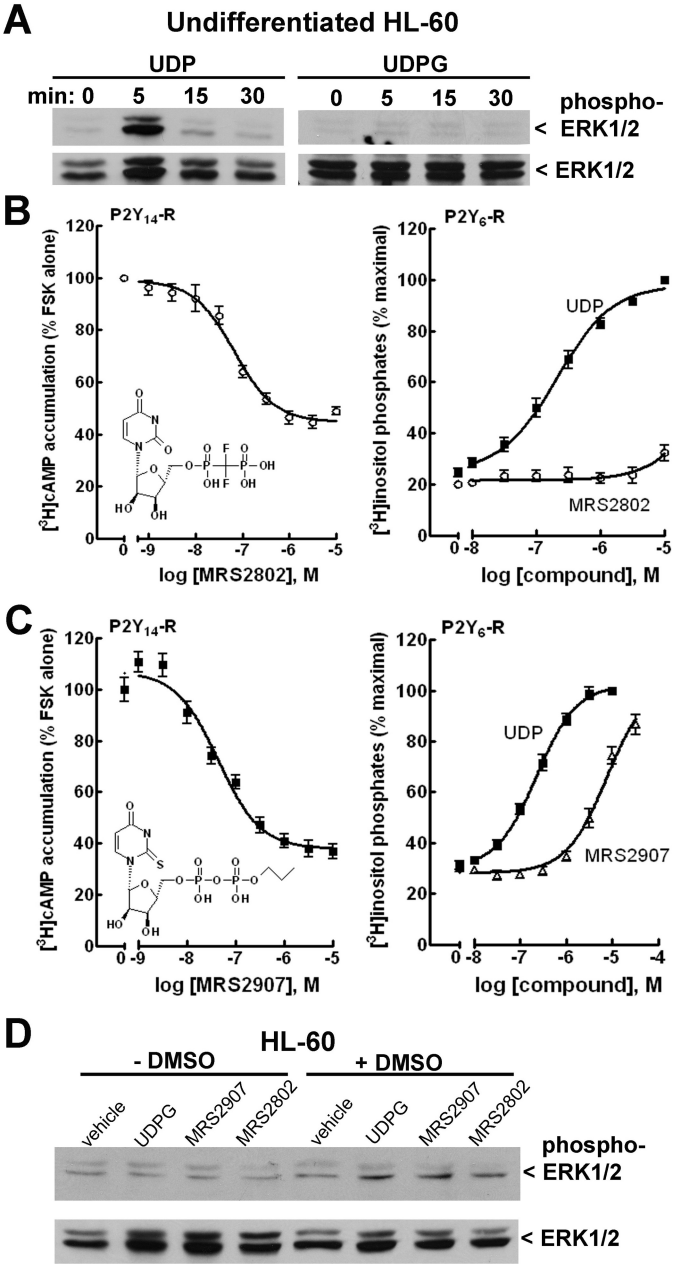

Fig. 7. Activation of a $P2Y_{14}$-R-dependent MAP kinase signaling response in differentiated human HL-60 promyeloleukemia cells. A, phosphorylation of ERK1/2 (phospho-ERK1/2) was analyzed in wild-type HL-60 cells after incubation for the indicated times with 10 μM UDP or 10 μM UDP-glucose (UDPG) as described under *Materials and Methods*. Levels of total ERK1/2 also are presented. B, concentration effect curves for MRS2802 for activation of $P2Y_{14}$-R in $P2Y_{14}$-HEK293 cells and activation of $P2Y_6$-R in 1321N1 human astrocytoma cells stably expressing the human $P2Y_6$-R were generated as described under *Materials and Methods*. C, concentration effect curves for MRS2907 for activation of $P2Y_{14}$-R in $P2Y_{14}$-HEK293 cells and activation of $P2Y_6$-R in 1321N1 human astrocytoma cells stably expressing the human $P2Y_6$-R were generated as described under *Materials and Methods*. D, phosphorylation of ERK1/2 (phospho-ERK1/2) was quantified in undifferentiated (−DMSO) or differentiated (+DMSO) HL-60 cells after incubation for 30 min with 10 μM UDP-glucose (UDPG), 1 μM MRS2907, or 10 μM MRS2802 as described under *Materials and Methods*. Levels of total ERK1/2 also are presented.

Discussion

The results of this study illustrate that UDP is a cognate agonist of the human $P2Y_{14}$-R. Agonist activity of UDP was observed in three different cell backgrounds in which the capacity of the human $P2Y_{14}$-R to promote inhibition of forskolin-stimulated adenylyl cyclase activity was quantified. Given that the potency of UDP is similar to that of UDP-glucose and other nucleotide sugars for activation, we conclude that UDP is a physiologically important agonist of the human $P2Y_{14}$-R.

Previous studies of $P2Y_{14}$-R have focused almost entirely on the activity of uridine nucleotide sugars. Chambers and colleagues (Chambers et al., 2000; Freeman et al., 2001) first reported that the orphan GPCR, KIAA0001, subsequently named the $P2Y_{14}$-R, is activated by UDP-glucose, UDP-galactose, UDP-glucuronic acid, and UDP-*N*-acetylglucosamine. In contrast, a broad range of other nucleotide sugars and nucleotides, including UDP, were reported to be inactive at this receptor. These initial pharmacological studies used three different systems to quantify agonist activities. A yeast transcription reporter assay system was applied that tested human $P2Y_{14}$-R-dependent activity during overnight incubation with potential agonists. The human $P2Y_{14}$-R also was transiently expressed in HEK293 cells with $G\alpha_{16}$, a heterotrimeric G protein that promiscuously couples to most agonist-activated GPCR and confers agonist-dependent activation of phospholipase C and mobilization of Ca^{2+} to these receptors. Finally, membranes were prepared from HEK293 cells that stably expressed the human $P2Y_{14}$-R, and UDP-glucose-promoted binding of [^{35}S]GTPγS to endogenous G proteins was quantified.

It is unclear why agonist activity of UDP was not observed

in early studies of the $P2Y_{14}$-R. The yeast transcription assay and assays that take advantage of promiscuous coupling of GPCR to $G\alpha_{16}$ provide robust assessments of GPCR activity. However, because neither of these systems measure coupling to the cognate heterotrimeric G protein known to mediate $P2Y_{14}$-R-promoted signaling responses, it is possible that a receptor conformation is favored in these test systems that does not fully reflect the ligand binding selectivities and agonist activities of the physiological receptor coupling to its cognate heterotrimeric G protein and signaling pathway. On the other hand, these early studies included assays of agonist-promoted binding of [^{35}S]GTPγS to endogenous G proteins in membranes from $P2Y_{14}$-R-expressing HEK293 cells, and UDP-glucose promoted increases in [^{35}S]GTPγS binding that were 40% greater than basal. This activity apparently reflected agonist-induced coupling to a Gα-subunit(s) of the G_i family because it was inhibited by pertussis toxin. It is noteworthy that concentration effect curves for four different nucleotides, including UDP, were generated with this test system, and that in contradistinction to the stimulatory effect of UDP-glucose, all four nucleotides inhibited [^{35}S]GTPγS binding in a concentration dependent manner. However, this effect was not dependent on expression of the $P2Y_{14}$-R, was not blocked by pertussis toxin, and therefore apparently represented a nonspecific effect on binding of [^{35}S]GTPγS to unspecified proteins. Given the magnitude of this inhibitory effect of nucleotides and the relatively small magnitude of $P2Y_{14}$-R-dependent UDP-glucose-promoted increases in [^{35}S]GTPγS binding, the sensitivity of this test system apparently was not sufficient to reveal an agonist action of UDP at the $P2Y_{14}$-R.

We recently reported that UDP exhibits competitive antagonist/partial agonist activity in measurements of [^{3}H]inositol phosphate accumulation in COS-7 cells transiently coexpressing the $hP2Y_{14}$-R with a chimeric G protein $G\alpha_{q/i}$ that confers a phospholipase C/Ca^{2+} signaling response to G_i-activating receptors (Fricks et al., 2008). It is noteworthy that UDP seemed to be a full agonist in parallel studies with the rat $P2Y_{14}$-R. Moreover, our unpublished studies examining activities of the human $P2Y_{14}$-R cotransfected with $G\alpha_{q/i}$ in COS-7 cells show that an antagonist/partial agonist action of UDP is observed only at very low levels of receptor expression. With higher receptor expression, UDP exhibits maximal agonist effects that are similar to those of UDP-glucose. As we speculated above concerning studies with $G\alpha_{16}$, a conformation of the $P2Y_{14}$-R may be favored when this receptor is coupled to $G\alpha_{q/i}$, which results in ligand binding selectivities and agonist activities that are not altogether consistent with activities of the receptor obtained when coupled to its cognate heterotrimeric G protein. The idea that relative receptor activation by ligands is influenced by both the levels of signaling proteins and the particular G protein signaling system studied is supported by previous studies of a variety of G protein-coupled receptors (Cordeaux et al., 2004; Kenakin, 2007; Urban et al., 2007). The work of Ault and Broach using directed evolution in yeast first suggested an interaction of UDP with the $hP2Y_{14}$-R, and their studies with various mutants of the human $P2Y_{14}$-R also suggested a range of activities of this diphosphate relative to activities observed with UDP-glucose (Ault and Broach, 2006).

Our data with $P2Y_{14}$-HEK293, $P2Y_{14}$-C6, and $P2Y_{14}$-CHO cells all reveal potent agonist activity of UDP. The maximal inhibitory effect on adenylyl cyclase observed with UDP was the same as that of UDP-glucose, which is consistent with the idea that UDP is a full agonist at the $hP2Y_{14}$-R. However, we cannot unequivocally rule out the possibility that under limiting receptor concentrations, UDP would prove to be a partial agonist. A radioligand is not available for quantification of $P2Y_{14}$-R; consequently, the density of receptors cannot be determined in these cells with current technology. Nonetheless, viral expression of GPCR predictably results in receptor levels that do not substantively exceed that of native receptors. We also have isolated a series of clonal $P2Y_{14}$-HEK293 cell lines that exhibit varying amounts of expression of $P2Y_{14}$-R quantified by surface epitope-tagging. UDP and UDP-glucose are similarly efficacious in each of these cell lines tested. Finally, the presence of an endogenous UDP-activated $P2Y_6$-R on HL-60 promyeloleukemia cells precludes straightforward testing of UDP as a $P2Y_{14}$-R agonist on these cells. However, the fact that $P2Y_{14}$-R-selective analogs of UDP promoted phosphorylation of ERK1/2 in differentiated, but not in undifferentiated, HL-60 cells also is consistent with the conclusion that UDP is an agonist of the $P2Y_{14}$-R.

Although the $P2Y_{14}$-R has been thought to be solely regulated by UDP sugars, we conclude that this signaling protein is also significantly activated by UDP. Lazarowski and colleagues have hypothesized that $P2Y_{14}$-R-activating nucleotide sugars arise from cellular protein secretory pathways (Lazarowski et al., 2003). Although we have illustrated that mechanical stimulation promotes release of UTP into the extracellular medium from a broad range of cells (Lazarowski et al., 1997; Lazarowski and Harden, 1999), the source of extracellular UDP remains unclear. One idea is that ecto-nucleotide triphosphate-diphosphohydrolase 2, which converts nucleoside triphosphates to nucleoside diphosphates (Zimmermann, 2000), converts UTP to UDP at the cell surface. For example, the pharmacological effects of ATP at the ADP-activated $P2Y_1$ receptor are markedly augmented by expression of ectoNTPDase2 (Alvarado-Castillo et al., 2005). Alternatively, UDP could be released from a secretory pathway, as suggested by Tatur et al. (2008). Because UDP and UDP-sugars participate in glycosylation reactions in the Golgi lumen, one speculation is that both species are released concomitantly during vesicle exocytosis. Perhaps a common source of the two agonists in part explains the evolution of a receptor that is dually activated by UDP and UDP sugars. The $P2Y_{14}$-R has been implicated in regulation of immune responses (Fumagalli et al., 2003; Skelton et al., 2003; Müller et al., 2005; Scrivens and Dickenson, 2005; Scrivens and Dickenson, 2006), and it will be important to establish that both UDP-glucose and UDP play important and possibly differing roles in this function.

An intriguing aspect of the discovery that UDP is an agonist of the $P2Y_{14}$-R is that the $P2Y_{14}$-R and the UDP-activated $P2Y_6$-R have overlapping cellular distributions (Burnstock and Knight, 2004). One of the best understood physiological responses mediated by P2Y-R is the ADP-promoted aggregation of platelets, which requires coordinated activation of both the G_i-coupled $P2Y_{12}$ receptor and the G_q-coupled $P2Y_1$ receptor (Gachet, 2006). We hypothesize that certain physiological responses to extracellular UDP also require coordinated activation of the G_q-activating $P2Y_6$-R and the G_i-activating $P2Y_{14}$-R. A current goal of our

research is to develop receptor selective ligands that allow pharmacological resolution of the activities of these two receptors in native tissues.

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, and Weisman GA (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* **58:**281–341.

Alvarado-Castillo C, Harden TK, and Boyer JL (2005) Regulation of $P2Y_1$ receptor-mediated signaling by the ectonucleoside triphosphate diphosphohydrolase isozymes NTPDase1 and NTPDase2. *Mol Pharmacol* **67:**114–122.

Ault AD and Broach JR (2006) Creation of GPCR-based chemical sensors by directed evolution in yeast. *Protein Eng Des Sel* **19:**1–8.

Besada P, Shin DH, Costanzi S, Ko H, Mathé C, Gagneron J, Gosselin G, Maddileti S, Harden TK, and Jacobson KA (2006) Structure-activity relationships of uridine 5′-diphosphate analogues at the human $P2Y_6$ receptor. *J Med Chem* **49:**5532–5543.

Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* **87:**659–797.

Burnstock G and Knight GE (2004) Cellular distribution and functions of P2 receptor subtypes in different systems. *Int Rev Cytol* **240:**31–304.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, et al. (2000) A G protein-coupled receptor for UDP-glucose. *J Biol Chem* **275:**10767–10771.

Cooper J, Hill SJ, and Alexander SP (1997) An endogenous A_{2B} adenosine receptor coupled to cyclic AMP generation in human embryonic kidney (HEK 293) cells. *Br J Pharmacol* **122:**546–550.

Cordeaux Y, Ijzerman AP, and Hill SJ (2004) Coupling of the human A_1 adenosine receptor to different heterotrimeric G proteins: evidence for agonist-specific G protein activation. *Br J Pharmacol* **143:**705–714.

Coward P, Chan SD, Wada HG, Humphries GM, and Conklin BR (1999) Chimeric G proteins allow a high-throughput signaling assay of G_i-coupled receptors. *Anal Biochem* **270:**242–248.

Freeman K, Tsui P, Moore D, Emson PC, Vawter L, Naheed S, Lane P, Bawagan H, Herrity N, Murphy K, et al. (2001) Cloning, pharmacology, and tissue distribution of G-protein-coupled receptor GPR105 (KIAA0001) rodent orthologs. *Genomics* **78:**124–128.

Fricks IP, Carter RL, Lazarowski ER, and Harden TK (2009) G_i-dependent cell signaling responses of the human $P2Y_{14}$-receptor in model cell systems. *J Pharmacol Exp Ther* **330:**162–168.

Fricks IP, Maddileti S, Carter RL, Lazarowski ER, Nicholas RA, Jacobson KA, and Harden TK (2008) UDP is a competitive antagonist at the human $P2Y_{14}$ receptor. *J Pharmacol Exp Ther* **325:**588–594.

Fumagalli M, Brambilla R, D'Ambrosi N, Volonté C, Matteoli M, Verderio C, and Abbracchio MP (2003) Nucleotide-mediated calcium signaling in rat cortical astrocytes: Role of P2X and P2Y receptors. *Glia* **43:**218–233.

Gachet C (2006) Regulation of platelet functions by P2 receptors. *Annu Rev Pharmacol Toxicol* **46:**277–300.

Harden TK, Scheer AG, and Smith MM (1982) Differential modification of the interaction of cardiac muscarinic cholinergic and beta-adrenergic receptors with a guanine nucleotide binding component(s). *Mol Pharmacol* **21:**570-580.

Kenakin T (2007) Functional selectivity through protean and biased agonism: who steers the ship? *Mol Pharmacol.* **72:**1393–1401.

Lazarowski ER and Harden TK (1999) Quantitation of extracellular UTP using a sensitive enzymatic assay. *Br J Pharmacol* **127:**1272–1278.

Lazarowski ER, Homolya L, Boucher RC, and Harden TK (1997) 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, Shea DA, Boucher RC, and Harden TK (2003) Release of cellular UDP-glucose as a potential extracellular signaling molecule. *Mol Pharmacol* **63:** 1190–1197.

Malmsjö M, Hou M, Harden TK, Pendergast W, Pantev E, Edvinsson L, and Erlinge D (2000) Characterization of contractile P2 receptors in human coronary arteries by use of the stable pyrimidines uridine 5′-O-thiodiphosphate and uridine 5′-O-3-thiotriphosphate. *J Pharmacol Exp Ther* **293:**755–760.

Müller T, Bayer H, Myrtek D, Ferrari D, Sorichter S, Ziegenhagen MW, Zissel G, Virchow JC Jr, Luttmann W, Norgauer J, et al. (2005) The $P2Y_{14}$ receptor of airway epithelial cells: coupling to intracellular Ca^{2+} and IL-8 secretion. *Am J Respir Cell Mol Biol* **33:**601–609.

Mundell SJ, Loudon RP, and Benovic JL (1999) Characterization of G protein-coupled receptor regulation in antisense mRNA-expressing cells with reduced arrestin levels. *Biochemistry* **38:**8723–8732.

Scrivens M and Dickenson JM (2005) Functional expression of the $P2Y_{14}$ receptor in murine T-lymphocytes. *Br J Pharmacol* **146:**435–444.

Scrivens M and Dickenson JM (2006) Functional expression of the $P2Y_{14}$ receptor in human neutrophils. *Eur J Pharmacol* **543:**166–173.

Skelton L, Cooper M, Murphy M, and Platt A (2003) Human immature monocyte-derived dendritic cells express the G protein-coupled receptor GPR105 (KIAA0001, $P2Y_{14}$) and increase intracellular calcium in response to its agonist, uridine diphosphoglucose. *J Immunol* **171:**1941–1949.

Tatur S, Kreda S, Lazarowski E, and Grygorczyk R (2008) Calcium-dependent release of adenosine and uridine nucleotides from A549 cells. *Purinergic Signal* **4:**139–146.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, et al. (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320:**1–13.

Wolff SC, Qi AD, Harden TK, and Nicholas RA (2005) Polarized expression of human P2Y receptors in epithelial cells from kidney, lung, and colon. *Am J Physiol Cell Physiol* **288:**C624–C632.

Zimmermann H (2000) Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol* **362:**299–309.

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