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**Quantification of Gi-mediated inhibition of adenylyl cyclase activity reveals that  
UDP is a potent agonist of the human P2Y<sub>14</sub> receptor**

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**Running Title:** UDP is a P2Y<sub>14</sub> receptor agonist

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Text Pages: 31

Tables: 1

Figures: 7

References: 30

Words in Abstract: 254

Words in Introduction: 345

Words in Discussion: 1315

**Abbreviations:** P2Y<sub>14</sub>-R, P2Y<sub>14</sub> receptor; P2Y<sub>6</sub>-R, P2Y<sub>6</sub> receptor; hP2Y<sub>14</sub>-R, human P2Y<sub>14</sub> receptor; P2Y<sub>14</sub>-HEK293 cells, human embryonic kidney 293 cells stably expressing the human P2Y<sub>14</sub> receptor; P2Y<sub>14</sub>-C6 cells, C6 rat glioma cells stably expressing the human P2Y<sub>14</sub> receptor; P2Y<sub>14</sub>-CHO cells, Chinese hamster ovary cells stably expressing the human P2Y<sub>14</sub> receptor; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; IBMX, isobutylmethylxanthine; DMSO, dimethylsulfoxide; DMEM, Dulbecco's modified Eagle's medium

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## Abstract

The P2Y<sub>14</sub> receptor was initially identified as a G protein-coupled receptor activated by UDP-glucose and other nucleotide-sugars. We recently developed several cell lines that stably express the human P2Y<sub>14</sub> receptor, allowing facile examination of its coupling to native Gi family G proteins and their associated downstream signaling pathways (Fricks et al., *J. Pharmacol. Exp. Ther.*, 2009). Here we examined P2Y<sub>14</sub> receptor-dependent inhibition of cyclic AMP accumulation in HEK293, C6 glioma, and CHO cells stably expressing this receptor. Not only was the human P2Y<sub>14</sub> 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<sub>50</sub> values (74 nM, 33 nM, and 29 nM) for UDP-dependent activation of the P2Y<sub>14</sub> receptor in HEK293, CHO and C6 glioma cells, respectively, were similar to the EC<sub>50</sub> values (323 nM, 132 nM, and 72 nM) observed for UDP-glucose. UDP and UDP-glucose also stimulated ERK1/2 phosphorylation in P2Y<sub>14</sub> receptor-expressing HEK293 cells but not in wild-type HEK293 cells. A series of analogues of UDP were potent P2Y<sub>14</sub> 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 analogues were identified that selectively activate the P2Y<sub>14</sub> receptor over the UDP-activated P2Y<sub>6</sub> receptor, and these molecules stimulated phosphorylation of ERK1/2 in differentiated human HL-60 promyeloleukemia cells, which natively express the P2Y<sub>14</sub> 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<sub>14</sub> receptor.

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## Introduction

The metabotropic P2Y receptors include a subgroup of five receptors, the P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, and P2Y<sub>11</sub> receptors, that primarily signal through Gq-activated signaling pathways, and a subgroup of three receptors, the P2Y<sub>12</sub>, P2Y<sub>13</sub>, and P2Y<sub>14</sub> receptors, that primarily signal by activating heterotrimeric G proteins of the Gi family (Abbracchio et al., 2006; Burnstock, 2007). The human P2Y<sub>1</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> receptors are activated by adenine nucleotides. The human P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors are activated by uridine nucleotides, and the P2Y<sub>2</sub> receptor is activated by both ATP and UTP.

The P2Y<sub>14</sub>-R exhibits the most unique agonist selectivity of the P2Y receptors extant since it was initially identified as an orphan G protein-coupled receptor that is activated by nucleotide sugars, such as UDP-glucose, UDP-galactose, N-acetylglucosamine, and N-glucuronic acid, rather than by adenine and/or uridine nucleotides (Chambers et al., 2000; Freeman et al., 2001). Although the P2Y<sub>14</sub>-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<sub>14</sub>-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 co-expressing recombinant P2Y<sub>14</sub>-R with a chimeric G protein, Gαq/i (Coward et al., 1999) that couples Gi-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 utilized. To address this issue, agonist activities were quantified in three different stable cell lines

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we developed that allow study of P2Y<sub>14</sub>-R-dependent inhibition of adenylyl cyclase activity. These experiments measuring a natural cell signaling response of the P2Y<sub>14</sub>-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<sub>14</sub>-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.

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## Methods

### *Cell lines stably expressing P2Y<sub>14</sub>-R*

Human embryonic kidney-293 cells stably expressing the hP2Y<sub>14</sub>-R (P2Y<sub>14</sub>-HEK293 cells) and C6 rat glioma cells stably expressing the hP2Y<sub>14</sub>-R (P2Y<sub>14</sub>-C6 cells) were generated as previously described (Fricks et al., 2009). Chinese hamster ovary cells stably expressing the hP2Y<sub>14</sub>-R (P2Y<sub>14</sub>-CHO cells) were produced using similar methodology. Briefly, hP2Y<sub>14</sub>-R cDNA was ligated into the retroviral expression vector pLXSN as described previously (Wolff et al., 2005), and recombinant retrovirus for hP2Y<sub>14</sub>-R expression was produced in PA317 cells. CHO cells were incubated with hP2Y<sub>14</sub>-R virus and 0.4 mg/ml G418 for two weeks to produce hP2Y<sub>14</sub>-R-expressing cells.

### *Cell Culture*

HEK293 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% geneticin at 37°C in a 5% CO<sub>2</sub> environment. C6 rat glioma cells were cultured in DMEM supplemented with 5% fetal bovine serum and 1% geneticin in a 5% CO<sub>2</sub> environment. CHO cells were grown in F-12 Medium supplemented with 10% fetal bovine serum and 1% geneticin at 37°C in a 5% CO<sub>2</sub> environment. 1321N1 Human astrocytoma cells were grown in DMEM supplemented with 5% fetal bovine serum in a 5% CO<sub>2</sub> environment. HL-60 promyeloleukemia cells were maintained in RPMI1641 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*

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

#### *Inositol Phosphate Accumulation*

P2Y<sub>6</sub>-R-dependent activation of phospholipase C was measured by quantifying [ $^3\text{H}$ ]inositol phosphate accumulation in 1321N1 human astrocytoma cells as previously described (Besada et al., 2006).

#### *MAP Kinase Activation Assays*

Phosphorylation of ERK1/2 was quantified as we have described in detail (Fricks et al, 2009). Briefly, wild-type HEK293 or P2Y<sub>14</sub>-HEK293 cells were serum-starved for 18 h prior to 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 prior to addition of agonist for 30 min at 37°C. Cell extracts were subjected to SDS-PAGE 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 (Pico West system, Thermo Fisher Scientific, Waltham, MA) and then was exposed to film. Membranes were stripped and re-probed with a primary antibody against total ERK1/2 to verify equal loading of lanes.

#### *Data Analysis*

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EC<sub>50</sub> values were determined using Prism software (GraphPad, San Diego, CA) and are presented as mean ± SE. All experiments were repeated at least three times.

### *Materials*

IBMX 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 (St. Louis, MO). HPLC analysis of UDP-glucose and all of the nucleotides revealed >95% purity. Pertussis toxin was purchased from List Biologicals (Campbell, CA). [<sup>3</sup>H]Adenine was purchased from American Radiolabeled Chemicals (St. Louis, MO). Geneticin, serum, and all cell culture medium were from Gibco (Invitrogen, Carlsbad, CA). Antibodies for phosphoERK1/2 and ERK1/2 were purchased from Cell Signaling Technologies (Beverly, MA).



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## 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 engineer 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 h $P2Y_{14}$ -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). Similarly, UDP had no effect in wild-type HEK293 cells (Fig. 1A), but promoted a concentration-dependent decrease in cyclic AMP accumulation in

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P2Y<sub>14</sub>-HEK293 cells. The potency (Table 1A) and maximal effect (Table 1B) 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<sub>14</sub>-HEK293 cells in which GPCR-promoted activation of Gi 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<sub>14</sub>-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<sub>14</sub>-R stably expressed in other cell backgrounds. Therefore, activity of UDP was assessed in a stable cell line, P2Y<sub>14</sub>-C6 cells, that we recently developed (Fricks et al., 2009), as well as in a third stable cell line, P2Y<sub>14</sub>-CHO cells, that was generated as described in 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<sub>14</sub>-C6 (Fig. 2B) and P2Y<sub>14</sub>-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 1B), and the EC<sub>50</sub> values of UDP and UDP-glucose also were similar (Table 1A). The effect of both UDP-glucose and UDP on cyclic AMP accumulation was blocked by preincubation of P2Y<sub>14</sub>-C6 cells (Fig. 3A) or P2Y<sub>14</sub>-CHO cells (Fig. 3B) with pertussis toxin.

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The agonist activity of other naturally occurring nucleoside diphosphates was tested in P2Y<sub>14</sub>-HEK293 cells. Both CDP and GDP exhibited agonist activities although the potencies observed were over 100-fold less than that observed with UDP (Fig. 4). The effect of ADP, which is the cognate agonist of the P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> receptors, also was examined. Incubation of P2Y<sub>14</sub>-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 back 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 only exhibited P2Y<sub>14</sub>-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<sub>14</sub>-HEK293 cells is that ectoenzymes convert ADP to adenosine, which in turn activates an endogenous A<sub>2B</sub> adenosine receptor known to be present on HEK293 cells (Cooper et al., 1997; Mundell et al., 1999).

Potential agonist activities of the UDP analogues, UDPβS (Malmsjö et al., 2000) and 2-methylthioUDP (Besada et al., 2006) also were examined in P2Y<sub>14</sub>-HEK293 cells. As is illustrated in Fig. 5, both UDPβS (EC<sub>50</sub> = 26 ± 11 nM, n = 3) and 2-methylthio-UDP (EC<sub>50</sub> = 2 ± 1 nM, n = 3) were potent agonists at the hP2Y<sub>14</sub>-R.

We recently reported that stable expression of the human P2Y<sub>14</sub> 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<sub>14</sub>-R-dependent phosphorylation of ERK1/2 also was examined. As is illustrated in Fig. 6, 10 μM UDP

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had no effect on the MAP kinase response in wild-type HEK293 cells, but promoted marked ERK1/2 phosphorylation in P2Y<sub>14</sub>-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<sub>14</sub> receptor.

The results presented thus far with the human P2Y<sub>14</sub>-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<sub>14</sub>-R is natively expressed in HL-60 promyeloleukemia cells (Fricks et al, 2009). Whereas neither P2Y<sub>14</sub>-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<sub>14</sub>-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<sub>14</sub>-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<sub>14</sub>-R expression (Fig. 7A). RT-PCR analyses revealed that the response observed to UDP in wild-type HL60 cells is likely due to the presence of a UDP-activated P2Y<sub>6</sub>-R since 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 Gi-coupled P2Y<sub>14</sub>-R versus Gq-coupled P2Y<sub>6</sub>-R in these cells, we have been unable to observe inhibition of adenylyl cyclase in response to UDP-glucose or

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formyl-Met-Leu-Phe, which activates a Gi-linked receptor for chemotactic peptides in HL-60 cells (Fricks et al, 2009).

In the absence of availability of another cell line that natively expresses the P2Y<sub>14</sub>-R, we considered a pharmacological approach to show that the P2Y<sub>14</sub>-R natively expressed on differentiated HL-60 cells responds to agonists in addition to nucleotide sugars. As illustrated above (Fig. 5), two UDP analogues activated the P2Y<sub>14</sub>-R stably expressed in HEK293 cells. Since these two molecules also are potent activators of the P2Y<sub>6</sub>-R (data not shown), they are not useful for selectively activating the P2Y<sub>14</sub>-R of HL-60 cells. However, our recent chemical syntheses<sup>1</sup> have identified analogues of UDP that activate the P2Y<sub>14</sub>-R without activating the P2Y<sub>6</sub>-R, and the relative activities of two of these, difluoro- $\alpha,\beta$ -methylene-UDP (MRS2802) and UDP- $\beta$ -propylester (MRS2907), at the P2Y<sub>14</sub>-R versus P2Y<sub>6</sub>-R are illustrated in Fig. 7. Difluoro- $\alpha,\beta$ -methylene-UDP inhibited forskolin-stimulated cyclic AMP accumulation in P2Y<sub>14</sub>-HEK293 cells with an EC<sub>50</sub> of approximately 50 nM (Fig. 7B, left panel), whereas little inositol phosphate response to this molecule was observed at concentrations up to 10  $\mu$ M in 1321N1 human astrocytoma cells stably expressing the human P2Y<sub>6</sub>-R (Fig. 7B, right panel). UDP- $\beta$ -propyl ester also selectively activated the P2Y<sub>14</sub>-R over the P2Y<sub>6</sub>-R (Fig 7C). Given the P2Y<sub>14</sub>-R selectivity of these two UDP analogues, we examined their capacity to stimulate ERK1/2 phosphorylation in wild-type and differentiated HL-60 cells. Neither difluoro- $\alpha,\beta$ -methylene-UDP nor UDP- $\beta$ -propylester 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 analogues was observed after induction of expression of the P2Y<sub>14</sub>-R by differentiation of these cells by treatment with DMSO. These results are

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consistent with the idea that UDP analogues, and by deduction UDP itself, are potent agonists of the P2Y<sub>14</sub>-R in a native environment.

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## Discussion

The results of this study illustrate that UDP is a cognate agonist of the human P2Y<sub>14</sub>-R. Agonist activity of UDP was observed in three different cell backgrounds in which the capacity of the human P2Y<sub>14</sub>-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<sub>14</sub>-R.

Previous studies of P2Y<sub>14</sub>-R have almost entirely focused on the activity of uridine nucleotide sugars. Chambers and his colleagues (Chambers et al., 2000; Freeman et al., 2001) first reported that the orphan GPCR, KIAA0001, subsequently named the P2Y<sub>14</sub>-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 utilized three different systems to quantify agonist activities. A yeast transcription reporter assay system was applied that tested human P2Y<sub>14</sub>-R-dependent activity during overnight incubation with potential agonists. The human P2Y<sub>14</sub>-R also was transiently expressed in HEK293 cells with Gα16, a heterotrimeric G protein that promiscuously couples to most agonist-activated GPCR and confers agonist-dependent activation of phospholipase C and mobilization of Ca<sup>2+</sup> to these receptors. Finally, membranes were prepared from HEK293 cells that stably expressed the human P2Y<sub>14</sub>-R, and UDP-glucose-promoted binding of [<sup>35</sup>S]GTPγS to endogenous G proteins was quantified.

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It is unclear why agonist activity of UDP was not observed in early studies of the P2Y<sub>14</sub>-R. The yeast transcription assay and assays that take advantage of promiscuous coupling of GPCR to Gα16 provide robust assessments of GPCR activity. However, since neither of these systems measure coupling to the cognate heterotrimeric G protein known to mediate P2Y<sub>14</sub>-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 [<sup>35</sup>S]GTPγS to endogenous G proteins in membranes from P2Y<sub>14</sub>-R-expressing HEK293 cells, and UDP-glucose promoted increases in [<sup>35</sup>S]GTPγS binding that were 40% greater than basal. This activity apparently reflected agonist-induced coupling to a Gα-subunit(s) of the Gi family since it was inhibited by pertussis toxin. It is notable 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 [<sup>35</sup>S]GTPγS binding in a concentration dependent manner. However, this effect was not dependent on expression of the P2Y<sub>14</sub>-R, was not blocked by pertussis toxin, and therefore, apparently represented a non-specific effect on binding of [<sup>35</sup>S]GTPγS to unspecified proteins. Given the magnitude of this inhibitory effect of nucleotides and the relatively small magnitude of P2Y<sub>14</sub>-R-dependent UDP-glucose-promoted increases in [<sup>35</sup>S]GTPγS binding, the sensitivity of this test system apparently was not sufficient to reveal an agonist action of UDP at the P2Y<sub>14</sub>-R.



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We recently reported that UDP exhibits competitive antagonist/partial agonist activity in measurements of [<sup>3</sup>H]inositol phosphate accumulation in COS-7 cells transiently coexpressing the hP2Y<sub>14</sub>-R with a chimeric G protein Gαq/i that confers a phospholipase C/Ca<sup>2+</sup> signaling response to Gi-activating receptors (Fricks et al., 2008). Interestingly, UDP appeared to be a full agonist in parallel studies with the rat P2Y<sub>14</sub>-R. Moreover, our unpublished studies examining activities of the human P2Y<sub>14</sub>-R cotransfected with Gαq/i in COS-7 cells show that an antagonist/partial agonist action of UDP only is observed 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 Gα16, a conformation of the P2Y<sub>14</sub>-R may be favored when this receptor is coupled to Gαq/i that 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<sub>14</sub>-R, and their studies with various mutants of the human P2Y<sub>14</sub>-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<sub>14</sub>-HEK293, P2Y<sub>14</sub>-C6, and P2Y<sub>14</sub>-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

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UDP is a full agonist at the hP2Y<sub>14</sub>-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<sub>14</sub>-R, and consequentially, 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<sub>14</sub>-HEK293 cell lines that exhibit varying amounts of expression of P2Y<sub>14</sub>-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<sub>6</sub>-R on HL-60 promyeloleukemia cells precludes straightforward testing of UDP as a P2Y<sub>14</sub>-R agonist on these cells. However, the fact that P2Y<sub>14</sub>-R-selective analogues 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<sub>14</sub>-R.

Although the P2Y<sub>14</sub>-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 his colleagues have hypothesized that P2Y<sub>14</sub>-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 and Harden, 1999; Lazarowski et al., 1997), the source of extracellular UDP remains unclear. One idea is that ectoNTPDase2, 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<sub>1</sub> receptor are markedly augmented by

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expression of ectoNTPDase2 (Alvarado-Castillo et al., 2005). Alternatively, UDP could be released from a secretory pathway, as recently suggested (Tatur et al., 2008). Since 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<sub>14</sub>-R has been implicated in regulation of immune responses (Fumagalli et al., 2003; Muller et al., 2005; Scrivens and Dickenson, 2005; Scrivens and Dickenson, 2006; Skelton et al., 2003), 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<sub>14</sub>-R is that the P2Y<sub>14</sub>-R and the UDP-activated P2Y<sub>6</sub>-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 Gi-coupled P2Y<sub>12</sub> receptor and the Gq-coupled P2Y<sub>1</sub> receptor (Gachet, 2006). We hypothesize that certain physiological responses to extracellular UDP also require coordinated activation of the Gq-activating P2Y<sub>6</sub>-R and the Gi-activating P2Y<sub>14</sub>-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.

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## Footnotes

<sup>1</sup>Arijit Das, Hyojin Ko, Matthem O. Barrett, Lauren B. Burianek,  
T. Kendall Harden, and Kenneth A. Jacobson, manuscript in preparation

This work was supported by grants from the National Institutes of Health (GM38213 and HL34322) and by Intramural Research Program of the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases.

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## Legends for Figures

**Figure 1.** UDP-glucose and UDP promote pertussis toxin-sensitive inhibition of cyclic AMP accumulation in P2Y<sub>14</sub>-HEK293 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 HEK293 cells as described in Methods. The data are mean ± SEM 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<sub>14</sub>-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 ± SEM of triplicate determinations and are representative of data from six experiments. **C**, Blockade of the P2Y<sub>14</sub>-R-dependent effects of UDP-glucose and UDP by pertussis toxin. P2Y<sub>14</sub>-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 ± SEM of results from three separate experiments.

**Figure 2.** P2Y<sub>14</sub>-R-dependent activities of UDP-glucose and UDP in P2Y<sub>14</sub>-C6 and P2Y<sub>14</sub>-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 in Methods. The data are mean ± SEM from triplicate determinations and are representative of results from three separate experiments. **B**, Concentration-dependent inhibition of forskolin-stimulated cyclic AMP

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accumulation by UDP-glucose and UDP in P2Y<sub>14</sub>-C6 cells. P2Y<sub>14</sub>-C6 cells were incubated in the absence (○) or 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 ± SEM 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<sub>14</sub>-CHO cells. P2Y<sub>14</sub>-CHO cells were incubated in the absence (○) or 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 ± SEM of triplicate determinations and are representative of data from six separate experiments.

**Figure 3.** Pertussis toxin sensitivity of the effects of UDP-glucose and UDP in P2Y<sub>14</sub>-C6 cells and P2Y<sub>14</sub>-CHO cells. **A**, Blockade of the P2Y<sub>14</sub>-R-dependent effects of UDP-glucose and UDP by pertussis toxin in P2Y<sub>14</sub>-C6 cells. P2Y<sub>14</sub>-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 ± SEM of results from three separate experiments. **B**, Blockade of the P2Y<sub>14</sub>-R-dependent effects of UDP-glucose and UDP by pertussis toxin in P2Y<sub>14</sub>-CHO cells. P2Y<sub>14</sub>-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 ± SEM of results from three separate experiments.

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**Figure 4.** Agonist activities of nucleoside diphosphate molecules in P2Y<sub>14</sub>-HEK293 cells. P2Y<sub>14</sub>-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 (Δ), or ADP (\*). The data are presented as mean ± SEM of results pooled from three separate experiments.

**Figure 5.** Agonist activities of 2-thioUDP and UDPβS in P2Y<sub>14</sub>-HEK293 cells. P2Y<sub>14</sub>-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-thioUDP (▼) or UDPβS (Δ). The data are presented as mean ± SEM of triplicate determinations and are representative of data from three separate experiments.

**Figure 6.** P2Y<sub>14</sub>-R-dependent activation of MAP kinase signaling by UDP. Empty vector or P2Y<sub>14</sub>-HEK293 cells were serum-starved for 18 h prior to incubation with vehicle, with 10 μM UDP, or 10 μM UDP-glucose for 15 min. Cell lysates were subjected to SDS-PAGE, the samples transferred to nitrocellulose membranes and the membranes probed with antibodies for phospho-ERK1/2 and total ERK1/2 as described in Methods. The results shown are representative of data from three individual experiments.

**Figure 7.** Activation of a P2Y<sub>14</sub>-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

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indicated times with 10  $\mu$ M UDP or 10  $\mu$ M UDP-glucose (UDPG) as described in Methods. Levels of total ERK1/2 also are presented. **B**, Concentration effect curves for MRS2802 for activation of P2Y<sub>14</sub>-R in P2Y<sub>14</sub>-HEK293 cells and activation of P2Y<sub>6</sub>-R in 1321N1 human astrocytoma cells stably expressing the human P2Y<sub>6</sub>-R were generated as described in Methods. **C**, Concentration effect curves for MRS2907 for activation of P2Y<sub>14</sub>-R in P2Y<sub>14</sub>-HEK293 cells and activation of P2Y<sub>6</sub>-R in 1321N1 human astrocytoma cells stably expressing the human P2Y<sub>6</sub>-R were generated as described in Methods. **D**, Phosphorylation of ERK1/2 (phospho-ERK1/2) was quantified in undifferentiated (-DMSO) or differentiated (+DMSO) HL-60 cells after incubation for the indicated times with 10  $\mu$ M UDP-glucose (UDPG), 1  $\mu$ M MRS2907, or 10  $\mu$ M MRS2802 as described in Methods. Levels of total ERK1/2 also are presented.

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**Table 1. Comparative potencies and maximal effects of UDP-glucose and UDP in cell lines stably expressing the human P2Y<sub>14</sub>-R.** **A.** Concentration effect curves for UDP-glucose- and UDP-promoted inhibition of forskolin-stimulated cyclic AMP accumulation were generated in P2Y<sub>14</sub>-HEK293, P2Y<sub>14</sub>-C6, and P2Y<sub>14</sub>-CHO cells as described in Methods. Assays were carried out in triplicate, and EC<sub>50</sub> values were determined from individual experiments by applying the Prism software package. The EC<sub>50</sub> values are presented as mean ± SEM averaged from 6 experiments each with C6 and CHO cells and from 21 experiments with HEK293 cells. **B.** The maximal percent inhibition of forskolin-stimulated cyclic AMP accumulation was calculated from the curves generated as described in part A.

**A. EC<sub>50</sub> values**

<u>Cell line</u>	<u>UDP-glucose</u>	<u>UDP</u>
P2Y <sub>14</sub> -HEK293 cells	323 ± 84 nM	74 ± 18 nM
P2Y <sub>14</sub> -CHO cells	132 ± 76 nM	33 ± 20 nM
P2Y <sub>14</sub> -C6 cells	72 ± 17 nM	29 ± 9 nM

**B. Percent maximal inhibition of forskolin-stimulated cyclic AMP accumulation**

<u>Cell line</u>	<u>UDP-glucose</u>	<u>UDP</u>
P2Y <sub>14</sub> -HEK293 cells	60 ± 6 %	58 ± 2 %
P2Y <sub>14</sub> -CHO cells	70 ± 10 %	68 ± 9 %
P2Y <sub>14</sub> -C6 cells	86 ± 3 %	91 ± 1 %

Figure 1

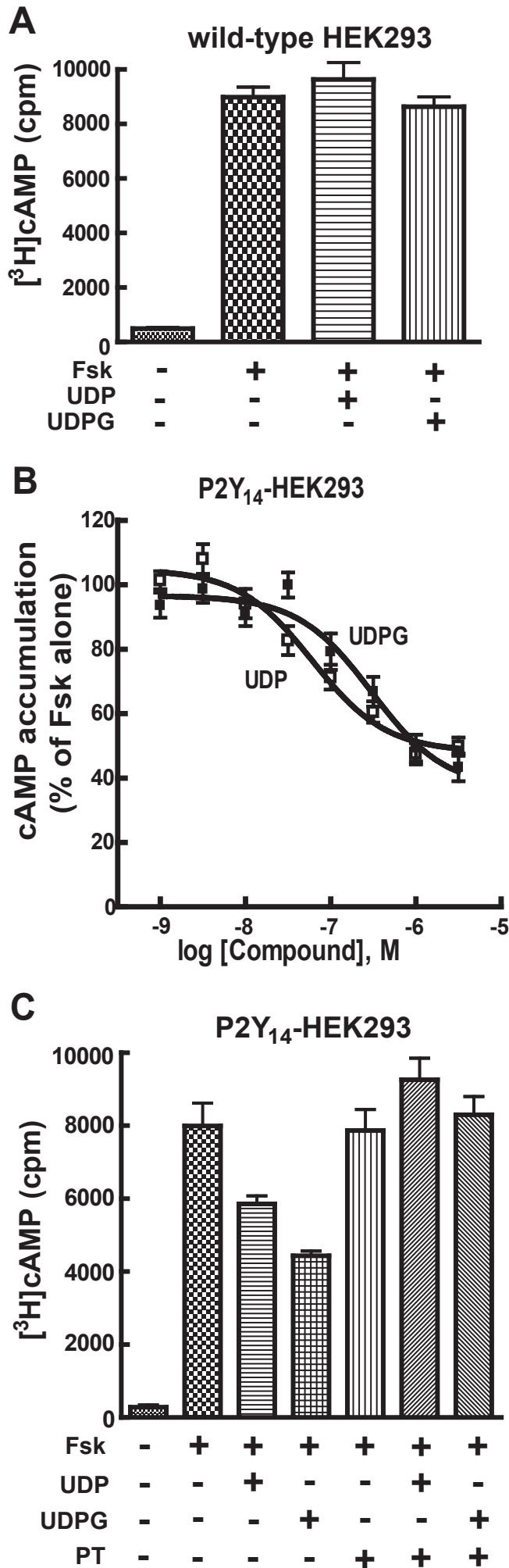




Figure 2

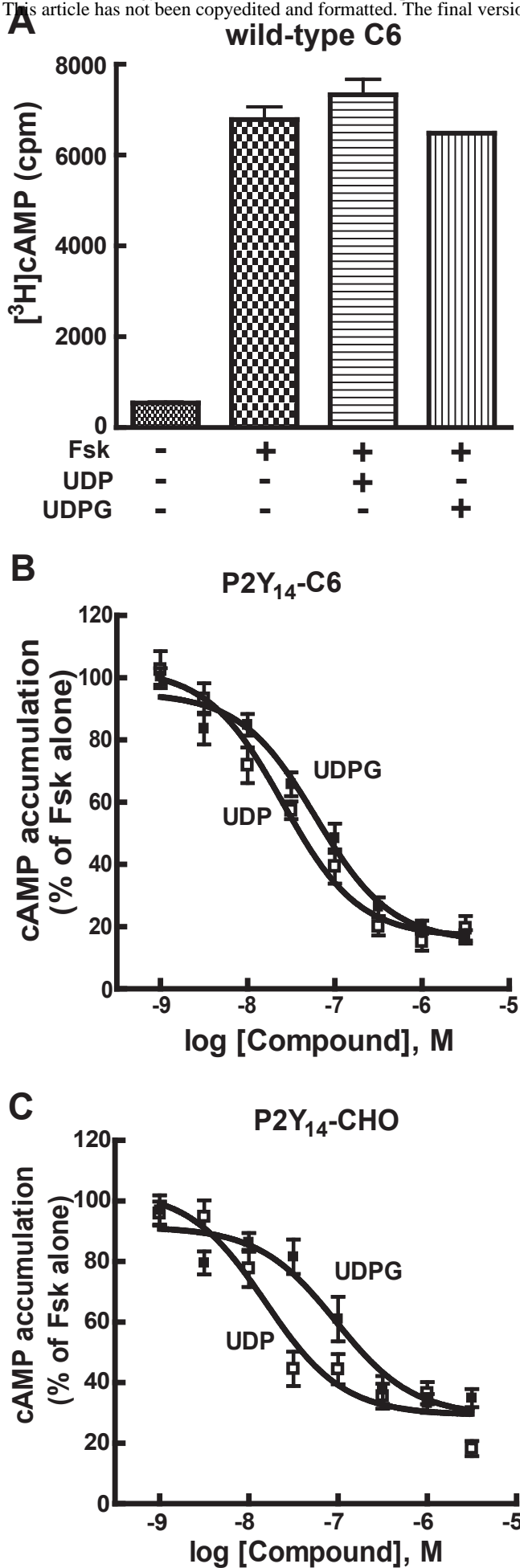


Figure 3

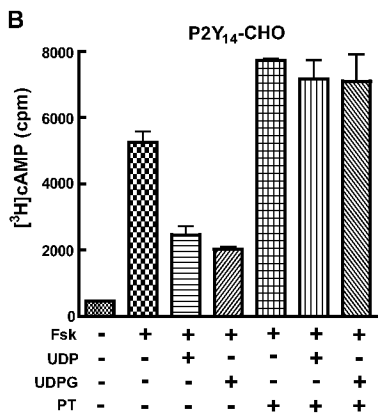
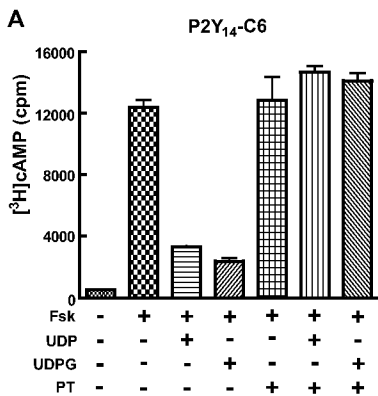


Figure 4

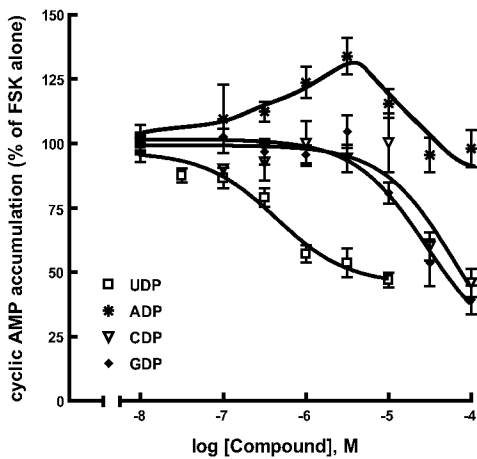
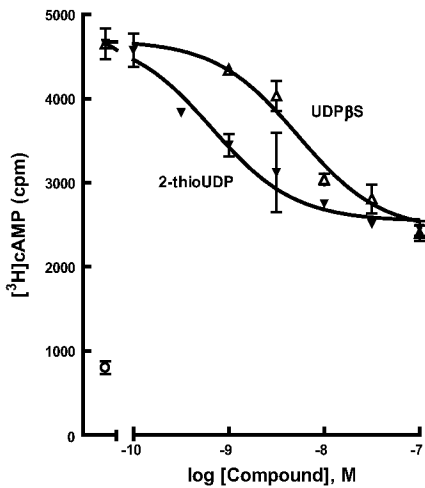
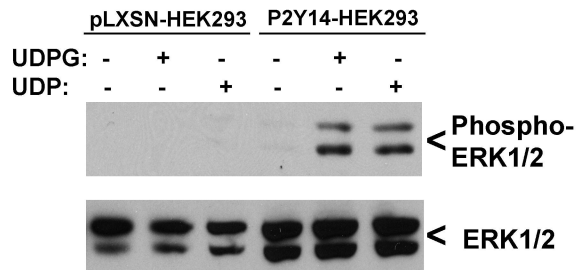


Figure 5



## Figure 6



**Figure 7**

