Molecular Pharmacology and Function of the $P2Y_{14}$ Receptor

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Abstract

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(Under the direction of T. Kendall Harden, Ph.D.)

P2Y receptors are a family of seven transmembrane spanning G protein-coupled receptors that are activated by nucleotides and nucleotide-sugars. The $P2Y_{14}$-R is activated by nucleotide sugars, although little is known about the physiological role(s) of this receptor. With a view toward generating pharmacological reagents for studies of $P2Y_{14}$-R, one goal of this dissertation was to apply a rational structure-activity relationship approach to develop novel ligands for the $P2Y_{14}$-R. Guided by molecular modeling studies of the $P2Y_{14}$-R, iterative design of synthetic ligands produced a multitude of compounds which were assessed for agonist activity at the $P2Y_{14}$-R. From these studies, several novel agonists were identified for the $P2Y_{14}$-R, including 2-thio-UDP-Glc, which exhibited greater than six-fold higher potency than UDP-Glc. Other novel agonists identified included several UDP-sugars. Using a COS-7 cell system in which recombinant $P2Y_{14}$-R were co-expressed with the chimeric G protein, Gαq/i, UDP was identified as a competitive antagonist at the $P2Y_{14}$-R. In contrast, in studies comparing the pharmacological selectivity of the rat $P2Y_{14}$-R to that of the human $P2Y_{14}$-R in the same cell system, UDP was found to be an agonist at the rat $P2Y_{14}$-R. Another goal of this work was to examine the signal transduction pathways downstream of $P2Y_{14}$-R activation, and for these studies, stable cell lines expressing $P2Y_{14}$-R in HEK293 and in C6 glioma cells were developed. This approach allowed study of $P2Y_{14}$-R coupled to native G proteins, and $P2Y_{14}$-R-dependent inhibition of adenylyl cyclase was observed.

$P2Y_{14}$-R activation also promoted pertussis toxin-sensitive phosphorylation of ERK1/2. Moreover, native $P2Y_{14}$-R were detected in differentiated HL-60 cells by RT-PCR, and in these cells, UDP-Glc promoted pertussis toxin-sensitive activation of ERK1/2. The work presented here provides a foundation for future development of pharmacological agents for the $P2Y_{14}$ receptor. Furthermore, this work establishes that the $P2Y_{14}$-R couples to Gi and the MAP kinase signaling pathway, and provides robust cell model systems for future studies of $P2Y_{14}$-R function.

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List of Abbreviations and Symbols

α, alpha

β, beta

γ, gamma

ADP, adenosine 5'-diphosphate

ATP, adenosine 5'-triphosphate

BSA, bovine serum albumin

cAMP, cyclic adenosine 3', 5'-monophosphate

DAG, diacylglycerol

DMEM, Dulbecco's Modified Eagle Medium

DMSO, dimethylsulfoxide

DTT, dithiothreitol

EC_{50}, effective concentration required to produce 50% of the maximal effect

EDTA, ethylenediaminetetraacetic acid

E-NPP, ectonucleoside pyrophosphatase/phosphodiesterase

ERK, extracellular-regulated kinase

FBS, fetal bovine serum

fMLP, formyl-methionine-leucine-phenylalanine

FPR, formyl peptide receptor

FSK, forskolin

GDI, GDP dissociation inhibitor

GDP, guanosine 5'-diphosphate

GEF, G protein exchange factor

GPCR, G protein copupled receptor

GRK, G protein coupled receptor kinase

GTP, guanosine 5'-triphosphate

HA, hemagglutinin

HBSS, Hank's Balanced Salt Solution

HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

IBMX, 3-isobutyl-1-methylxanthine

IP_3, inositol trisphosphate

JNK, Jun N-terminal kinase

KO, knockout

LARG, leukemia-associated Rho GEF

LPA, lysophosphatidyl acid

MAPK, mitogen-activated protein kinase

MCMM, Monte Carlo Multiple Minimums

mRNA, messenger ribonucleic acid

NDPK, nucleoside diphosphokinase

NTPDase, nucleotide triphosphate diphosphohydrolase

PAR, protease-activated receptor

PBS, phosphate buffered saline

PI3K, phosphatidylinositol-3-kinase

PIP_2, phosphatidylinositol 4,5-bisphosphate

PKA, protein kinase A

pK_B, log dissociation constant for antagonist/receptor complex

PKC, protein kinase C

PLC, phospholipase C

PPADS, pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid

PPi, inorganic pyrophosphate

PTX, pertussis toxin

RGS, regulator of G protein signaling

RNA, ribonucleic acid

RT-PCR, reverse transcriptase polymerase chain reaction

SAR, structure-activity relationship

SE, standard error

TCA, trichloroacetic acid

U, units

UDP, uridine 5’-diphosphate

UDP-Glc, uridine 5’-diphosphoglucose

UTP, uridine 5’-triphosphate

Chapter I. Introduction

Nucleotides are important extracellular signaling molecules that activate cell surface receptors to regulate many physiological processes such as neurotransmission, vasoconstriction/ dilation, cell differentiation, platelet aggregation, and also ion transport in epithelial cells. Previous to Burnstock's observations in the 1970's, ATP was generally accepted to function only in intracellular processes where it acts as the energy source for the cell. Burnstock originally hypothesized that ATP was released from cells and could act on cell surface receptors to contribute to second messenger signaling, based on evidence that ATP mediated responses to nerve stimulation independently from cholinergic or adrenergic nerve responses (Burnstock, 1972). We now know that ATP produces autocrine/ paracrine signaling in non-excitatory cells as well as functions as an important neurotransmitter, involved in a broad range of cellular and organ functions. Currently, ATP and ADP, as well as UTP, UDP, and UDP-glucose are well accepted as extracellular signaling molecules that act on a diverse family of nucleotide binding receptors, and extracellular concentrations of nucleotides are precisely regulated by cellular release and metabolism by ectoenzymes.

The following pages will overview the source of extracellular nucleotides and their metabolism by ectoenzymes. Subsequent to discussion of nucleotides present in the extracellular space, a general introduction to purinergic receptors will be presented. A more detailed description of members of the P2Y receptor family will follow, with an expanded focus on the $P2Y_{14}$-R, the topic of this dissertation.

Cellular release of nucleotides

Regulated release of ATP through exocytosis from excitatory cells is a phenomenon that has been known to occur for many decades. More recently, nucleotide release from non-excitatory cells has been established, and evidence for the release of other nucleotides including UTP and UDP-glucose has been reported. Sources for extracellular nucleotides include constitutive release from resting cells, release after hormone stimulation or mechanical stress, and cell lysis. Elucidating the mechanism(s) of release is currently the topic of investigation in many labs.

Assays for quantification of ATP have been developed using a luciferase assay, allowing spatiotemporal resolution of ATP release and hydrolysis in the medium on cultured cells (Dubyak and el-Moatassim, 1993; Taylor et al., 1998; Beigi et al., 1999). Luciferin is a substrate for luciferase, and the catalytic reaction produces luminescence in the presence of ATP. These assays are very sensitive methods for quantification of ATP in the bulk medium and have even been adapted to measure cell surface concentrations of ATP with a luciferase engineered to be anchored at the plasma membrane (Beigi et al., 1999). Sensitive assays detecting released uridine nucleotides and nucleotide-sugars from cultured cells have been developed by Lazarowski and colleagues (Lazarowski et al., 1997a; Lazarowski and Harden, 1999; Lazarowski et al., 2003). Such detection assays have informed us about circumstances of nucleotide release and also about relative rates of release versus degradation of nucleotides.

Differences in measurements of ATP with soluble luciferase compared with plasma membrane-associated luciferase suggest that measurements from the bulk medium are likely

to grossly underestimate the concentration at the receptor level (Beigi et al., 1999). One possible explanation for this may be that the microenvironment at the cell surface has a higher concentration of nucleotide than is measured in the bulk medium. That is, higher concentrations of nucleotides are retained at the surface of cells in close proximity to cell surface receptors, whereas the bulk medium contains a diffused, lower concentration of nucleotide due to ectonucleotidase activity. This may occur because the rates of release and accumulation of nucleotide at the cell surface is more rapid than the rate of diffusion throughout the bulk medium, or it is possible that hydrolyzing enzymes are localized on the cell surface or as soluble enzymes in the medium in an orientation that allows degradation of nucleotides away from the site of release and receptor expression, allowing receptors to interact with nucleotides immediately upon release before degradation of the nucleotide occurs.

Cellular release of ATP, UTP, or UDP-glucose after mechanical stimulation of cells has been well established (Gordon, 1986; Lazarowski et al., 1997a). Levels of ATP and UTP measured in the bulk medium on several types of cultured cells rise sharply within five minutes of stimulation. In contrast to the rapid rise in extracellular concentrations of other nucleotides on mechanically stimulated cells, UDP-Glc increases little. However, UDP-glucose in the medium on 1321N1 human astrocytoma cells was observed to gradually increase to a maximal concentration at 1 h after stimulation of the cells and remain at that level for a subsequent 2 h (Lazarowski et al., 2003). Extracellular UDP-glucose is apparently metabolized at a slower rate than ATP in several cell lines and furthermore, the concentration reported for UDP-glucose on resting cells was 2-6 fold higher than the ATP concentration,

suggesting that UDP-glucose is released constitutively from cells as well as upon mechanical stimulation.

In addition to constitutive release of nucleotides and after mechanical stimulation of cells, multiple reports have established that nucleotide release also occurs as a result of hormone activation of some GPCRs. Thrombin treatment of many cell types promotes ATP release (Pearson and Gordon, 1979; Joseph et al., 2003), relevant to the work described in this dissertation, thrombin promotes UDP-Glc release from astrocytoma cells (Kreda *et al.*, 2008). Bradykinin, acetylcholine, and serotonin have all been shown to induce release of ATP from several types of cells (Yang et al., 1994; Ostrom et al., 2000; Buxton et al., 2001). Hormone activation of cells leads to nucleotide release, and multiple signaling pathways may be activated in a cascade from hormone activation and subsequent activation by nucleotides.

Cell lysis due to tissue damage is another source for extracellular nucleotides. After insults such as ischemia in the brain or heart, nucleotides are released from damaged cells and are present at concentrations sufficient to activate P2 receptors (Cook and McCleskey, 2002; Melani et al., 2005). Induced ischemia in pig artery increased plasma levels of ATP and UTP, and also correlated with vasodilation (Erlinge et al., 2005). Patients with coronary heart disease were found to have higher than normal plasma levels of UTP (Wihlborg et al., 2006). Intracellular nucleotides that are released as a course of cell lysis from tissue damage are likely to activate nearby P2 receptors.

The mechanisms for regulated release of nucleotides have not been established. The proposed mechanisms for nucleotide release from epithelial cells are exocytosis of ATP-containing vesicles, release through ATP-permeable channels, or some combination of these two processes. Observations of ATP-containing vesicles in pancreatic cells support the

hypothesis that exocytosis is a mechanism for ATP release (Sorensen and Novak, 2001). Additionally, findings from several groups support exocytotic release of ATP as a result of cell swelling, and implicate the involvement of phosphatidylinositol-3-kinase (PI3K) (Merlin et al., 1996; Feranchak et al., 1999; Gatof et al., 2004). Several anion channels have been reported to be permeable to ATP under certain experimental conditions, although a selective ATP channel has not been identified (Wang et al., 1996; Roman et al., 1997; Bell et al., 2003; Okada et al., 2004).

Interest in connexin and pannexin hemichannels as potential release mechanisms is growing. Connexins and pannexins have four transmembrane domains with the amino and carboxyl termini on the intracellular side of the plasma membrane. Six subunits (connexons) assemble to form hemichannels (Shestopalov and Panchin, 2008). Connexin hemichannels are important components of gap junctions, which allow the flow of ions between cells and have even been shown to be permeable to ATP (Penuela et al., 2007). Pannexins, however, do not form gap junctions and furthermore, while they form channels that are permeable to ATP, their function has been reported to be inhibited by extracellular ATP (reviewed in Dubyak, 2009). While the exact mechanisms for cellular release of nucleotides remain to be elucidated, it seems likely that some combination of vesicular release and channel-regulated release are involved in nucleotide release from cells.

Clearly, metabolism of extracellular nucleotides regulates purinergic receptor function. The specifics of how P2 receptors and ectonucleotidases coordinate physiological functions remain largely undefined. Precise regulation of nucleotide action is likely specific to the tissue or cell type and stage of cell development. Spatiotemporal resolution of ectonucleotidase and receptor expression together with accurate measurements of nucleotide

concentrations will bring us closer to understanding the biological processes dependent on nucleotide signaling.

Ectonucleotidases

In addition to release of nucleotides from cells, extracellular concentrations of ATP, UTP, ADP, UDP, and UDP-glucose are precisely controlled by cell surface enzymes catalyzing nucleotide hydrolysis and phosphorylation (Fig. 1). Ectoenzymes remove nucleotide agonists from the cell surface, contributing an important mechanism for terminating P2Y-R signal transduction. Along with cell surface receptors, most cell types also express ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) and other enzymes that metabolize nucleotides or interconvert nucleotides. In addition to ectoNTPDases, this cadre of enzymes includes ecto-nucleotide pyrophosphatase phosphodiesterase (E-NPP), alkaline phosphatase, adenylate kinase, and nucleoside diphosphate kinase (Zimmermann, 2000; Robson et al., 2006) (Fig. 2). Although some ecto-enzymes degrade nucleosides in addition to nucleotides, other enzymes that metabolize only nucleosides also are expressed on the cell surface, and these include adenosine deaminase, purine nucleoside phosphorylase (PNP), and 5'-nucleotidase, which exists as a dimer and is anchored to the cell surface through a glycophosphatidylinositol (GPI)-linkage, hydrolyzing AMP $\rightarrow$ Ado + P_i.

NTPDases

Of the eight members of the NTPDase family, only NTPDase 1, 2, 3, and 8 are expressed at the cell surface, exhibiting an extracellular catalytic site and two transmembrane domains. These four enzymes catalyze nucleotides with a K_M in the micromolar range, and require divalent cations for their catalytic activity (Kukulski *et al.*, 2005).

NTPDase 1/ CD39 hydrolyzes both di- and triphosphate nucleotides to monophosphate nucleotides. Studies investigating the relationship between NTPDase1 and $P2Y_1$-R used an engineered fusion protein that expressed NTPDase1 with the $P2Y_1$-R as a single construct on CHO cells (Alvarado-Castillo et al., 2002). The expression of the NTPDase in tandem with the receptor caused a shift to the right in the ADP concentration effect curve, indicating that ectoenzymes can regulate concentrations of nucleotides with physiological consequences. In previous studies with the $P2Y_1$-R, receptor activation in the absence of added agonist was observed. Basal levels of activation of the $P2Y_1$-R was not observed with the fusion protein, suggesting cellular release of nucleotide was autocrinely activating receptor.

While NTPDase1 is expressed mainly in vascular smooth muscle and endothelial cells (Enjyoji *et al.*, 1999), it has also been detected in the promyeloid cell line HL-60 and increases expression with differentiation of the myeloid leukocyte (Clifford *et al.*, 1997). Studies of the NTPDase1/ $CD39^{-/-}$ knockout mouse first implicated this enzyme as an important component of platelet biology (Enjyoji et al., 1999), and more recently of other physiological processes as well. Although the knockout mice did not exhibit any gross defects in development or viability as compared with wild-type mice, a host of disorders have been reported. Bleeding times in the $NTPDase1^{-/-}$ mice were prolonged (Enjyoji et al., 1999), suggestive of a deficiency in platelet activation. This bleeding disorder phenotype was rescued following injection of knockout mice with the soluble potato-derived ATPDase apyrase. Likewise, platelets isolated from $NTPDase1^{-/-}$ mice were deficient in their ability to aggregate in response to ADP, however the $NTPDase1^{-/-}$ platelets could be activated by ADP plus serotonin, suggesting that a Gq-coupled, ADP-activated receptor was desensitized in

platelets lacking NTPDase1. Furthermore, when platelets were treated with apyrase, platelet activation in response to ADP was restored, indicating that the $P2Y_1$-R was desensitized with chronic agonist stimulation, and NTPDase was necessary for removal of agonist. Additional disorders reported for the NTPDase1$^{-/-}$ mice include impaired neutrophil chemotaxtis (Corriden et al., 2008), decreased insulin sensitivity (Enjyoji et al., 2008), and deficiencies in microglial migration (Farber et al., 2008), among others. The broad impact of NTPDase1 loss in mice indicated that nucleotide/nucleoside signaling has critical roles in many biological functions.

NTPDase2 is expressed on vascular smooth muscle cells as well as on stromal cells (Zimmermann, 1999). NTPDase 2 has a much higher affinity for nucleotide triphosphates over diphosphates (Fig. 2). Studies with NTPDase1 and NTPDase2 highlighted the effects of ectoenzyme isotype expression on the observed pharmacological activity of the $P2Y_1$-R (Alvarado-Castillo et al., 2005). When $P2Y_1$-R and NTPDase2 were co-expressed, the EC_{50} of ADP was similar to that of $P2Y_1$-R alone. However, when NTPDase1 was coexpressed with the receptor, the ADP curve shifted to the right, likely as a function of ADP hydrolysis reducing the available agonist. In addition to illustrating how ectoenzymes may regulate extracellular nucleotide concentrations and thus altering the potency of agonist at a P2Y-R, this work also proposed the notion that ectoenzyme expression may indirectly regulate receptor expression levels, for example, by inhibiting desensitization of P2Y-R when ectoenzymes hydrolyze constitutively-released nucleotides.

NTPDases 3 and 8 prefer nucleotide triphosphates to diphosphates (Fausther *et al.*, 2007). In contrast to the membrane-bound NTPDases (1, 2, 3, and 8), NTPDase5 and NTPDase6 are expressed in golgi of monocytes and cardiocytes, respectively, and were

observed to be secreted from the cell upon heterologous expression. Both enzymes hydrolyze UDP and GDP (Chadwick and Frischauf, 1998; Ivanenkov et al., 2003). NTPDase formation of homo-oligomers has been reported, although it is unclear how oligomerization may affect catalytic activity (Grinthal and Guidotti, 2002).

E-NPPs

The E-NPP family of enzymes bear a single transmembrane domain with an extracellular C-terminus. The catalytic domain lies in the C-terminal portion of the protein, a region that also harbors an EF-hand sequence likely responsible for binding divalent cations that are necessary for catalytic activity. While there exist seven E-NPP enzymes, only three isoenzymes in this family hydrolyze nucleotides and dinucleotides. E-NPPs generally recognize a broad array of substrates, including nucleic acids, NAD, lysophospholipids, and cAMP. Furthermore, they can hydrolyze pyrophosphate bonds of nucleotide sugars (Zimmermann, 2000). E-NPP family members are also called PC-1 (E-NPP1), PDNP, and autotoxin (E-NPP2). E-NPP1 can undergo proteolysis causing the C-terminal portion of the enzyme to become soluble, a state in which it retains catalytic activity (Belli *et al.*, 1993; Clair *et al.*, 1997). The knockout mouse lacking the E-NPP1 gene develops bone abnormalities, indicating that E-NPP1 functions in bone formation biology. E-NPP2 does not have a transmembrane domain, but is secreted from the cell. E-NPP2 knockout mice are embryonic lethal (Yegutkin, 2008), suggesting a critical role in developmental processes. K_M values for ENPPs at ATP are in the 50 μM range, similar to that of NTPDases (Zimmermann, 2000).

Phosphatases and Kinases

Other enzymes that are expressed on the cell surface also use nucleotides as substrates. Alkaline phosphatase hydrolyzes tri-, di-, and monophosphate adenine nucleotides through cleavage of the terminal phosphate, ultimately yielding nucleoside plus inorganic phosphate. Alkaline phosphatase also cleaves the phosphate group from other molecules, such as glucose-phosphate and pyrophosphate (Yegutkin, 2008). The K_M of alkaline phosphatase for nucleotides is in the millimolar range. Alkaline phosphatases can be soluble or plasma membrane-associated through a glycophosphatidylinositol (GPI)-linkage (Low and Saltiel, 1988; Zimmermann, 2000). Additionally, nucleoside diphosphokinase (NDPK) was identified as a transphosphorylating enzyme that uses ATP to phosphorylate UDP or GDP in a reversible reaction, and reciprocally, UTP as substrate will yield ATP + UDP (Harden et al., 1997; Lazarowski et al., 1997b; Grobben et al., 1999; Lazarowski et al., 2000). The gamma phosphate of the nucleotide triphosphate is cleaved, and subsequently used to phosphorylate a nucleotide diphosphate. Another kinase, adenylyl kinase, converts ATP + AMP into 2 ADP molecules (Yegutkin et al., 2002; Picher and Boucher, 2003).

Nucleotide concentrations on the extracellular space are a dynamic function of cellular release and nucleotide metabolism, which includes degradation, phosphorylation, and interconversion. One could imagine that the differential signaling effects regulated by nucleosides and nucleotides are dependent on the nucleotidase isotype expressed on the cell surface in addition to the P2Y-R expression. For example, distinct signal transduction pathways are activated by ATP versus ADP versus adenosine, and perhaps the relative ratios of nucleotides on the cells are just as critical to physiological homeostasis as is which P2Y-R may be expressed on a particular tissue type.

Purinergic Receptors

Purinergic signaling encompasses responses initiated by nucleosides and nucleotides. P1 receptors define the family of G protein coupled receptors that are activated by adenosine, and include the subtypes A_1, A_{2A}, A_{2B}, and A_3. P2 receptors are a class of nucleotide-activated cell surface receptors and are categorized into two families of receptors: P2X receptors and P2Y receptors.

P2X receptors are ligand-gated ion channels, and all are activated by ATP. The seven receptors in the P2X family are: $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$. P2X receptors are comprised of three subunits and can exist as homomeric or heteromeric receptor assemblies. When ATP binds, the channel opens, allowing cations to pass through to the intracellular space. $P2X_{1\text{-}5,7}$ are permeable to Na^+, K^+, and Ca^{2+} ions. $P2X_6$ cannot form a homomeric receptor, but can form a functional heteromeric receptor with other P2X subunits (Ralevic and Burnstock, 1998; Abbracchio et al., 2006).

P2Y receptors are metabotropic receptors that couple to heterotrimeric G-proteins. There are eight identified P2Y receptors: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$. The nomenclature assigned includes numbers up to fourteen, reflecting a repeated problem in the field of P2Y receptor research. The missing P2Y receptor numbers are due to receptors that were mistakenly included in this family of receptors. The receptors that were erroneously assigned P2Y numbers have since been re-classified as either non-mammalian P2Y orthologs, or belonging to other receptor families, including leukotriene receptors and lysophosphatidic acid (LPA) receptors (Yokomizo et al., 1997; Li et al., 1998; Noguchi et al., 2003).

G Protein Signal Transduction

P2Y receptors, like other G protein-coupled receptors, couple to heterotrimeric G proteins comprised of α, β, and γ subunits. When agonist binds the cell-surface receptor, conformational changes in the receptor coordinate subsequent conformational rearrangements in the G protein. Guanosine 5'-triphosphate (GTP) then replaces guanosine 5'-diphosphate (GDP) on the Gα subunit and the result is disengagement of the βγ dimer from the α subunit. The GTP-bound Gα is the activated form, and so initiates downstream signaling events. The Gβγ dimer also activates downstream signaling targets such as phospholipases, adenylyl cyclases, phosphatidylinositol-3-kinase γ, and ion channels. Additional proteins and pathways may also be regulated by Gβγ-dependent signaling, as evidenced by the number of Gβγ-binding proteins that have been identified (Smrcka 2008). G protein-induced signaling ceases when the intrinsic GTPase activity of the Gα subunit causes hydrolysis of GTP to GDP, and the heterotrimeric G protein reassembles.

The main signaling pathways activated by GPCRs are represented by at least four families of heterotrimeric G proteins: Gq, Gs, Gi, and G12. The Gq family of G proteins includes $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{15}$, and $G\alpha_{16}$, and these subunits activate phospholipase C-β, which hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) into inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts on receptors at the endoplasmic reticulum to release from its intracellular calcium stores. DAG activates protein kinase C (PKC). Gq also has been shown to bind to the regulator of G protein signaling (RGS) domain of G protein receptor kinase-2 (GRK2) (Carman et al., 1999), and evidence shows that GRK2 is an important hub of signaling for the Gq signal transduction pathway, as it binds a number of elements in the cascade (Tesmer et al., 2005). Additionally, guanine nucleotide exchange

factors (GEFs) for Rho family proteins interact with Gq. Rho family proteins are small G protein GTPases that control myriad cellular functions such as contraction, motility, and proliferation. At least three Rho GEFs, p63-RhoGEF, Trio, and PDZ-RhoGEF, are known to interact with GTP-bound Gq and lead to RhoA activation (Lutz et al., 2005; Rojas et al., 2007), establishing a link between heterotrimeric G protein signaling and Rho signaling pathways.

The Gs family includes $G\alpha_s$ and the related olfactory system expressed $G\alpha_{olf}$. Gs family proteins activate adenylyl cyclase, which generates cyclic adenosine 5'-monophosphate (cAMP) from ATP. Protein kinase A (PKA) is downstream of adenylyl cyclase and is activated by intracellular cAMP. Conversely, Gi proteins inhibit some isoforms of adenylyl cyclase (I, III, V, VI, and VIII) and also regulate ion channels (Gilman, 1987; Birnbaumer, 1992; Clapham and Neer, 1997), which appears to be through released $G\beta\gamma$ subunits. The Gi family is comprised of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_t$, $G\alpha_z$, and $G\alpha_{gust}$. Tissue-specific expression was established for $G\alpha_t$ in the retina, $G\alpha_{gust}$ in the gustatory system, and $G\alpha_z$ in platelets and brain.

G12 family proteins ($G\alpha_{12}$, $G\alpha_{13}$) are ubiquitously expressed. G12 proteins activate guanine nucleotide exchange factors (GEFs) for RhoA family members such as PDZ-RhoGEF, p115RhoGEF, and leukemia-associated RhoGEF (LARG) (Kozasa et al., 1998; Suzuki et al., 2003). G12 G proteins may also interact with other signaling molecules such as cadherins, protein phosphatases, and non-receptor tyrosine kinases to modulate other signaling pathways. Studies of mouse models suggest that the functions of $G\alpha_{12}$ and $G\alpha_{13}$ are distinct. $G\alpha_{13}^{-/-}$ mice do not survive more than a few days, while $G\alpha_{12}^{-/-}$ mice survive into

adulthood, suggesting differential roles for G12 proteins in developmental processes (Offermanns et al., 1997).

Termination of agonist-promoted GPCR signaling occurs when G protein receptor kinases (GRK) phosphorylate residues on intracellular domains of the receptor. This acts as a signal for β-arrestin recruitment to the plasma membrane, where it uncouples GPCR from G proteins. Arrestins also mediate internalization of some GPCRs, acting as a scaffold for the receptor with endocytosis machinery. Endocytosed receptors may be either recycled to the plasma membrane, or degraded via the lysosome (Krupnick and Benovic, 1998).

P2Y Receptors

P2Y receptors are grouped into two categories based on amino acid sequence homology and proposed G-protein coupling selectivity (Fig. 3). The $P2Y_1$-R subgroup includes $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$, all of which couple to $G\alpha_q$ and lead to activation of PLC. The $P2Y_{11}$ receptor also couples to $G\alpha_s$ to increase intracellular cAMP. The $P2Y_{12}$-R subgroup is comprised of $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$, all of which are proposed to couple to $G\alpha_i$ to inhibit formation of cAMP (Abbracchio *et al.*, 2006). GPR87, an orphan receptor, exhibits high amino acid sequence homology to the $P2Y_{14}$-R, (Schoneberg *et al.*, 2007), suggesting that GPR87 likely shares a common ancestor with the $P2Y_{12}$-like P2Y-R. However, GPR87 was recently reported to be activated by lysophosphatidic acid (LPA) (Tabata et al., 2007).

The $P2Y_1$ receptor is activated by ADP. The $P2Y_2$ receptor is activated by UTP and ATP, while the human $P2Y_4$ receptor is activated only by UTP. The only receptor known to be activated by UDP is the $P2Y_6$ receptor. The $P2Y_{11}$ receptor is activated by ATP. In the

$P2Y_{12}$-R subgroup, both $P2Y_{12}$ and $P2Y_{13}$ receptors are activated by ADP, and the $P2Y_{14}$ receptor is activated by UDP-glucose and other nucleotide-sugars.

While the lack of selective, high affinity antagonists has impeded research on P2Y receptors, several labs have ongoing studies to identify competitive antagonists at each of the P2Y receptors, and progress has been greatest for the $P2Y_1$-R and $P2Y_{12}$-R. In fact, a prodrug, clopidogrel, that metabolizes into a $P2Y_{12}$-R antagonist, is used therapeutically to prevent thrombosis (Foster et al., 2001; Hollopeter et al., 2001). Antagonists for the $P2Y_2$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{13}$ receptors have been described and can be used as templates for the development of high affinity compounds that will be useful for pharmacological studies of P2Y receptors (Jacobson *et al.*, 2008). Although ATP is an antagonist at the human $P2Y_4$-R (Herold *et al.*, 2004), no selective antagonists have been identified for the $P2Y_4$ and $P2Y_{14}$ receptors. Several non-selective acting antagonists for P2Y receptors have been identified. These include PPADS, suramin, and reactive blue 2 (Ralevic and Burnstock, 1998; Brown and Brown, 2002).

$P2Y_1$-like Receptors

$P2Y_1$-R

The $P2Y_1$ and $P2Y_{12}$ receptors have a well established function in platelet biology (Dubyak et al., 1993; Savi et al., 1998; Hechler et al., 1998a; Leon et al., 1999; Fabre et al., 1999). The role of ADP in platelet aggregation involves both the Gq-activating $P2Y_1$-R as well as the Gi-activating $P2Y_{12}$-R. Knockout mice for $P2Y_1$-R exhibited increased bleeding time after tail amputation as compared with wild-type mice. Platelets from these mice lost the ability to undergo shape change in response to ADP. However, platelets treated with ADP retained the capacity to inhibit adenylyl cyclase (Fabre et al., 1999). Although the

$P2Y_1$-R is one element of the platelet clotting response to injury, the $P2Y_{12}$-R governs the aggregation component of platelet activation only after Gq-dependent shape change has occurred (Enjyoji et al., 1999; Bourdon et al., 2006). Pharmacological studies using $P2Y_1$-R-selective ligands in human platelets revealed that the $P2Y_1$-R undergoes rapid desensitization upon activation (Bourdon et al., 2006).

$P2Y_2$-R

The $P2Y_2$-R has been reported to have an important role in ion secretion and absorption in airway epithelial cells. Upon activation of $P2Y_2$-R by UTP or ATP, Cl^- secretion increases and Na^+ absorption is inhibited. Improvement of mucociliary clearance in cystic fibrosis patients was demonstrated after treatment with inhaled $P2Y_2$ receptor agonists (Kellerman et al., 2002). In the $P2Y_2$-R knockout mouse, the effect of UTP on inositol phosphate levels and calcium mobilization in tracheal and nasal epithelial cells was abrogated. Likewise, UTP- and ATP-dependent Cl^- transport was disrupted in trachea from the $P2Y_2$-$R^{-/-}$ mouse (Cressman et al., 1999; Homolya et al., 1999). Additionally, multiple studies have identified $P2Y_2$-R mRNA in immune cells, where ATP and UTP effects have been reported (Chen et al., 2006; Myrtek and Idzko, 2007). Moreover, the $P2Y_2$-R is implicated in atherosclerosis (Seye et al., 2002), suggesting that $P2Y_2$-R may have a role in inflammation processes. In rats overexpressing the $P2Y_2$-R, lesions were observed on their lacrimal glands, which regulate fluid secretion to the eyes, and also on the kidney by 3 months of age (Agca et al, 2008), supporting the notion that $P2Y_2$-R also regulates fluid secretion in the eye and kidney.

$P2Y_4$-R

The $P2Y_4$-R is activated by UTP. ATP acts as an antagonist at the human $P2Y_4$-R, and it was determined also to be an agonist at the rat ortholog receptor (Kennedy et al., 2000). In humans, $P2Y_4$-R are expressed in brain, intestine, lung, liver, and placenta (Communi et al., 1995). Chloride secretion in the epithelium of the small and large intestines is dependent on $P2Y_4$-R activation, as determined by studies with $P2Y_4$-R knockout mice (Robaye et al., 2003; Ghanem et al., 2005). Just as the $P2Y_2$-R is important for chloride secretion in lung, the $P2Y_4$-R is likely the primary P2Y-R involved in chloride secretion in intestine.

$P2Y_6$-R

UDP-activated $P2Y_6$-R are expressed throughout the body, specifically in heart, lung, spleen, and intestine, as well as in monocyte-derived immune cells (Communi et al., 1996). Recently, cooperative signal transduction of the $P2Y_6$-R with the cysteinyl leukotriene receptor $CysLT_1R$ was reported in human mast cells, such that when one receptor was inhibited via shRNA technology or with the use of a selective antagonist, the other receptor also lost its function (Jiang et al., 2009). Although the relevance of such an effect is unknown, the overlapping receptor expression distribution and the possibility that the two signal transduction pathways are linked suggest that the $P2Y_6$-R may have an important role in innate immune responses. While the physiological function of $P2Y_6$-R is unknown, reports of UDP-dependent phagocytosis in rat microglia and upregulation of $P2Y_6$-R mRNA 72 h after neuronal damage suggest an immunoprotective role for $P2Y_6$-R (Koizumi et al., 2007). A recently developed knockout mouse for the $P2Y_6$-R strongly supports the role of $P2Y_6$-R in immune responses (Bar et al., 2008). While no obvious abnormalities were

observed on the $P2Y_6$-$R^{-/-}$ mouse, macrophages isolated from the mouse lacked UDP-promoted signal transduction and cytokine production. Additional studies with the $P2Y_6$-R knockout mouse suggested that $P2Y_6$-R may regulate vasoconstriction in aorta in response to UDP treatment. The $P2Y_6$-R was recently reported to be involved in the induction of cardiac fibrosis through $G_{12/13}$ signaling to Rho in mice (Nishida et al., 2008), prompting further investigation of the $P2Y_6$-R as a potential therapeutic target for heart disease.

$P2Y_{11}$-R

$P2Y_{11}$-R mRNA has been detected in placenta, brain, and lymphocytes (Communi et al., 1997; Moore et al., 2001). Unique among the P2Y receptors, the $P2Y_{11}$-R gene has several introns (Communi et al., 2001b). Another distinctive characteristic of the $P2Y_{11}$-R is that it couples to both Gq to activate PLC as well as to Gs to activate adenylyl cyclase (Qi et al., 2001). Additionally, species-specific pharmacological differences have been identified for the $P2Y_{11}$-R. While ADP may be a weak partial agonist at the human $P2Y_{11}$-R, ADP acts as a potent full agonist at the canine $P2Y_{11}$-R (Qi et al., 2001). ATP was reported to induce differentiation of HL-60 cells into neutrophil-like cells, and conventional inducers of differentiation such as DMSO and dibutyryl-cAMP increased the mRNA expression of $P2Y_{11}$-R. The $P2Y_{11}$-R has been implicated for involvement in maturation and migration of dendritic cells (Wilkin et al., 2001; Marteau et al., 2004; Idzko et al., 2007). Taken together, it appears that the $P2Y_{11}$-R may be involved in hematopoiesis.

$P2Y_{12}$-like receptors

The three P2Y receptors in the $P2Y_{12}$-R subgroup share amino acid sequence homology of approximately 44%. The $P2Y_{12}$-like receptor genes are clustered on chromosome 3, at 3q24-25 (Nomura *et al.*, 1994). Evolution of the $P2Y_{12}$-R family has been

fairly conserved across species. The $P2Y_{12}$-like receptors are found in almost all vertebrate classes, and have not been identified in non-vertebrates (Schoneberg *et al.*, 2007). In some fishes and amphibians, orthologous receptors share close homology to two receptors from the $P2Y_{12}$-like subgroup. For example, one ortholog is related to $P2Y_{12/13}$ and another is related to $P2Y_{14}$/GPR87, suggesting a point of evolutionary distinction for these receptors.

$P2Y_{12}$-R

The $P2Y_{12}$-R is the most investigated of the subgroup due to its clinical relevance in anti-blood clotting therapeutics. Evidence for a critical physiological role of the $P2Y_{12}$-R in aggregation of platelets is well-accepted. Knockout mice lacking the $P2Y_{12}$-R exhibited prolonged bleeding times (Foster et al, 2001). Platelets from $P2Y_{12}$-$R^{-/-}$ mice responded to ADP with shape change as did the platelets from wild-type mice. However, platelets lacking $P2Y_{12}$-R did not aggregate and further analysis revealed they did not inhibit adenylyl cyclase in response to ADP. Studies with the knockout mouse underscore the role of the $P2Y_{12}$-R as a critical component in platelet biology. Antithrombotic therapeutics successfully interrupt platelet aggregation in response to P2Y-R signaling, as evidenced by the widely-prescribed drug clopidogrel, an active metabolite of which acts as an irrevesible $P2Y_{12}$-R antagonist (Quinn and Fitzgerald, 1999; Gachet, 2005; Savi and Herbert, 2005).

In addition to platelets, $P2Y_{12}$-R are expressed throughout brain and in smooth muscle cells (Burnstock and Knight, 2004). The importance of the $P2Y_{12}$-R in microglial response to tissue injury was highlighted by a study comparing wild-type mice to $P2Y_{12}$-$R^{-/-}$ mice (Haynes et al., 2006). The $P2Y_{12}$-R was detected at the protein and RNA levels expressed on microglia. Wild-type microglial cultures, but not those from $P2Y_{12}$-$R^{-/-}$ mice, responded to ADP with lamelipodial extensions. Furthermore, chemotaxis of $P2Y_{12}$-R-

expressing cells toward an ADP gradient was demonstrated with microglial cultures, and process extension and migration of microglia were observed in response to laser-induced tissue damage in living mice, but were significantly delayed in $P2Y_{12}$-$R^{-/-}$ mice. This study and others define the $P2Y_{12}$-R as a crucial element in microglial development and response to injury (Davalos et al., 2005).

$P2Y_{13}$-R

The $P2Y_{13}$-R is similar to the $P2Y_{12}$-R in amino acid sequence, in G protein coupling, and in agonist profile. The $P2Y_{13}$-R also couples to ERK1/2 activation in CHO-K1 cells. In contrast to the $P2Y_{12}$-R, expression of the $P2Y_{13}$-R is reported in spleen, small intestine, liver, kidney, brain, and on peripheral immune cells (Communi et al., 2001a; Fumagalli et al., 2004; Wang et al., 2004), but the physiological significance of the $P2Y_{13}$-R has not yet been examined. An analogue of PPADS, MRS2211, recently has been described as a selective, competitive antagonist at the $P2Y_{13}$-R (Wirkner et al., 2004; Kim et al., 2005).

$P2Y_{14}$-R

The $P2Y_{14}$-R is the eighth member of the P2Y receptor family. It shares 44% amino acid sequence homology to the $P2Y_{12}$-R and $P2Y_{13}$-R, compared to only 22% with the $P2Y_1$-R; it is accordingly grouped into the $P2Y_{12}$-like receptor sub-family. The intronless $P2Y_{14}$-R gene encodes 338 amino acids (Fig. 5). While the $P2Y_{14}$-R was first cloned from the human myeloid cell line KG-1 (Nomura *et al.*, 1994) and relatively high levels of $P2Y_{14}$-R mRNA have been detected in neutrophils, expression of the $P2Y_{14}$-R has also been reported in the brain, lung, stomach, heart, placenta, and adipose tissue (Moore *et al.*, 2003). Although a functional role for the $P2Y_{14}$-R has not yet been identified, evidence suggests that one function of the receptor may be contributing to immune system homeostasis.

$P2Y_{14}$-R Tissue Distribution

There have been several antibodies generated to recognize the $P2Y_{14}$-R, although none has been fully characterized. For example, a commercially available antibody is reported to recognize the second extracellular loop of the receptor, a region that is conserved in mouse and rat sequences (Alomone, Jerusalem, Israel). This antibody was used to detect expression of glycosylated $P2Y_{14}$-R in glioma C6 cells (Krzeminski *et al.*, 2008), and the authors reported that non-glycosylated receptor became predominantly expressed upon serum starvation of the cells. However, results discussed in Chapter 4 of this dissertation contradicts the expression of $P2Y_{14}$-R in C6 glioma cells. Another $P2Y_{14}$-R antibody was generated against the first extracellular loop of the receptor (Lee *et al.*, 2003) and was used to detect expression of the $P2Y_{14}$-R on human fetal bone marrow cells. Additionally, an antibody against the C-terminus of the $P2Y_{14}$-R was generated for immunohistochemical studies in which $P2Y_{14}$-R expression was reported throughout the brain, but only in sub-populations of glial cells (Moore *et al.*, 2003). The specificity of these antibodies is unknown because results for recognition of other P2Y-R or of non-specific immunoreactivity in each experimental system were not reported. Until the usefulness of these antibodies has been validated, we still lack reliable means for detecting $P2Y_{14}$-R directly.

In their report of glial $P2Y_{14}$-R expression, Moore and colleagues also used RT-PCR to demonstrate the presence of $P2Y_{14}$-R transcripts in neutrophils, lymphocytes, and also in the leukocyte cell lines M-07e and UT7-Epo, as well as in HEK293 cells (Moore *et al.*, 2003). The $P2Y_{14}$-R message has been detected in platelets (Moore et al., 2003; Dovlatova et al., 2008). However, the receptor has not been shown to be functional in such cells. Other reports of tissue distribution based on RT-PCR studies include highest levels in placental and

adipose tissue, and also in stomach, intestine, lung, and heart (Chambers *et al.*, 2000). Reports of $P2Y_{14}$-R mRNA upregulation in mouse uterus after estradiol treatment for seven days (Crabtree et al., 2006; Crabtree et al., 2008) suggest that $P2Y_{14}$-R expression may be regulated by circulating hormone levels.

Other reports of $P2Y_{14}$-R expression include microglia and astrocytes (Charlton *et al.*, 1997), non-neuronal spinal cord cells (Kobayashi *et al.*, 2006), and synoviocytes from human patients suffering from rheumatoid arthritis (Caporali *et al.*, 2008), which together may indicate a neuroprotective role for the $P2Y_{14}$-R.

Regulation of the $P2Y_{14}$-R

Expression and function of GPCRs are dynamically regulated through several mechanisms, from post-translational modifications, cellular localization and cell-stage-dependent expression to signaling events and internalization regulated by motifs and domains harbored within the receptor's sequence. Although little is known about the regulation of the $P2Y_{14}$-R, we can surmise that it has many potential mechanisms for regulation based on what is known of other GPCRs and shared sequence characteristics of the $P2Y_{14}$-R.

Like other GPCRs, the $P2Y_{14}$-R exhibits several distinctive amino acid sequence characteristics. At the intracellular region of transmembrane 3, the sequence includes the residues aspartate-arginine-tyrosine. This "DRY motif" is highly conserved among seven transmembrane GPCRs. In other GPCRs, these residues are suspected to interact with acidic residues in the third intracellular loop and such interactions are thought to regulate receptor activation (Ballesteros *et al.*, 2001). However, the $P2Y_{14}$-R does not have any acidic residues in the third intracellular loop, and 30% of seven transmembrane GPCRs, including all of the known chemokine receptors, do not exhibit an acidic residue at the expected point of

interaction. In fact, the $P2Y_{14}$-R sequence in the third intracellular loop is rich in positively charged residues, so the significance of the DRY motif in this receptor may be different from other GPCRs. Additionally, GPCRs are known to exhibit two conserved cysteine residues in the extracellular domains that form disulfide bonds for stabilizing the receptor's tertiary structure. Indeed, the $P2Y_{14}$-R has a cysteine residue in each of the four extracellular domains. The $P2Y_{14}$-R also exhibits potential consensus sequences for phosphorylation by protein kinase A (PKA) and protein kinase C (PKC) in its third intracellular loop, but there is no evidence available that either of these protein kinases interact with this receptor.

Similar to all other P2Y-R, potential N-glycosylation sites occur on extracellular domains of the $P2Y_{14}$-R. Glycosylation of the receptor was reported to modulate its function (Krzeminski *et al.*, 2008). Indeed, the glycosylation state of the $P2Y_{12}$-R has also been reported to bear functional consequences (Zhong *et al.*, 2004). While glycosylation of GPCRs is thought to be important for insertion into the lipid bilayer, it is possible that the glycosylation state has an impact on receptor expression or otherwise promotes a conformational orientation of the receptor that compromises ligand binding or G-protein coupling or activation. However, it remains to be determined whether glycosylation is an important mechanism for regulating the $P2Y_{14}$-R.

Cellular localization of the receptor may also be an important means for regulating receptor function. Confocal microscopy studies of HA-epitope tagged $P2Y_{14}$-R suggest that the receptor localizes to the basolateral membrane of polarized epithelial cells (Wolff *et al.*, 2005). In light of the reported $P2Y_{14}$-R expression in lung and and in immune cells, it is possible that the $P2Y_{14}$-R has a specific function related to its basolateral localization in cells. The $P2Y_2$-R, for example, exhibits apical localization in lung epithelial cell lines (Qi *et al.*,

2005; Wolff *et al.*, 2005), and has been demonstrated to be important in mucociliary clearance (Cressman *et al.*, 1999; Donaldson *et al.*, 2000).

Desensitization is an important mechanism for GPCRs to terminate receptor signaling in the presence of chronic agonist exposure. Because it has been demonstrated that UDP-Glc is released from cells constitutively as well as after mechanical and pharmacological stimulation, it is likely that UDP-Glc is constantly present in the extracellular space. Therefore, the question arises whether the $P2Y_{14}$-R desensitizes and how this may impact UDP-Glc-dependent signal transduction. Other P2Y-R are regulated by desensitization, and exhibit various rates of desensitization upon agonist stimulation. The $P2Y_6$-R, for example is reported to desensitize after prolonged agonist stimulation (Brinson and Harden, 2001), while the $P2Y_1$-R and $P2Y_{12}$-R desensitize quickly in platelets after agonist activation leads to shape change and aggregation (Hardy et al., 2005; Bourdon et al., 2006). Future studies will determine whether desensitization plays a role in $P2Y_{14}$-R-dependent processes.

As we gain insight into the functional role of the $P2Y_{14}$-R, we will undoubtedly learn more about how this receptor is regulated. Myriad mechanisms have been demonstrated for regulation of other P2Y receptor functions, and the significance of amino acid sequence signals, post-translational modifications and desensitization on $P2Y_{14}$-R function will await further investigation.

Pharmacology and Signal Transduction

UDP-Glc has long been known as a compound that is concentrated in the ER/Golgi apparatus and is a component of the protein synthesis quality control machinery (Parodi, 2000). With the discovery that the $P2Y_{14}$-R is activated by UDP-Glc and three other UDP-sugars (Fig. 5), the notion of nucleotide-sugars as extracellular signaling molecules was

introduced. The rank order of potency of $P2Y_{14}$-R agonists has been reported as follows: UDP-glucose ≥ UDP-galactose ≥ UDP-glucuronic acid > UDP-N-acetylglucosamine (Chambers *et al.*, 2000). While UDP-Glc is considered the endogenous agonist, the other UDP-sugars have been reported to have varying degrees of efficacy at the $P2Y_{14}$ receptor. There have not been any reports of antagonists for this receptor.

UDP-Glc promoted GTPγS binding on HEK293 membranes expressing recombinant $P2Y_{14}$-R. Additionally, UDP-Glc promoted calcium mobilization when the $P2Y_{14}$-R was co-expressed with the promiscuous Gα16 (Chambers *et al.*, 2000). The $P2Y_{14}$-R also couples to the chimeric $G\alpha_{q/i}$ (Lazarowski *et al.*, 2003; Moore *et al.*, 2003). This chimeric G protein is a Gq that has been engineered to couple Gi-coupled receptors to PLC (Coward et al., 1999). The $P2Y_{14}$-R is expected to couple to $G\alpha_i$ leading to inhibition of adenylyl cyclase, but this has yet to be shown.

In some experimental systems, investigators have observed UDP-Glc-dependent Ca^{2+} mobilization, indicating that the $P2Y_{14}$-R may couple to PLCβ isoforms, likely through release of Gβγ upon activation of Gi. UDP-glucose- and UDP-galactose-dependent increases in Ca^{2+} in rat cortical astrocytes were reported (Fumagalli *et al.*, 2003). UDP-Glc-dependent increases in Ca^{2+} were also observed in human immature dendritic cells (Skelton *et al.*, 2003).

Physiological Significance

While the physiological role of the $P2Y_{14}$-R remains unclear, the reportedly high expression levels in leukocytes in conjunction with several reports of UDP-Glc-dependent activity in immune response assays suggests that there may be a potential role for the $P2Y_{14}$-R in immune system homeostasis. $P2Y_{14}$-R mRNA was demonstrated to be upregulated in

rat brain after immunologic challenge with lipopolysaccharide (Moore *et al.*, 2003), suggesting that the $P2Y_{14}$-R may be involved in immune responses to bacterial insult, as LPS treatment has been shown to modulate release of cytokines and upregulate expression of chemokine receptors (Palin et al., 2001; Banisadr et al., 2002). $P2Y_{14}$-R mRNA has also been detected in immature human dendritic cells, which are immune cells that mature and migrate to a site of inflammation in response to insult or injury (Skelton *et al.*, 2003). UDP-Glc affected a calcium response in immature dendritic cells, suggesting that the $P2Y_{14}$-R may have a role in induction of maturation of dendritic cells in response to high concentrations of ligand released from injured tissues. Furthermore, Scrivens and Dickenson (2005, 2006) reported $P2Y_{14}$-R expression in T-lymphocytes from mice and in human neutrophils, and reported UDP-Glc-, but not other UDP-sugar-dependent inhibition of adenylyl cyclase stimulation in both cell types (Scrivens and Dickenson, 2005; Scrivens and Dickenson, 2006). In T-lymphocytes, the authors reported that all four UDP-sugar agonists partially inhibited IL-2- and anti-CD3-induced cell proliferation, suggesting that perhaps the $P2Y_{14}$-R is involved in T-lymphocyte biology. The authors observed no $P2Y_{14}$-R-dependent effect on elastase release, which is an assay for neutrophil degranulation. While it seems likely there will be a role for the $P2Y_{14}$-R in immune response physiology, it is too early to speculate on details related to which cell processes may be modulated by $P2Y_{14}$-R function.

Aims of this dissertation

The work presented in the following pages addresses specific aims of research regarding molecular pharmacology and signal transduction of the $P2Y_{14}$-R. In Chapter 2, studies to develop novel ligands for the $P2Y_{14}$-R will be discussed. Rhodopsin-based homology modeling was used to simulate theoretical interactions of known $P2Y_{14}$-R agonists

with the receptor. Analyses revealed the putative involvement of several residues interacting with the hexose moiety, suggesting that the glucose structure may be an important part of the molecule for receptor activation. For these studies, a cellular assay system was developed that employs the chimera $G\alpha_{q/i}$ and over-expressed $P2Y_{14}$-R in COS-7 cells. Novel, selective ligands for the $P2Y_{14}$-R have been developed based on structure-activity relationships, and we are continuing in this endeavor to identify high affinity agonists and antagonists that can be used as templates to propel the development of pharmacological research tools for the $P2Y_{14}$-R. A specifically acting ligand will augment the currently available methods for studying the $P2Y_{14}$-R and distinguishing its individual physiological role relative to other P2Y receptors.

In Chapter 3, the identification of a competitive antagonist for the $P2Y_{14}$-R in a heterologous system is reported. Few selective antagonists are available for any of the P2Y receptors, causing difficulties in conclusive establishment of a physiological role for individual receptor subtypes. The lack of selective antagonists for the $P2Y_{14}$-R has impeded investigation into the signaling properties and tissue distribution of this receptor. The identification of UDP as an antagonist at the $P2Y_{14}$-R contributes to our knowledge of $P2Y_{14}$-R pharmacology and will further our work toward developing a high affinity, non-hydrolyzable competitive antagonist for the receptor.

An additional element of Chapter 3 includes an investigation into the molecular pharmacology of the rat ortholog of the $P2Y_{14}$-R, and a comparison of known ligands to determine whether the rat $P2Y_{14}$-R functions similarly to the human receptor. Precedence for divergent pharmacological activation profiles among species orthologs has been reported for the $P2Y_4$-R and for the $P2Y_{11}$-R. The studies presented in Chapter 3 reveal differences in the

pharmacological profile between the human and rat $P2Y_{14}$-R and will be necessary criteria for assessing studies of the $P2Y_{14}$-R in murine model systems.

Chapter 4 details the findings that the $P2Y_{14}$-R couples to inhibition of adenylyl cyclase and activates ERK1/2. One goal of these studies was to generate stable cell lines for examining $P2Y_{14}$-R signal transduction through its natively coupling G protein. Studies performed in HEK293 epithelial cells and in C6 rat glioma cells have established that the $P2Y_{14}$-R couples to the Gi family of G proteins. Furthermore, the identification of a functional $P2Y_{14}$-R endogenously expressed in differentiated HL-60 cells provides a system for examining this receptor at expression levels and with signal transduction pathways that are likely to be similar to its native physiological activity. While $P2Y_{14}$-R expression has been reported in various tissues and brain regions, we are still far from understanding the physiological role of this receptor, and have barely begun to uncover the signaling networks downstream of receptor activation. With no explicit pathophysiologies in mouse or man to hint at potential functions for this receptor, model cell systems will be critical for furthering our knowledge of signal transduction and biological responses from $P2Y_{14}$-R activation.

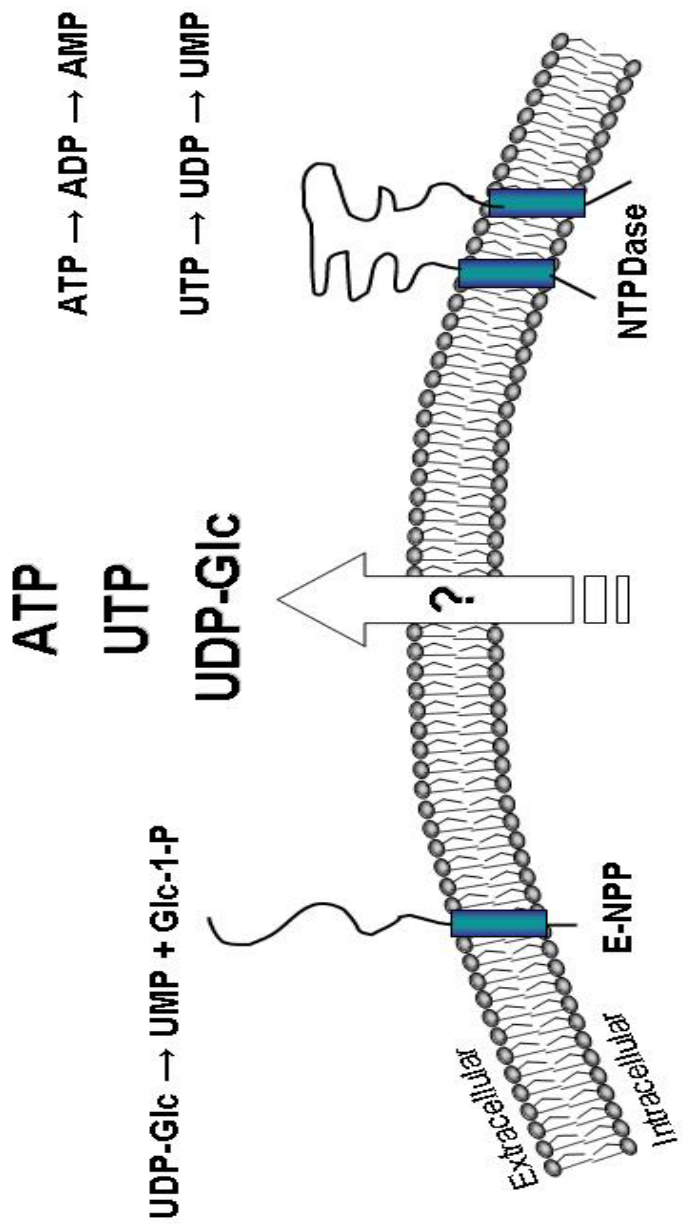
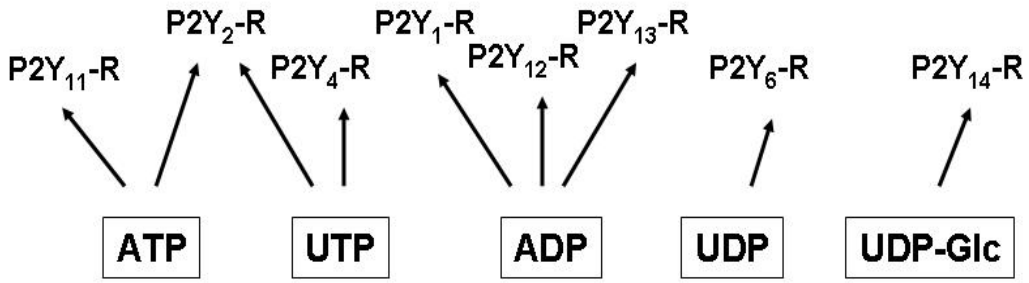


Figure 1. Nucleotides are released from cells and are metabolized by ectoenzymes. ATP, UTP, and UDP-Glc undergo regulated release from cells via an unknown mechanism. Extracellular nucleotides and nucleotide-sugars are metabolized by plasma membrane-anchored enzymes such as E-NPPs and NTPDases. Nucleotide triphosphates are degraded into nucleotide diphosphates, and subsequently into nucleotide monophosphates. UDP-Glc is metabolized by E-NPP family of ectoenzymes, resulting in UMP and glucose-1-P.



NTPDase1:	NTP → NMP + 2Pi	NDP → NMP + Pi	
NTPDase2:	NTP → NDP + Pi		
NTPDase3:	NTP → NMP + 2Pi	NDP → NMP + Pi	
NTPDase5:		NDP → NMP + Pi	
NTPDase8:	NTP → NDP + Pi	NDP → NMP + Pi	
E-NPPs (1-3):	NTP → NMP + PPi	NDP → NMP + Pi	UDP-Glc → UMP+G-1-P
Alkaline phosphatase:	ATP → ADP + Pi	ADP → AMP + Pi	
NDPK:	NDP + N_1TP ↔ NTP + N_1DP		
Adenylyl kinase:	ATP + AMP ↔ 2ADP		

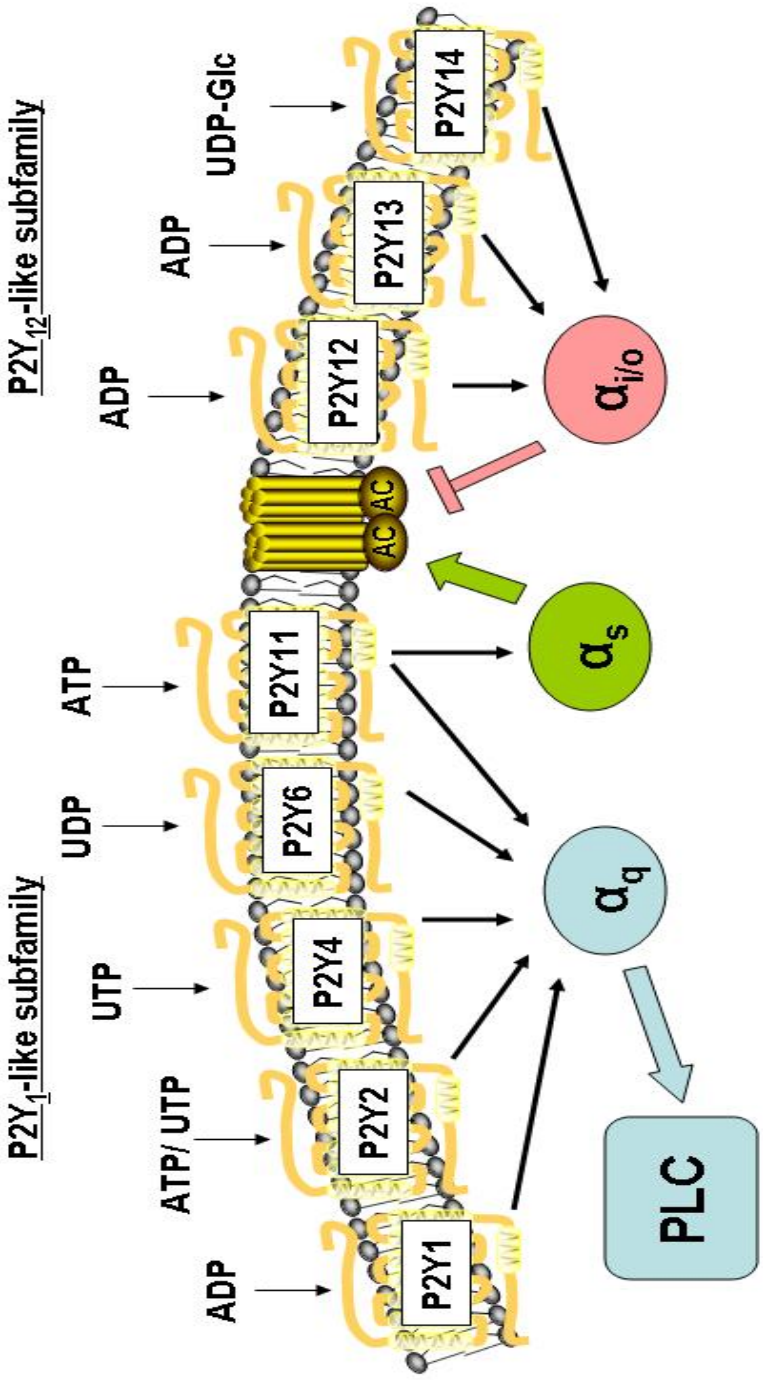


Figure 3. P2Y-R agonists and G protein coupling schematic. The metabotropic P2Y receptor family is comprised of eight members and is divided into two subfamilies, P2Y1-like and P2Y12-like, based on amino acid sequence homology and proposed G-protein coupling. $P2Y_1$-R, $P2Y_2$-R, $P2Y_4$-R, $P2Y_6$-R, and $P2Y_{11}$-R activate Gq, and $P2Y_{11}$-R also activates Gs. $P2Y_{12}$-R, $P2Y_{13}$-R, and $P2Y_{14}$-R activate Gi. P2Y-R are activated by nucleotides and nucleotide sugars.

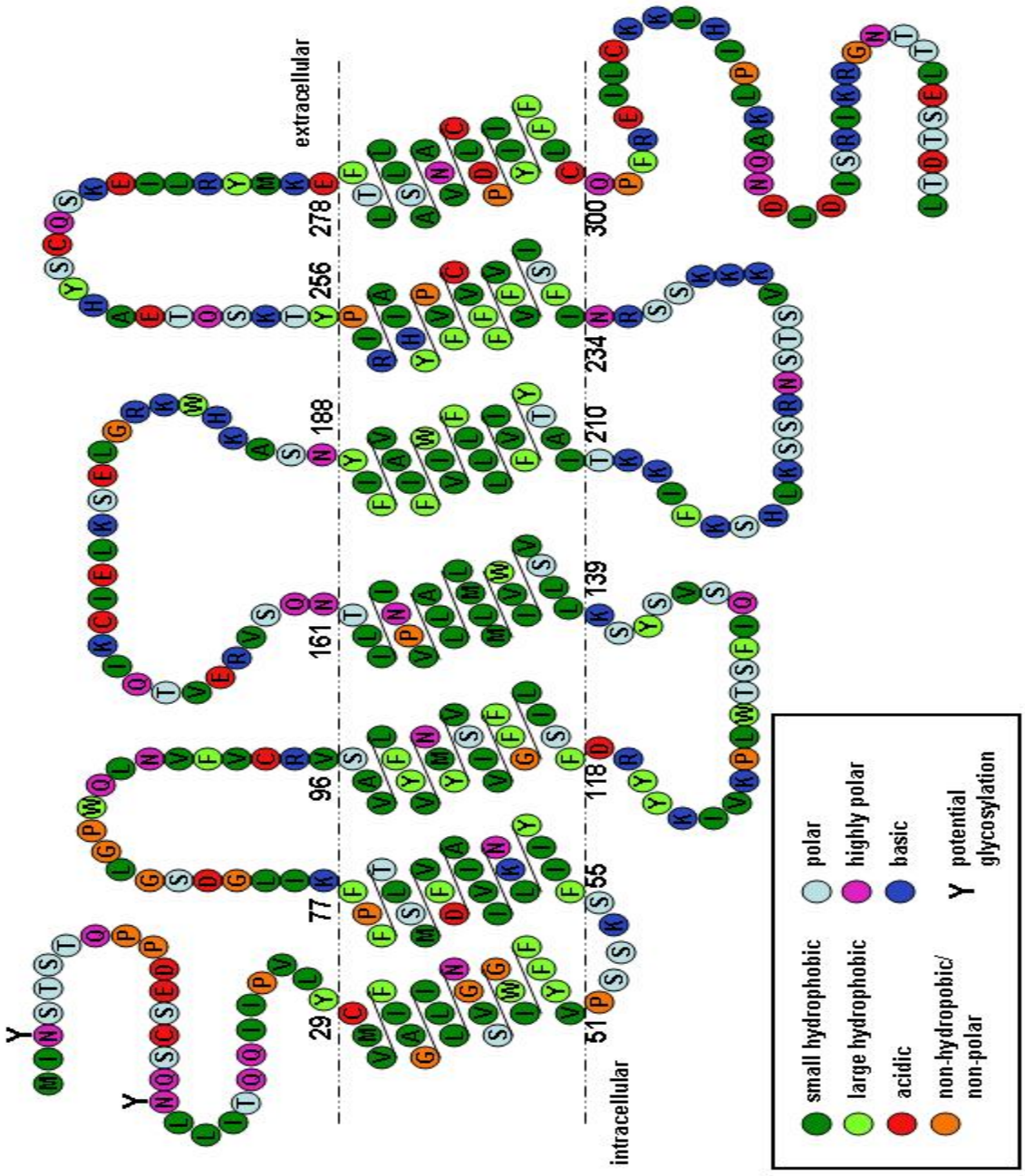


Figure 4. $P2Y_{14}$-R serpentine model. The $P2Y_{14}$-R has 338 amino acids with seven predicted transmembrane domains. The $P2Y_{14}$-R exhibits protein sequence features common to other P2Y-R, such as potential N-glycosylation sites at the amino-terminal region, four extracellular cysteine residues that are predicted to form 2 disulfide bridges, and aspartate118-arginine119-tyrosine120 form a "DRY motif" at the intracellular cusp of transmembrane 3.

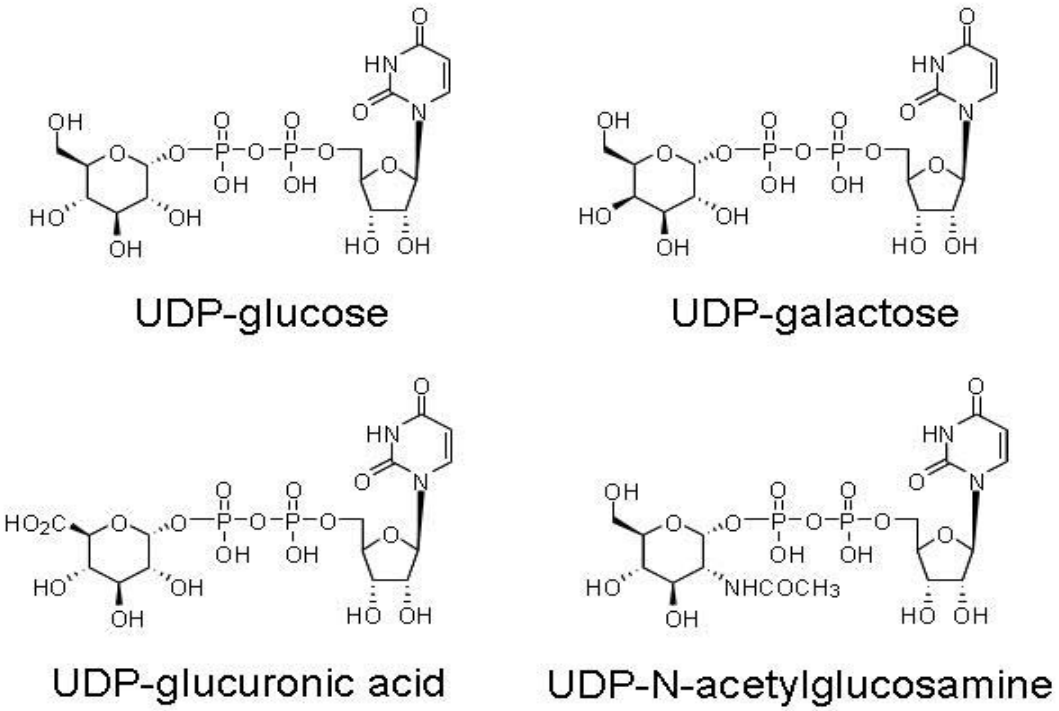


Figure 5. **The P2Y$_{14}$-R is activated by UDP-sugars.** Four agonists possessing similar chemical structures have been identified. Compared with UDP-glucose, UDP-galactose has an inverted chirality of the hydroxyl group at position 4 of the hexose ring. UDP-glucuronic acid has a carboxylic acid substituted for the hydroxyl group in UDP-glucose at position 6 of the hexose ring. UDP-N-acetylglucosamine exhibits a bulky acetamide group at position 2 of the hexose ring in comparison with the hydroxyl group of UDP-glucose.

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.

Alvarado-Castillo C, Lozano-Zarain P, Mateo J, Harden TK and Boyer JL (2002) A fusion protein of the human $P2Y_1$ receptor and NTPDase1 exhibits functional activities of the native receptor and ectoenzyme and reduced signaling responses to endogenously released nucleotides. *Mol Pharmacol* **62**:521-528.

Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U and Javitch JA (2001) Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *J Biol Chem* **276**:29171-29177.

Banisadr G, Queraud-Lesaux F, Boutterin MC, Pelaprat D, Zalc B, Rostene W, Haour F and Parsadaniantz SM (2002) Distribution, cellular localization and functional role of CCR2 chemokine receptors in adult rat brain. *J Neurochem* **81**:257-269.

Bar I, Guns PJ, Metallo J, Cammarata D, Wilkin F, Boeynams JM, Bult H and Robaye B (2008) Knockout mice reveal a role for P2Y6 receptor in macrophages, endothelial cells, and vascular smooth muscle cells. *Mol Pharmacol* **74**:777-784.

Beigi R, Kobatake E, Aizawa M and Dubyak GR (1999) Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. *Am J Physiol* **276**:C267-278.

Bell PD, Lapointe JY, Sabirov R, Hayashi S, Peti-Peterdi J, Manabe K, Kovacs G and Okada Y (2003) Macula densa cell signaling involves ATP release through a maxi anion channel. *Proc Natl Acad Sci U S A* **100**:4322-4327.

Belli SI, van Driel IR and Goding JW (1993) Identification and characterization of a soluble form of the plasma cell membrane glycoprotein PC-1 (5'-nucleotide phosphodiesterase). *Eur J Biochem* **217**:421-428.

Birnbaumer L (1992) Receptor-to-effector signaling through G proteins: roles for beta gamma dimers as well as alpha subunits. *Cell* **71**:1069-1072.

Bourdon DM, Mahanty SK, Jacobson KA, Boyer JL and Harden TK (2006) (N)-methanocarba-2MeSADP (MRS2365) is a subtype-specific agonist that induces rapid desensitization of the $P2Y_1$ receptor of human platelets. *J Thromb Haemost* **4**:861-868.

Brinson AE and Harden TK (2001) Differential regulation of the uridine nucleotide-activated P2Y4 and P2Y6 receptors. SER-333 and SER-334 in the carboxyl terminus are involved in agonist-dependent phosphorylation desensitization and internalization of the P2Y4 receptor. *J Biol Chem* **276**:11939-11948.

Burnstock G (1972) Purinergic nerves. *Pharmacol Rev* **24**:509-581.

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

Buxton IL, Kaiser RA, Oxhorn BC and Cheek DJ (2001) Evidence supporting the Nucleotide Axis Hypothesis: ATP release and metabolism by coronary endothelium. *Am J Physiol Heart Circ Physiol* **281**:H1657-1666.

Caporali F, Capecchi PL, Gamberucci A, Lazzerini PE, Pompella G, Natale M, Lorenzini S, Selvi E, Galeazzi M and Laghi Pasini F (2008) Human rheumatoid synoviocytes express functional P2X7 receptors. *J Mol Med* **86**:937-949.

Carman CV, Parent JL, Day PW, Pronin AN, Sternweis PM, Wedegaertner PB, Gilman AG, Benovic JL and Kozasa T (1999) Selective regulation of Galpha(q/11) by an RGS domain in the G protein-coupled receptor kinase, GRK2. *J Biol Chem* **274**:34483-34492.

Chadwick BP and Frischauf AM (1998) The CD39-like gene family: identification of three new human members (CD39L2, CD39L3, and CD39L4), their murine homologues, and a member of the gene family from Drosophila melanogaster. *Genomics* **50**:357-367.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S and Livi GP (2000) A G protein-coupled receptor for UDP-glucose. *J Biol Chem* **275**:10767-10771.

Charlton ME, Williams AS, Fogliano M, Sweetnam PM and Duman RS (1997) The isolation and characterization of a novel G protein-coupled receptor regulated by immunologic challenge. *Brain Res* **764**:141-148.

Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA and Junger WG (2006) ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* **314**:1792-1795.

Clair T, Lee HY, Liotta LA and Stracke ML (1997) Autotaxin is an exoenzyme possessing 5'-nucleotide phosphodiesterase/ATP pyrophosphatase and ATPase activities. *J Biol Chem* **272**:996-1001.

Clapham DE and Neer EJ (1997) G protein beta gamma subunits. *Annu Rev Pharmacol Toxicol* **37**:167-203.

Clifford EE, Martin KA, Dalal P, Thomas R and Dubyak GR (1997) Stage-specific expression of P2Y receptors, ecto-apyrase, and ecto-5'-nucleotidase in myeloid leukocytes. *Am J Physiol* **273**:C973-987.

Communi D, Gonzalez NS, Detheux M, Brezillon S, Lannoy V, Parmentier M and Boeynaems JM (2001a) Identification of a novel human ADP receptor coupled to Gi. *J Biol Chem* **276**:41479-41485.

Communi D, Govaerts C, Parmentier M and Boeynaems JM (1997) Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J Biol Chem* **272**:31969-31973.

Communi D, Parmentier M and Boeynaems JM (1996) Cloning, functional expression and tissue distribution of the human P2Y6 receptor. *Biochem Biophys Res Commun* **222**:303-308.

Communi D, Pirotton S, Parmentier M and Boeynaems JM (1995) Cloning and functional expression of a human uridine nucleotide receptor. *J Biol Chem* **270**:30849-30852.

Communi D, Suarez-Huerta N, Dussossoy D, Savi P and Boeynaems JM (2001b) Cotranscription and intergenic splicing of human P2Y11 and SSF1 genes. *J Biol Chem* **276**:16561-16566.

Cook SP and McCleskey EW (2002) Cell damage excites nociceptors through release of cytosolic ATP. *Pain* **95**:41-47.

Corriden R, Chen Y, Inoue Y, Beldi G, Robson SC, Insel PA, and Junger WG (2008) Ecto-nucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1/CD39) regulates neutrophil chemotaxis by hydrolyzing released ATP to adenosine. *J Biol Chem* **283**:28480-6.

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

Crabtree JS, Peano BJ, Zhang X, Komm BS, Winneker RC and Harris HA (2008) Activity of three selective estrogen receptor modulators on hormone-dependent responses in the mouse uterus and mammary gland. *Mol Cell Endocrinol* **287**:40-46.

Crabtree JS, Zhang X, Peano BJ, Zhang Z, Winneker RC and Harris HA (2006) Development of a mouse model of mammary gland versus uterus tissue selectivity using estrogen- and progesterone-regulated gene markers. *J Steroid Biochem Mol Biol* **101**:11-21.

Cressman VL, Lazarowski E, Homolya L, Boucher RC, Koller BH and Grubb BR (1999) Effect of loss of $P2Y_2$ receptor gene expression on nucleotide regulation of murine epithelial Cl^- transport. *J Biol Chem* **274**:26461-26468.

Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML and Gan WB (2005) ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci* **8**:752-758.

Donaldson SH, Lazarowski ER, Picher M, Knowles MR, Stutts MJ and Boucher RC (2000) Basal nucleotide levels, release, and metabolism in normal and cystic fibrosis airways. *Mol Med* **6**:969-982.

Dovlatova N, Wijeyeratne YD, Fox SC, Manolopoulos P, Johnson AJ, White AE, Latif ML, Ralevic V and Heptinstall S (2008) Detection of P2Y(14) protein in platelets and investigation of the role of P2Y(14) in platelet function in comparison with the EP(3) receptor. *Thromb Haemost* **100**:261-270.

Dubyak GR (2009) Both sides now: multiple interactions of ATP with pannexin-1 hemichannels. Focus on "A permeant regulating its permeation pore: inhibition of pannexin 1 channels by ATP". *Am J Physiol Cell Physiol* **296**:C235-241.

Dubyak GR and el-Moatassim C (1993) Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol* **265**:C577-606.

Enjyoji K, Sevigny J, Lin Y, Frenette PS, Christie PD, Esch JS, 2nd, Imai M, Edelberg JM, Rayburn H, Lech M, Beeler DL, Csizmadia E, Wagner DD, Robson SC and Rosenberg RD (1999) Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. *Nat Med* **5**:1010-1017.

Enjyoji K, Kotani K, Thukral C, Blumel B, Sun X, Wu Y, Imai M, Friedman D, Csizmadia E, Bleibel W, Kahn BB, and Robson SC (2008) Deletion of cd39/entpd1 results in hepatic insulin resistance *Diabetes* **57**:2311-20.

Erlinge D, Harnek J, van Heusden C, Olivecrona G, Jern S and Lazarowski E (2005) Uridine triphosphate (UTP) is released during cardiac ischemia. *Int J Cardiol* **100**:427-433.

Fabre JE, Nguyen M, Latour A, Keifer JA, Audoly LP, Coffman TM and Koller BH (1999) Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat Med* **5**:1199-1202.

Färber K, Markworth S, Pannasch U, Nolte C, Prinz V, Kronenberg G, Gertz K, Endres M, Bechmann I, Enjyoji K, Robson SC, and Kettenmann H (2008) The ectonucleotidase cd39/ENTPDase1 modulates purinergic-mediated microglial migration. *Glia* **56**:331-41.

Fausther M, Lecka J, Kukulski F, Levesque SA, Pelletier J, Zimmermann H, Dranoff JA and Sevigny J (2007) Cloning, purification, and identification of the liver canalicular ecto-ATPase as NTPDase8. *Am J Physiol Gastrointest Liver Physiol* **292**:G785-795.

Feranchak AP, Roman RM, Doctor RB, Salter KD, Toker A and Fitz JG (1999) The lipid products of phosphoinositide 3-kinase contribute to regulation of cholangiocyte ATP and chloride transport. *J Biol Chem* **274**:30979-30986.

Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, Zhang FL, Gustafson E, Monsma FJ, Jr., Wiekowski MT, Abbondanzo SJ, Cook DN, Bayne ML, Lira SA and Chintala MS (2001) Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest* **107**:1591-1598.

Fumagalli M, Brambilla R, D'Ambrosi N, Volonte 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**:218-203.

Fumagalli M, Trincavelli L, Lecca D, Martini C, Ciana P and Abbracchio MP (2004) Cloning, pharmacological characterisation and distribution of the rat G-protein-coupled P2Y(13) receptor. *Biochem Pharmacol* **68**:113-124.

Gachet C (2005) The platelet P2 receptors as molecular targets for old and new antiplatelet drugs. *Pharmacol Ther.*

Gatof D, Kilic G and Fitz JG (2004) Vesicular exocytosis contributes to volume-sensitive ATP release in biliary cells. *Am J Physiol Gastrointest Liver Physiol* **286**:G538-546.

Ghanem E, Robaye B, Leal T, Leipziger J, Van Driessche W, Beauwens R and Boeynaems JM (2005) The role of epithelial $P2Y_2$ and $P2Y_4$ receptors in the regulation of intestinal chloride secretion. *Br J Pharmacol* **146**:364-369.

Gilman AG (1987) G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56**:615-649.

Gordon JL (1986) Extracellular ATP: effects, sources and fate. *Biochem J* **233**:309-319.

Grinthal A and Guidotti G (2002) Transmembrane domains confer different substrate specificities and adenosine diphosphate hydrolysis mechanisms on CD39, CD39L1, and chimeras. *Biochemistry* **41**:1947-1956.

Grobben B, Anciaux K, Roymans D, Stefan C, Bollen M, Esmans EL and Slegers H (1999) An ecto-nucleotide pyrophosphatase is one of the main enzymes involved in the extracellular metabolism of ATP in rat C6 glioma. *J Neurochem* **72**:826-834.

Harden TK, Lazarowski ER and Boucher RC (1997) Release, metabolism and interconversion of adenine and uridine nucleotides: implications for G protein-coupled P2 receptor agonist selectivity. *Trends Pharmacol Sci* **18**:43-46.

Hardy AR, Conley PB, Luo J, Benovic JL, Poole AW and Mundell SJ (2005) P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood* **105**:3552-3560.

Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB and Julius D (2006) The $P2Y_{12}$ receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci* **9**:1512-1519.

Herold CL, Qi AD, Harden TK and Nicholas RA (2004) Agonist versus antagonist action of ATP at the $P2Y_4$ receptor is determined by the second extracellular loop. *J Biol Chem* **279**:11456-11464.

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D and Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* **409**:202-207.

Homolya L, Watt WC, Lazarowski ER, Koller BH and Boucher RC (1999) Nucleotide-regulated calcium signaling in lung fibroblasts and epithelial cells from normal and P2Y(2) receptor (-/-) mice. *J Biol Chem* **274**:26454-26460.

Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MA, Muskens F, Hoogsteden HC, Luttmann W, Ferrari D, Di Virgilio F, Virchow JC, Jr. and Lambrecht BN (2007) Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat Med* **13**:913-919.

Ivanenkov VV, Murphy-Piedmonte DM and Kirley TL (2003) Bacterial expression, characterization, and disulfide bond determination of soluble human NTPDase6 (CD39L2) nucleotidase: implications for structure and function. *Biochemistry* **42**:11726-11735.

Jacobson KA, Ivanov AA, de Castro S, Harden TK and Ko H (2008) Development of selective agonists and antagonists of P2Y receptors. *Purinergic Signal*.

Jiang Y, Borrelli L, Bacskai BJ, Kanaoka Y and Boyce JA (2009) P2Y6 receptors require an intact cysteinyl leukotriene synthetic and signaling system to induce survival and activation of mast cells. *J Immunol* **182**:1129-1137.

Joseph SM, Buchakjian MR and Dubyak GR (2003) Colocalization of ATP release sites and ecto-ATPase activity at the extracellular surface of human astrocytes. *J Biol Chem* **278**:23331-23342.

Kellerman D, Evans R, Mathews D and Shaffer C (2002) Inhaled P2Y2 receptor agonists as a treatment for patients with Cystic Fibrosis lung disease. *Adv Drug Deliv Rev* **54**:1463-1474.

Kennedy C, Qi AD, Herold CL, Harden TK and Nicholas RA (2000) ATP, an agonist at the rat $P2Y_4$ receptor, is an antagonist at the human $P2Y_4$ receptor. *Mol Pharmacol* **57**:926-931.

Kim YC, Lee JS, Sak K, Marteau F, Mamedova L, Boeynaems JM and Jacobson KA (2005) Synthesis of pyridoxal phosphate derivatives with antagonist activity at the P2Y13 receptor. *Biochem Pharmacol* **70**:266-274.

Kobayashi K, Fukuoka T, Yamanaka H, Dai Y, Obata K, Tokunaga A and Noguchi K (2006) Neurons and glial cells differentially express P2Y receptor mRNAs in the rat dorsal root ganglion and spinal cord. *J Comp Neurol* **498**:443-454.

Koizumi S, Shigemoto-Mogami Y, Nasu-Tada K, Shinozaki Y, Ohsawa K, Tsuda M, Joshi BV, Jacobson KA, Kohsaka S and Inoue K (2007) UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis. *Nature* **446**:1091-1095.

Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G and Sternweis PC (1998) p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. *Science* **280**:2109-2111.

Kreda SM, Seminario-Vidal L, Heusden C and Lazarowski ER (2008) Thrombin-promoted release of UDP-glucose from human astrocytoma cells. *Br J Pharmacol* **153**:1528-1537.

Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38**:289-319.

Krzeminski P, Pomorski P and Baranska J (2008) The P2Y14 receptor activity in glioma C6 cells. *Eur J Pharmacol* **594**:49-54.

Kukulski F, Levesque SA, Lavoie EG, Lecka J, Bigonnesse F, Knowles AF, Robson SC, Kirley TL and Sevigny J (2005) Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. *Purinergic Signal* **1**:193-204.

Lazarowski E, Boucher RC and Harden TK (2000) Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. *J Biol Chem* **275**:31061-31068.

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 (1997a) 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, Homolya L, Boucher RC and Harden TK (1997b) Identification of an ecto-nucleoside diphosphokinase and its contribution to interconversion of P2 receptor agonists. *J Biol Chem* **272**:20402-20407.

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.

Lee BC, Cheng T, Adams GB, Attar EC, Miura N, Lee SB, Saito Y, Olszak I, Dombkowski D, Olson DP, Hancock J, Choi PS, Haber DA, Luster AD and Scadden DT (2003) P2Y-like receptor, GPR105 ($P2Y_{14}$), identifies and mediates chemotaxis of bone-marrow hematopoietic stem cells. *Genes Dev* **17**:1592-1604.

Li Q, Olesky M, Palmer RK, Harden TK and Nicholas RA (1998) Evidence that the p2y3 receptor is the avian homologue of the mammalian P2Y6 receptor. *Mol Pharmacol* **54**:541-546.

Low MG and Saltiel AR (1988) Structural and functional roles of glycosyl-phosphatidylinositol in membranes. *Science* **239**:268-275.

Lutz S, Freichel-Blomquist A, Yang Y, Rumenapp U, Jakobs KH, Schmidt M and Wieland T (2005) The guanine nucleotide exchange factor p63RhoGEF, a specific link between Gq/11-coupled receptor signaling and RhoA. *J Biol Chem* **280**:11134-11139.

Marteau F, Communi D, Boeynaems JM and Suarez Gonzalez N (2004) Involvement of multiple P2Y receptors and signaling pathways in the action of adenine nucleotides diphosphates on human monocyte-derived dendritic cells. *J Leukoc Biol* **76**:796-803.

Melani A, Turchi D, Vannucchi MG, Cipriani S, Gianfriddo M and Pedata F (2005) ATP extracellular concentrations are increased in the rat striatum during in vivo ischemia. *Neurochem Int* **47**:442-448.

Merlin D, Guo X, Martin K, Laboisse C, Landis D, Dubyak G and Hopfer U (1996) Recruitment of purinergically stimulated Cl- channels from granule membrane to plasma membrane. *Am J Physiol* **271**:C612-619.

Moore DJ, Chambers JK, Wahlin JP, Tan KB, Moore GB, Jenkins O, Emson PC and Murdock PR (2001) Expression pattern of human P2Y receptor subtypes: a

quantitative reverse transcription-polymerase chain reaction study. *Biochim Biophys Acta* **1521**:107-119.

Moore DJ, Murdock PR, Watson JM, Faull RL, Waldvogel HJ, Szekeres PG, Wilson S, Freeman KB and Emson PC (2003) GPR105, a novel Gi/o-coupled UDP-glucose receptor expressed on brain glia and peripheral immune cells, is regulated by immunologic challenge: possible role in neuroimmune function. *Brain Res Mol Brain Res* **118**:10-23.

Myrtek D and Idzko M (2007) Chemotactic activity of extracellular nucleotideson human immune cells. *Purinergic Signal* **3**:5-11.

Nishida M, Sato Y, Uemura A, Narita Y, Tozaki-Saitoh H, Nakaya M, Ide T, Suzuki K, Inoue K, Nagao T and Kurose H (2008) P2Y6 receptor-Galpha12/13 signalling in cardiomyocytes triggers pressure overload-induced cardiac fibrosis. *Embo J* **27**:3104-3115.

Noguchi K, Ishii S and Shimizu T (2003) Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J Biol Chem* **278**:25600-25606.

Nomura N, Miyajima N, Sazuka T, Tanaka A, Kawarabayasi Y, Sato S, Nagase T, Seki N, Ishikawa K and Tabata S (1994) Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1. *DNA Res* **1**:27-35.

Offermanns S, Mancino V, Revel JP and Simon MI (1997) Vascular system defects and impaired cell chemokinesis as a result of Galpha13 deficiency. *Science* **275**:533-536.

Okada SF, O'Neal WK, Huang P, Nicholas RA, Ostrowski LE, Craigen WJ, Lazarowski ER and Boucher RC (2004) Voltage-dependent anion channel-1 (VDAC-1) contributes to ATP release and cell volume regulation in murine cells. *J Gen Physiol* **124**:513-526.

Ostrom RS, Gregorian C and Insel PA (2000) Cellular release of and response to ATP as key determinants of the set-point of signal transduction pathways. *J Biol Chem* **275**:11735-11739.

Palin K, Pousset F, Verrier D, Dantzer R, Kelley K, Parnet P and Lestage J (2001) Characterization of interleukin-1 receptor antagonist isoform expression in the brain of lipopolysaccharide-treated rats. *Neuroscience* **103**:161-169.

Parodi AJ (2000) Protein glucosylation and its role in protein folding. *Annu Rev Biochem* **69**:69-93.

Pearson JD and Gordon JL (1979) Vascular endothelial and smooth muscle cells in culture selelctively release adenine nucleotides. *Nature* **281**:384-386.

Penuela S, Bhalla R, Gong XQ, Cowan KN, Celetti SJ, Cowan BJ, Bai D, Shao Q and Laird DW (2007) Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins. *J Cell Sci* **120**:3772-3783.

Picher M and Boucher RC (2003) Human airway ecto-adenylate kinase. A mechanism to propagate ATP signaling on airway surfaces. *J Biol Chem* **278**:11256-11264.

Qi AD, Kennedy C, Harden TK and Nicholas RA (2001) Differential coupling of the human $P2Y_{11}$ receptor to phospholipase C and adenylyl cyclase. *Br J Pharmacol* **132**:318-326.

Qi AD, Wolff SC and Nicholas RA (2005) The apical targeting signal of the P2Y2 receptor is located in its first extracellular loop. *J Biol Chem* **280**:29169-29175.

Quinn MJ and Fitzgerald DJ (1999) Ticlopidine and clopidogrel. *Circulation* **100**:1667-1672.

Ralevic V and Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* **50**:413-492.

Robaye B, Ghanem E, Wilkin F, Fokan D, Van Driessche W, Schurmans S, Boeynaems JM and Beauwens R (2003) Loss of nucleotide regulation of epithelial chloride transport in the jejunum of P2Y4-null mice. *Mol Pharmacol* **63**:777-783.

Robson SC, Sevigny J and Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal* **2**:409-430.

Rojas RJ, Yohe ME, Gershburg S, Kawano T, Kozasa T and Sondek J (2007) Galphaq directly activates p63RhoGEF and Trio via a conserved extension of the Dbl homology-associated pleckstrin homology domain. *J Biol Chem* **282**:29201-29210.

Roman RM, Wang Y, Lidofsky SD, Feranchak AP, Lomri N, Scharschmidt BF and Fitz JG (1997) Hepatocellular ATP-binding cassette protein expression enhances ATP release and autocrine regulation of cell volume. *J Biol Chem* **272**:21970-21976.

Savi P and Herbert JM (2005) Clopidogrel and ticlopidine: P2Y12 adenosine diphosphate-receptor antagonists for the prevention of atherothrombosis. *Semin Thromb Hemost* **31**:174-183.

Schoneberg T, Hermsdorf T, Engemaier E, Engel K, Liebscher I, Thor D, Zierau K, Rompler H and Schulz A (2007) Structural and functional evolution of the P2Y(12)-like receptor group. *Purinergic Signal* **3**:255-268.

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.

Seye CI, Kong Q, Erb L, Garrad RC, Krugh B, Wang M, Turner JT, Sturek M, Gonzalez FA and Weisman GA (2002) Functional P2Y2 nucleotide receptors mediate uridine 5'-triphosphate-induced intimal hyperplasia in collared rabbit carotid arteries. *Circulation* **106**:2720-2726.

Shestopalov VI and Panchin Y (2008) Pannexins and gap junction protein diversity. *Cell Mol Life Sci* **65**:376-394.

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.

Sorensen CE and Novak I (2001) Visualization of ATP release in pancreatic acini in response to cholinergic stimulus. Use of fluorescent probes and confocal microscopy. *J Biol Chem* **276**:32925-32932.

Suzuki N, Nakamura S, Mano H and Kozasa T (2003) Gα 12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. *Proc Natl Acad Sci U S A* **100**:733-738.

Tabata K, Baba K, Shiraishi A, Ito M and Fujita N (2007) The orphan GPCR GPR87 was deorphanized and shown to be a lysophosphatidic acid receptor. *Biochem Biophys Res Commun* **363**:861-866.

Taylor AL, Kudlow BA, Marrs KL, Gruenert DC, Guggino WB and Schwiebert EM (1998) Bioluminescence detection of ATP release mechanisms in epithelia. *Am J Physiol* **275**:C1391-1406.

Tesmer VM, Kawano T, Shankaranarayanan A, Kozasa T and Tesmer JJ (2005) Snapshot of activated G proteins at the membrane: the Gαq-GRK2-Gβγ complex. *Science* **310**:1686-1690.

Wang L, Jacobsen SE, Bengtsson A and Erlinge D (2004) P2 receptor mRNA expression profiles in human lymphocytes, monocytes and CD34+ stem and progenitor cells. *BMC Immunol* **5**:16.

Wang Y, Roman R, Lidofsky SD and Fitz JG (1996) Autocrine signaling through ATP release represents a novel mechanism for cell volume regulation. *Proc Natl Acad Sci U S A* **93**:12020-12025.

Wihlborg AK, Balogh J, Wang L, Borna C, Dou Y, Joshi BV, Lazarowski E, Jacobson KA, Arner A and Erlinge D (2006) Positive inotropic effects by uridine triphosphate (UTP) and uridine diphosphate (UDP) via P2Y2 and P2Y6 receptors on cardiomyocytes and release of UTP in man during myocardial infarction. *Circ Res* **98**:970-976.

Wilkin F, Duhant X, Bruyns C, Suarez-Huerta N, Boeynaems JM and Robaye B (2001) The P2Y11 receptor mediates the ATP-induced maturation of human monocyte-derived dendritic cells. *J Immunol* **166**:7172-7177.

Wirkner K, Schweigel J, Gerevich Z, Franke H, Allgaier C, Barsoumian EL, Draheim H and Illes P (2004) Adenine nucleotides inhibit recombinant N-type calcium channels via G protein-coupled mechanisms in HEK 293 cells; involvement of the P2Y13 receptor-type. *Br J Pharmacol* **141**:141-151.

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**:C624-632.

Yang S, Cheek DJ, Westfall DP and Buxton IL (1994) Purinergic axis in cardiac blood vessels. Agonist-mediated release of ATP from cardiac endothelial cells. *Circ Res* **74**:401-407.

Yegutkin GG (2008) Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta* **1783**:673-694.

Yegutkin GG, Henttinen T, Samburski SS, Spychala J and Jalkanen S (2002) The evidence for two opposite, ATP-generating and ATP-consuming, extracellular pathways on endothelial and lymphoid cells. *Biochem J* **367**:121-128.

Yokomizo T, Izumi T, Chang K, Takuwa Y and Shimizu T (1997) A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. *Nature* **387**:620-624.

Zhong X, Kriz R, Seehra J and Kumar R (2004) N-linked glycosylation of platelet P2Y12 ADP receptor is essential for signal transduction but not for ligand binding or cell surface expression. *FEBS Lett* **562**:111-117.

Zimmermann H (1999) Two novel families of ectonucleotidases: molecular structures, catalytic properties and a search for function. *Trends Pharmacol Sci* **20**:231-236.

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

Chapter II. Identification of $P2Y_{14}$-R ligands using structure-activity relationships and molecular modeling

Introduction

The diverse family of P2Y-R are widely distributed and have emerged as therapeutic targets for several pathophysiologies (Volonte et al., 2006). The $P2Y_1$-R and $P2Y_{12}$-R play a vital role in platelet activation, and a metabolite of the drug clopidogrel, clinically used as an antithrombotic, is an irreversible antagonist at the $P2Y_{12}$-R (Foster et al., 2001; Hollopeter et al., 2001). Also, the $P2Y_2$-R is an essential regulator of chloride transport in lung and other tissues. In cystic fibrosis patients, the $P2Y_2$-R is a therapeutic target for improvement of mucociliary clearance (Kellerman et al., 2002). Although evidence for P2Y-R involvement in a number of physiological processes is robust, research on this receptor family has been impeded by the lack of selectively acting pharmacological agents. Further complicated by cellular release of nucleotides and the metabolism of extracellular nucleotides, characterization of P2Y-R has relied primarily on studies of recombinant receptors and detection of receptor mRNA in tissues.

Detection of P2Y receptors has been primarily based on quantification of mRNA due to the lack of reliable radioligand binding assays. Because levels of mRNA expression do not always equate to levels of expressed protein, studies attributing receptor function to presence of mRNA must be interpreted cautiously. Additionally, reports of species-specific pharmacological profiles for P2Y-R underscore the necessity to validate findings from

animal models in human tissues as well. For example, ATP was demonstrated to be an antagonist at the human $P2Y_4$-R and an agonist at the rat $P2Y_4$-R (Kennedy et al., 2000).

Ectonucleotidases are expressed on the cell surface, and they metabolize agonist for some P2Y-R subtypes even while generating agonist for another. Extracellular nucleotide concentrations are dependent on expression of ectonucleotidases, thus impacting the potency for a particular nucleotide at a given receptor. The expression of different types of ectoenzymes on the cell surface may selectively permit the activation of a subset of P2Y-R due to the availability of each nucleotide. Another inherent challenge to studies of P2Y receptors is that nucleotides are released from cells upon mechanical stimulation, with changes in the pH of cell growth medium, after hormone stimulation of cells, and upon cell lysis (Fitz, 2007).

Commercial preparations of nucleotides with a high level of purity are sometimes difficult to obtain. ATP that is contaminated with UTP, for example, would produce confounding results in studies of multiple P2Y receptors. When uridine nucleotide receptors were first cloned, the selectivities of the agonists were only clarified when contaminating UTP was converted to UDP with the use of hexokinase (Nicholas et al., 1996). Likewise, ATP that has degraded into ADP would complicate studies of receptors targeted by ATP, or mislead the investigator to believe that ATP has an effect at ADP-activated receptors. A study investigating $P2Y_{14}$-R function in N9 microglial cells describes different effects of UDP-Glc depending on the source of the material, underscoring potential technical complications that may occur due to contaminated compounds purchased commercially (Brautigam *et al.*, 2008).

Novel P2Y receptor ligands exhibiting high affinity and receptor selectivity have been developed with the use of high throughput screens, receptor mutagenesis studies, structure-activity relationships (SAR), and molecular modeling (Jacobson et al., 2008). High throughput screening has been limited in its usefulness for identifying P2Y receptor ligands, but in contrast, molecular modeling has recently become an effective method in conjunction with activity studies to guide development of new structural compounds.

Ligand development studies have identified novel agonists and antagonists for the $P2Y_2$-R, $P2Y_6$-R, and the $P2Y_{12}$-R. Greatest progress has been realized, however, with ligand development for the $P2Y_1$-R. The finding that the adenosine bisphosphate molecules (A3P5PS) and (A3P5P) are competitive antagonists at the $P2Y_1$-R was the impetus for launching a series of studies to develop high affinity antagonists for the $P2Y_1$-R (Boyer et al., 1996). These compounds were used in studies to delineate the role of the $P2Y_1$-R in platelet biology and also to distinguish the effects of the $P2Y_{12}$-R from those of the $P2Y_1$-R in signal transduction leading to platelet activation (Hechler et al., 1998; Jin et al., 1998). These bisphosphate molecules were not ideal P2Y-R ligands because of their low affinity for the $P2Y_1$-R and their structure was easily metabolized by ectoenzymes, so molecular modeling of the $P2Y_1$-R in conjunction with SAR studies produced synthetic ligands with more desirable properties, using A3P5P as a template. A selective and higher affinity antagonist, N^6-methyl 2'-deoxyadenosine 3',5'-bisphosphate (N6MABP), also known as MRS2179, was developed subsequently, although it also was vulnerable to hydrolysis (Boyer et al., 1998). MRS2179 was widely used in studies characterizing the $P2Y_1$-R in platelets and in astrocytes. [^{33}P]MRS2179 was used for quantifying $P2Y_1$-R in platelets, verifying $P2Y_1$-R

function in platelets, and MRS2179 also was assessed for its effects on platelet aggregation when systemically administered to mice (Baurand et al., 2001).

Following success with MRS2179, ligand development studies for the $P2Y_1$-R became focused on non-hydrolyzable ligands that retained selectivity and high affinity at the receptor. A constrained bicyclo-hexane ring moiety was introduced in place of the ribose, and through molecular modeling studies, the Northern (N) conformation of the ring was found to be tolerated at the $P2Y_1$-R. One of the molecules that emerged from these studies, 2-chloro-N^6-methyl-(N)-methanocarba-2'-deoxyadenosine 3',5'-bis-phosphate, also called MRS2279, was the first non-nucleotide competitive antagonist at the $P2Y_1$-R that was widely used for characterization of the $P2Y_1$-R without agonist degradation (Boyer et al., 2002). [^{3}H]MRS2279 was used in radioligand binding assays to quantify $P2Y_1$-R expression on various types of cells (Waldo et al., 2002). An analogous molecule with a 2-iodo substitution, MRS2500, exhibited an affinity at the $P2Y_1$-R of 1 nM, ten-fold higher than that of MRS2279 (Kim et al., 2003). With the development of [^{32}P]MRS2500, distribution of the $P2Y_1$-R was quantified in rat tissues and also in human platelets (Houston et al., 2006).

In addition to the development of high affinity competitive antagonists, development of high affinity selective agonists for the $P2Y_1$-R has also met with success. Upon determination of the high affinity and hydrolysis-resistant properties conferred by the (N)-methanocarba modification in antagonists, (N)-methanocarba-2-methylthio-ADP (MRS2365) was developed as a high affinity non-nucleotide agonist for the $P2Y_1$-R that was inactive at the ADP-activated $P2Y_{12}$-R and $P2Y_{13}$-R (Chhatriwala et al., 2004). These newest synthetic ligands for the $P2Y_1$-R will prove useful in further studies of $P2Y_1$-R function not only in platelets, but also in other tissues expressing functional $P2Y_1$-R.

Propelled by successes with ligand development for the $P2Y_1$-R, we have applied a similar rational SAR approach to discovery of high affinity ligands for the $P2Y_{14}$-R. Characterization of the $P2Y_{14}$-R has been primarily based on RNA-level receptor expression and also with studies reporting biological consequences of extracellular UDP-Glc treatment on cultured cells, but has been impaired by the lack of high affinity agonists and competitive antagonists. What we know of $P2Y_{14}$-R function and tissue expression in native tissues is limited, and could benefit from the development of non-hydrolyzable, selective, high affinity ligands.

The $P2Y_{14}$-R was first described as a UDP-glucose receptor after the receptor was cloned and expressed in a heterologous cell system, and screened against multiple potential agonists (Chambers et al., 2000). In addition to UDP-glucose, three other UDP-sugars were identified as agonists at the $P2Y_{14}$-R: UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine. This initial discovery provided the framework for a series of studies in a collaboration between our lab and the lab of Dr. Kenneth A. Jacobson at the National Institutes of Health. The goal of this work is to develop selective, high affinity agonists and antagonists for the $P2Y_{14}$-R. Molecular modeling studies have guided syntheses of UDP-Glc analogues examined for agonist action at the $P2Y_{14}$-R. These studies have been published (Ivanov et al., 2007; Ko et al., 2007; Ko et al., 2009), and will be reviewed herein.

Methods

Cell Culture. COS-7 cells were grown on 12-well culture dishes and maintained in DMEM supplemented with 10% FBS and 4 mM L-glutamine at 37°C in a 10% CO_2 environment. Cells were transfected 48 h prior to assay with pcDNA3.1 expression vectors encoding the human $P2Y_{14}$ receptor. Transfections also included a pcDNA3.1-$G\alpha_{q/i}$, a vector that directs

expression of a chimera of Gq containing the last five amino acids of Gi. This chimeric G protein promotes activation of phospholipase C through Gi-coupled receptors (Coward et al., 1999). The levels of basal inositol phosphates increase markedly in COS-7 cells upon expression of human $P2Y_{14}$-R and $G\alpha_{q/i}$. Since we have previously shown that this activity is dependent on release of cellular UDP-sugars (Lazarowski et al., 2003), in some experiments pcDNA3.1 expressing ectonucleotide pyrophosphatase/ phosphodiesterase-1 (ENPP1) was co-transfected with the goal of lowering basal inositol phosphate signaling and therefore increasing fold response to the agonist. FuGENE 6 (Roche Applied Science, Indianapolis, IN) was used as the transfection reagent following the manufacturer's protocol.

Inositol Phosphate Accumulation Assay. Cells were labeled 18 h prior to assay with 1 μCi/ well [^{3}H]-*myo*-inositol (American Radiolabeled Chemicals, St. Louis, MO) in inositol-free and serum-free DMEM. The assay was started with the addition of 10 mM LiCl with or without drugs and cells were incubated for 45 min at 37°C. The reaction was stopped by aspiration of medium and addition of ice-cold 50 mM formic acid. After neutralization with 150 mM ammonium hydroxide, [^{3}H]inositol phosphates were isolated by Dowex column chromatography as described previously (Nakahata and Harden, 1987).

Summary of Results

The $P2Y_{14}$-R was subjected to rhodopsin-based homology modeling to estimate an overall structure for the receptor, and UDP-Glc was modeled into the putative binding pocket of the $P2Y_{14}$-R to predict points of contact in the ligand-receptor complex. From these studies, extrapolations regarding regions of the pharmacophore that would tolerate modifications as well as enhance potency at the receptor were made. Structural analogs of UDP-Glc were synthesized and pharmacologically assessed at recombinant $P2Y_{14}$-R. The

receptor was co-expressed with a chimeric Gq/i in COS-7 cells. Gq/i is an engineered chimeric construct that replaces the five carboxyl terminal residues of Gq with those of Gi (Coward et al., 1999). Thus, $P2Y_{14}$-R were artificially coupled to PLC activation, and measurement of $P2Y_{14}$-R function was accomplished by quantifying accumulated [^{3}H]inositol phosphates.

Structure of the $P2Y_{14}$-R by molecular modeling

When all of the P2Y receptors were modeled to rhodopsin, general interpretations were made regarding similarities and differences among P2Y receptors. All of the P2Y receptors, including $P2Y_{14}$-R have intermolecular hydrogen bonds among the transmembrane regions that help to stabilize the protein: TM1 – TM7; TM3 – TM6, TM7; TM2 – TM4. Specific differences in the binding sites between $P2Y_1$-like and $P2Y_{12}$-like receptors were also identified (Costanzi et al., 2004).

The initial modeling study of the P2Y-R used rhodopsin as a template and built a homology model of the $P2Y_{14}$-R (Costanzi et al., 2004). Homology modeling is a useful tool to estimate the three dimensional orientation of a protein, and can provide information about intermolecular interactions and putative ligand binding sites. The modeling study of the $P2Y_{14}$-R was refined by molecular dynamics simulation, and furthermore, UDP-Glc was used in studies to identify the ligand binding site on the $P2Y_{14}$-R by automatic molecular docking to the $P2Y_{14}$-R model and then Monte Carlo Multiple Minimum (MCMM) analyses were performed (Ivanov et al., 2007; Ko et al., 2007).

The molecular dynamic simulation revealed that the first extracellular loop and the third intracellular loop exhibited the greatest flexibility in the molecule, while the second extracellular loop exhibited virtually no movement. The constrained nature of the second

extracellular loop was verified with the identification of multiple interactions among residues in that region of the protein. Hydrogen bonds can form between Arg165 and Glu166, and also between Arg165 and Lys176. Arg165 is also in proximity to Glu12 in the N-terminal region, suggesting a potential interaction between these two residues. Other potential interactions between EL2 residues and other regions of the receptor include hydrogen bonds between Glu174 and Arg253, and also between Glu166 and Lys277.

When UDP-Glc was docked into the putative binding site of the $P2Y_{14}$-R model, multiple potential points of interaction between ligand and receptor were identified (Fig. 6). The two possible conformations of the ribose ring (Northern versus Southern) of UDP-Glc were compared to determine which may be favored. In modeling studies of other P2Y-R-ligand complexes, the Northern (N) conformation of the ribose ring was preferred, with the exception of the $P2Y_6$-R (Kim et al., 2002). The 3'-hydroxyl group of the ribose ring of UDP-Glc did not form hydrogen bonds with the receptor in either (N)-UDP-Glc or (S)-UDP-Glc. The 2'-hydroxyl group, however, formed hydrogen bonds with different residues in each of the conformations. With (N)-UDP-Glc, the 2'-hydroxyl group formed a hydrogen bond with Asn104. In contrast, the 2'-hydroxyl group of (S)-UDP-Glc formed a hydrogen bond with Asn287. The modeling studies indicated that either of the conformations of UDP-Glc may be tolerated, and this hypothesis was probed experimentally with UDP-Glc containing a carbocyclic analogue of the ribose, a methanocarba ring, and results of which will be discussed below.

The uracil ring of UDP-Glc was in close proximity to several residues, suggesting potential interactions between ligand and receptor at these points (Fig. 6). Tyr29 is a highly conserved residue among P2Y receptors, and has been identified as a residue that may

interact with the uracil moiety in other P2Y-R (Costanzi et al., 2004). Likewise, it may also be important in ligand binding at the $P2Y_{14}$-R, as the oxygen atom at position 4 of the uracil ring in UDP-Glc was close to Tyr29 in the $P2Y_{14}$-R model. Additional interactions are predicted to occur between the oxygen atom at position 2 of the uracil ring with Asn287 and/or Val288. Other potentially important interactions for receptor binding to the uracil moiety may be between the 3-NH group of uracil and Val32 and Val288.

The phosphate groups of UDP-Glc were also analyzed for potential interactions with the $P2Y_{14}$-R (Fig. 6). The α-phosphate of UDP-Glc associated with the hydroxyl groups of Ser284 and with residue Thr280, which is highly conserved among P2Y-R. Another proposed receptor interaction with the phosphate chain of UDP-Glc is with Lys171, which is located in the second extracellular loop of the $P2Y_{14}$-R. Other $P2Y_{12}$-like receptors also have been proposed to interact with the ligand phosphate chain at an EL2 lysine. However, other proposed interactions between the phosphate chain and residues in TM6 and TM7 in the $P2Y_{12}$-like receptors do not appear to be critical for phosphate group interactions with the $P2Y_{14}$-R. The hexose moiety of UDP-Glc is predicted to form many hydrogen bonds with residues in the $P2Y_{14}$-R, most of which are located in the second extracellular loop and transmembrane regions (Fig. 6). Hydroxyl groups on the hexose ring likely form hydrogen bonds with one or more of the following residues when the $P2Y_{14}$-R binds UDP-Glc: Arg253 (TM6), Lys277 (TM7), Lys171 (EL2), Glu174 (EL2), and Glu166 (EL2).

Other UDP-sugars that are known agonists were used in modeling studies of the $P2Y_{14}$-R (Ko et al., 2009). UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine are all similar in structure to UDP-glucose except at the sugar moiety (Table 1). UDP-galactose has a hydroxyl group at position 4 of the hexose ring like UDP-

glucose, but the hydroxyl group has an inverted chirality. UDP-glucuronic acid is like UDP-glucose except that there is a carboxylic acid group at position 6 of the hexose, while UDP-glucose has only a hydroxyl group at that position. UDP-N-acetylglucosamine is the least like the other UDP-sugars. At the 2 position of the hexose ring, UDP-Glc has a hydroxyl group, but UDP-N-acetylglucosamine has a bulky acetamide group. UDP-N-acetylglucosamine has been observed to exhibit the least potency of the four UDP-sugars at the $P2Y_{14}$-R (Table 1), and molecular modeling may give us an idea as to why that may be true. When each of the UDP-sugars was docked into the $P2Y_{14}$-R model, they all assumed a relatively similar position to UDP-Glc in the putative binding site and were proposed to maintain most of the hydrogen bonds observed for UDP-Glc. However, in the case of UDP-N-acetylglucosamine, the bulky acetamide group did not appear to form any interactions with the receptor and caused a slight shift in the orientation of the receptor in that region. While it is not clear whether an altered receptor orientation is responsible for the lower potency of UDP-N-acetylglucosamine at the $P2Y_{14}$-R, it is likely that the acetamide group at position 2 of the hexose ring is responsible for steric occlusion of the receptor binding site. Further docking studies substituted other sugar moieties for glucose in an attempt to guide design of novel agonists at the $P2Y_{14}$-R.

Molecular modeling studies of the $P2Y_{14}$-R were informative with regard to identifying the putative ligand binding site of the receptor and assisted in predictions of receptor conformation within the lipid bilayer and potential intermolecular interactions. These studies revealed that the hexose moiety of UDP-Glc appears to be the most flexible region of the molecule for purposes of synthesizing novel ligands for the $P2Y_{14}$-R, and this prediction is well supported by results from biological assays as described below.

Structure-Activity Relationships of UDP-glucose analogs at the $P2Y_{14}$-R

a. Uracil and Ribose Modifications

Most of the modifications made to UDP-glucose on the uracil or ribose rings yielded an inactive molecule at the $P2Y_{14}$-R (Table 2). Specifically, substitution of the uracil moiety with the other bases cytidine, guanine, or adenine produced a compound with no effect. Modifications at the 5-position of the uracil ring (iodo-, azido-, amino-) of UDP-Glc abolished agonist activity. Two uracil modifications were tolerated. A 4-thio substitution on the uracil ring retained agonist activity and 4-thio-UDP-Glc was equipotent to UDP-Glc (Fig. 7A). A methylated thio group at the same position was not tolerated, resulting in a compound with no effect. A thio substitution at the 2 position, 2-thio-UDP-Glc, resulted in a compound that exhibited at least six-fold greater potency than UDP-Glc (Fig. 7B).

More than a dozen ribose-modified compounds were synthesized. These modifications included 2'- and 3'-deoxy compounds, substitutions at varying positions on the ring, and replacement of the ribose with a rigid methanocarba moiety, in either the Southern or Northern conformation. All of the ribose modified compounds had no effect at the $P2Y_{14}$-R (Table 2), indicating that this part of the molecule is important for maintaining stability of the ligand-receptor complex. Additionally, UMP, UDP, UTP, and several dinucleotides were tested for agonist activity, and no effect of any of these compounds was observed. From these results we have determined that modifications to the ribose moiety of UDP-Glc are not tolerated, and while more permissive, few modifications to the uracil ring are tolerated that retain agonist activity at the $P2Y_{14}$-R. This restrictive SAR of the $P2Y_{14}$-R is in sharp contrast to that of other P2Y receptors, since modifications to the base or ribose moieties were mostly tolerated and retained efficacy, although not necessarily potency.

b. Hexose Modifications

Informed by molecular modeling studies of the $P2Y_{14}$-R in complex with various potential ligands, compounds with substitutions at the glucose ring were synthesized and tested as novel ligands. Generally, substitutions of other sugars for glucose were well tolerated (Table 3). UDP-fructose, UDP-mannose, and UDP-inositol were identified as novel agonists at the $P2Y_{14}$-R with potencies similar to that of UDP-Glc. Additional sugar substitutions were made to synthesize UDP-arabinose, UDP-fucose, and UDP-ribose, which all retained activity at the $P2Y_{14}$-R. Thus, it appears that most simple sugar substitutions are tolerated in place of the hexose position and exhibit agonist action at the $P2Y_{14}$-R.

It is clear that chirality of the hydroxyl groups on the hexose ring is critical in ligand recognition. UDP-galactose is structurally similar to UDP-Glc. However, the inverted chirality of the hydroxyl group at position 4 slightly reduces the potency of UDP-galactose at the $P2Y_{14}$-R (Table 1). Similarly, while a chiral inversion at position 1 of the hexose ring, UDP-β-glucose, decreased the potency of UDP-Glc at the $P2Y_{14}$-R only two-fold, an inverted chirality of the hydroxyl group at position 2 resulted in UDP-mannose, a compound with three-fold lower potency than UDP-Glc (Table 3).

More in depth studies were undertaken to probe the pharmacophore with particular consideration to the hexose moiety. Fluoro-substitutions for the hydroxyl moieties at each of the hexose ring positions revealed reduced potencies upon fluoro substitution at the 2' and 6' positions, likely destablilizing the receptor-ligand complex due to the loss of a hydrogen bond. The importance of the hydrogen bond at the 2' position was further demonstrated by the observations that inversion of the 2'-hydroxyl chirality reduced the potency of UDP-Glc at the $P2Y_{14}$-R, and 2'-deoxy-UDP-Glc exhibited greatly reduced efficacy (Table 3).

To explore the possibility of attaching a bulky chain onto a $P2Y_{14}$-R ligand, a functional congener approach was employed (Li et al., 1999). An amide-linked chain was attached to the carboxylic acid moiety of UDP-glucuronic acid. As was predicted from modeling studies, these UDP-glucuronic acid analogues exhibited agonist activity at the $P2Y_{14}$-R with similar potencies compared to UDP-glucuronic acid (Table 3). We concluded that position 6 of the hexose moiety is a tractable portion of the pharmacophore, and thus, it may be possible to conjugate large molecules to a $P2Y_{14}$-R agonist through a carefully-placed functional group without losing affinity for the receptor. These findings provide an opportunity to synthesize new $P2Y_{14}$-R ligands with bulky chemical groups, compounds which may be utilized in pharmacological studies. Fluorophore-conjugated ligands that are selective for the $P2Y_{14}$-R would be useful in characterizing receptor expression in tissues and cells. Likewise, attachment of probes that increase affinity or selectivity for the receptor would provide a useful tool for quantifying and further characterizing the $P2Y_{14}$-R in various tissues.

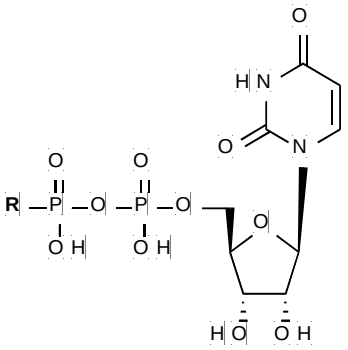
Conclusions

Molecular modeling functions as a rational starting point to guide objectives of ligand structure-activity relationship studies for the purpose of developing novel ligands for the $P2Y_{14}$-R. Molecular modeling has predicted UDP-Glc contact sites at the uracil, phosphate, and hexose moieties. The SAR studies, however, expanded our understanding of the nature of those interactions with regard to the role of each putative contact point in stabilizing the receptor-ligand complex. Furthermore, studies of UDP-Glc analogues informed us about how modifications at each part of the molecule impacts potency at the receptor. The second extracellular loop of the $P2Y_{14}$-R appears to play an important role in stabilizing the hexose

moiety of the UDP-Glc molecule, and transmembrane domains three, six, and seven also harbor contact points for the phosphate and uracil moieties.

Agonist binding pockets of other P2Y receptors have also been predicted to form among residues in the TM3, TM6, TM7, and the EL2 (Costanzi et al., 2004), although different orientations of agonist in each receptor's binding pocket dictates the particular residues that are important for agonist binding for each receptor. Evidence from comparisons modeling ADP in the binding sites of the $P2Y_1$-R or the $P2Y_{12}$-R suggests that the same agonist uses non-correlative residues in each of the distinctive binding pockets. While 44% shared sequence homology between the $P2Y_{14}$-R and the $P2Y_{12}$-R may ostensibly indicate closely aligned binding sites, differences in ligand selectivity undoubtedly make differences in binding modes necessary. Comparisons to previous modeling studies with the $P2Y_{12}$-R and $P2Y_{13}$-R suggest that the $P2Y_{14}$-R uses some analogous residues – an arginine in TM6, a lysine in TM7, and another lysine in EL2 - in forming a binding pocket. These studies are ongoing, and as we learn more about the structure of the $P2Y_{14}$-R and its ligand selectivity, we can pursue further the development of high affinity ligands for use in characterizing $P2Y_{14}$-R function and expression.

Table 1. $P2Y_{14}$-R agonists. Table of $P2Y_{14}$-R agonists previously identified with corresponding EC_{50} values measured by phosphatidylinositol hydrolysis assays in COS-7 cells. *Left,* structure of UDP-sugars, and R denotes the sugar substitution shown in the table.



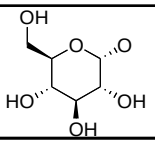
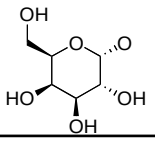
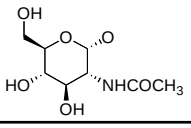
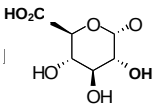
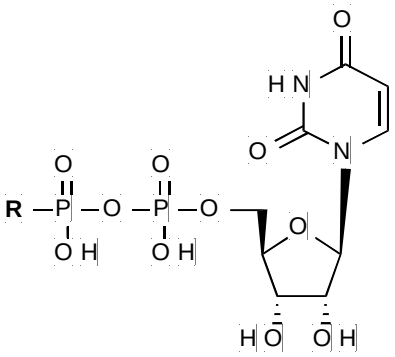
Agonist	R	EC_{50}, μM ± SE
UDP-glucose		0.261 ± 0.053
UDP-galactose		0.670 ± 0.090
UDP-N-Ac-glucosamine		4.38 ± 1.05
UDP-glucuronic acid		0.370 ± 0.070

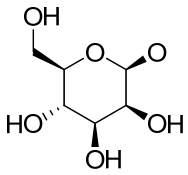
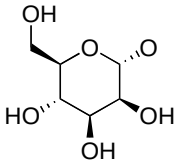
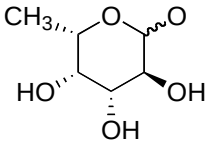
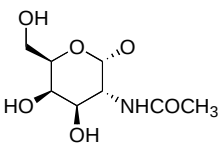
Table 2. Ribose and uracil modifications on UDP-Glc are largely inactive at the $P2Y_{14}$-R. Compounds assessed for agonist activity at the $P2Y_{14}$-R include ribose-modified, base-modified, ribose- and base-modified UDP-Glc analogues, and also dinucleotides. EC_{50} values reported are derived from phosphatidylinositol hydrolysis assays in COS-7 cells transiently expressing the $P2Y_{14}$-R and $G_{q/i}$ as described in Methods. (NE= no effect at 10 μM)

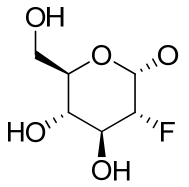
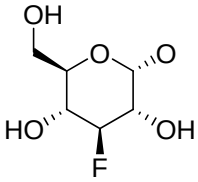
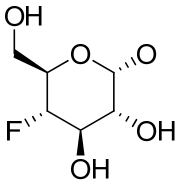
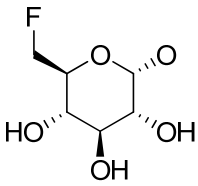
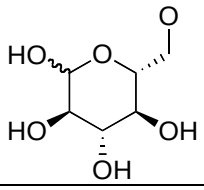
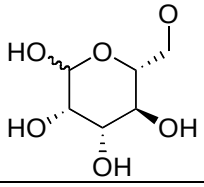
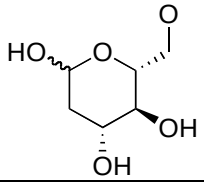
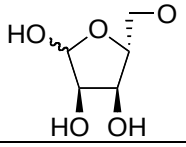
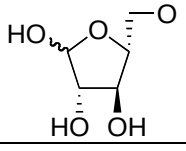
Modification	EC_{50} at $hP2Y_{14}$ receptor, μM ± SE
Ribose modified	
2′-deoxy	NE
2′-deoxy-2′-azido	NE
2′-deoxy-2′-amino	NE
cyclic-2′-deoxy-2′-aminocarbonyl-3′-O	NE
3′-deoxy	NE
2′,3′-dideoxy-2′-methoxy-carbonyl	NE
2′-fluoro-2′-deoxyara	NE
(S)-methanocarba	NE
(N)-methanocarba	NE
(S)-mc-2′-deoxy	NE
carbocyclic	NE
Base-modified	
4-thio	0.29 ± 0.16
4-methylthio	>10
2-thio	0.049 ± 0.02
5-iodo	NE
5-azido	NE
5-amino	NE

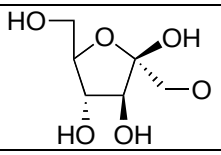
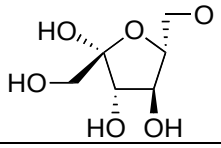
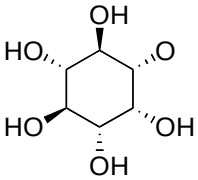
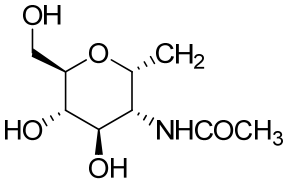
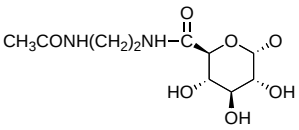
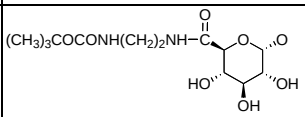
Base = C	NE
Base = A	NE
Base = G	NE
N^4-methoxy-CDP-glucose	<50% max at 10 μM
Ribose- and Base-modified	
2′-deoxy-C	NE
2′-deoxy-T	NE
2′-deoxy- 5-F-U	NE
Dinucleotides	
Up_2U	NE
Cp_2C	NE

Table 3. Modifications at the hexose moiety of UDP-Glc are largely tolerated as agonists at the $P2Y_{14}$-R. All of the simple sugars substituted for the glucose moiety retain agonist activity at the $P2Y_{14}$-R, most with similar potency as UDP-Glc. EC_{50} values were determined from phosphatidylinositol assays in COS-7 cells transiently expressing the $P2Y_{14}$-R. 2'-Substitutions or deletion of the hydroxyl group reduce potency or efficacy. Functional congener substitutions are the last two compounds in the table. *Inset,* structure of UDP-sugar, and R denotes the hexose substitution shown in the table.



Modification	Structure R =	EC_{50} at $hP2Y_{14}$ receptor, µM ± SE
UDP-β-[1]glucose		0.588 ± 0.130
UDP-[1]mannose		0.910 ± 0.150
UDP-[1]fucose		0.562 ± 0.173
UDP-N-Ac-galactosamine		0.810 ± 0.090

UDP-2′-F-[1]glucose		2.5 ± 0.9
UDP-3′-F-[1]glucose		0.361 ± 0.094
UDP-4′-F-[1]glucose		0.567 ± 0.156
UDP-6′-F-[1]glucose		0.905 ± 0.429
UDP-[6]glucose		0.373 ± 0.073
UDP-[6]mannose		0.658 ± 0.022
UDP-[6]2′-deoxyglucose		<50% max at 10 μM
UDP-[5]ribose		0.238 ± 0.084
UDP-[5]arabinose		0.460 ± 0.057

UDP-[1]fructose		0.880 ± 0.210
UDP-[6]fructose		0.323 ± 0.069
UDP-inositol		1.88 ± 1.10
UDP-[2-(acetylamino)-2-deoxy α-D-glucopyranosyl] methyl phosphonyl uridine 5´yl phosphate		<50% max at 10 μM
UDP-glucuronyl-ED-Ac		0.496 ± 0.067
UDP-glucuronyl-ED-Boc		0.951 ± 0.277

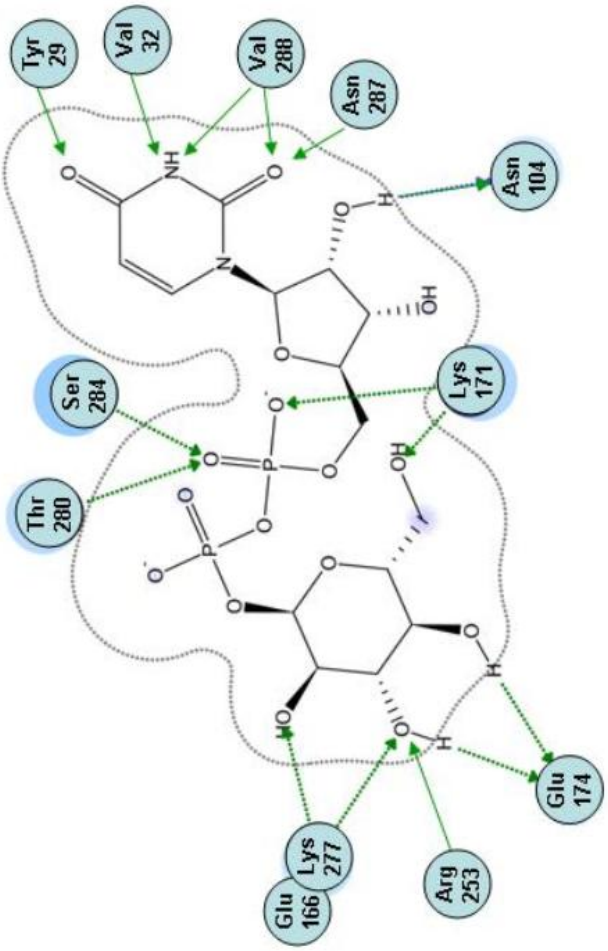


Figure 6. **Potential points of interaction between UDP-Glc and the P2Y14-R.** Diagram of putative binding site for UDP-Glc depicting amino acid side chains that may form bonds with various atoms on the UDP-Glc molecule. Tyr29 and Val32 lie in the amino terminal end; Asn104 is in TM3; Arg253 is in TM6; Thr280, Ser284, Asn287, and Val288 are in TM7; Glu166, Lys171 and Glu174 are in EL2; Lys277 is in EL3.

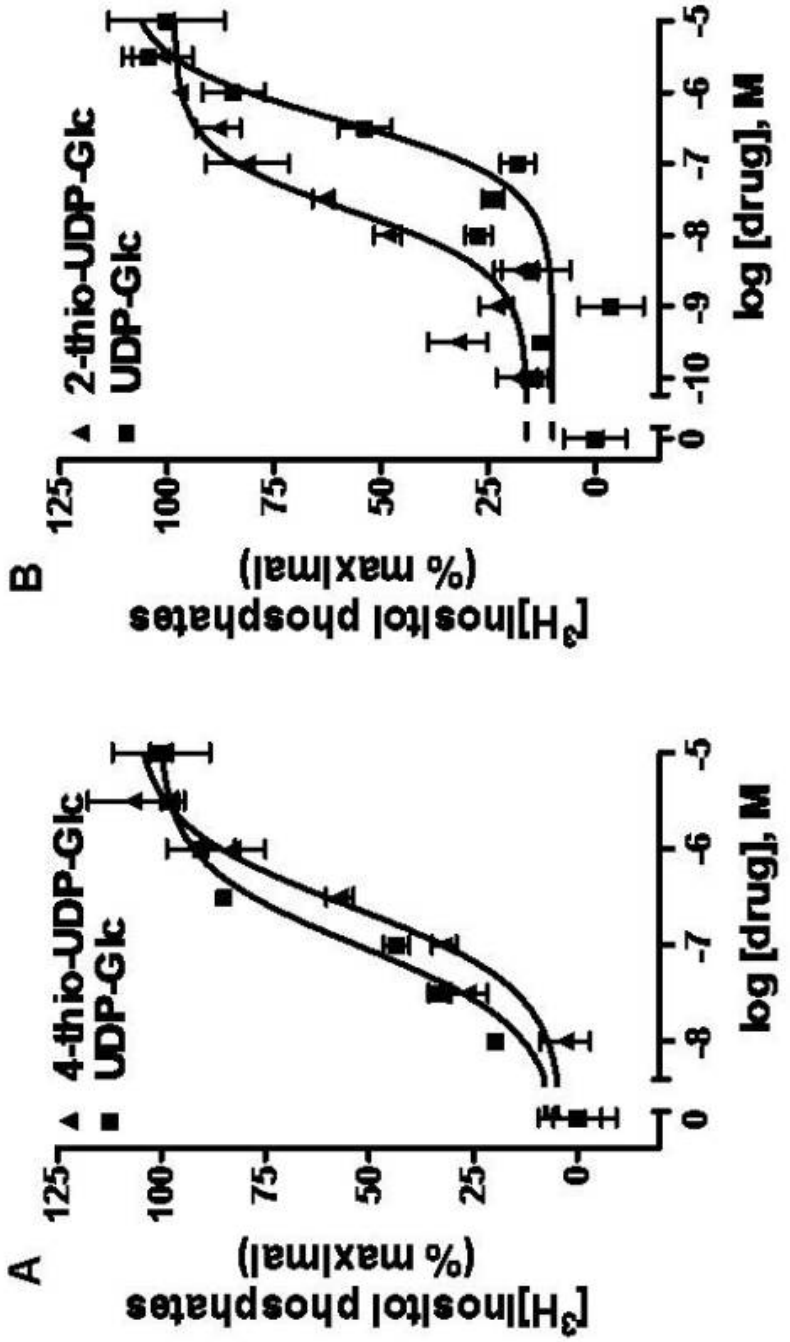


Figure 7. **UDP-Glc analogues with 4'- and 2'-uracil ring modifications are novel agonists at the P2Y$_{14}$-R.** COS-7 cells expressing P2Y$_{14}$-R and Gq/i were radiolabeled with [^{3}H]inositol, and incubated with varying concentrations of indicated compound for 45 min. *A*, 4-thio-UDP-Glc is equipotent to UDP-Glc. *B*, 2-thio-UDP-Glc is >6X more potent than UDP-Glc at the P2Y$_{14}$-R.

References

Baurand A, Raboisson P, Freund M, Leon C, Cazenave JP, Bourguignon JJ and Gachet C (2001) Inhibition of platelet function by administration of MRS2179, a P2Y1 receptor antagonist. *Eur J Pharmacol* **412**:213-221.

Boyer JL, Adams M, Ravi RG, Jacobson KA and Harden TK (2002) 2-Chloro N^6-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate is a selective high affinity $P2Y_1$ receptor antagonist. *Br J Pharmacol* **135**:2004-2010.

Boyer JL, Mohanram A, Camaioni E, Jacobson KA and Harden TK (1998) Competitive and selective antagonism of $P2Y_1$ receptors by N^6-methyl 2'-deoxyadenosine 3',5'-bisphosphate. *Br J Pharmacol* **124**:1-3.

Boyer JL, Romero-Avila T, Schachter JB and Harden TK (1996) Identification of competitive antagonists of the $P2Y_1$ receptor. *Mol Pharmacol* **50**:1323-1329.

Brautigam VM, Dubyak GR, Crain JM and Watters JJ (2008) The inflammatory effects of UDP-glucose in N9 microglia are not mediated by P2Y14 receptor activation. *Purinergic Signal* **4**:73-78.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S and Livi GP (2000) A G protein-coupled receptor for UDP-glucose. *J Biol Chem* **275**:10767-10771.

Chhatriwala M, Ravi RG, Patel RI, Boyer JL, Jacobson KA and Harden TK (2004) Induction of novel agonist selectivity for the ADP-activated $P2Y_1$ receptor versus the ADP-activated $P2Y_{12}$ and $P2Y_{13}$ receptors by conformational constraint of an ADP analog. *J Pharmacol Exp Ther* **311**:1038-1043.

Costanzi S, Mamedova L, Gao ZG and Jacobson KA (2004) Architecture of P2Y nucleotide receptors: structural comparison based on sequence analysis, mutagenesis, and homology modeling. *J Med Chem* **47**:5393-5404.

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

Fitz JG (2007) Regulation of cellular ATP release. *Trans Am Clin Climatol Assoc* vb**118**:199-208.

Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, Zhang FL, Gustafson E, Monsma FJ, Jr., Wiekowski MT, Abbondanzo SJ, Cook DN, Bayne ML, Lira SA and Chintala MS (2001) Molecular identification and characterization of the platelet

ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest* **107**:1591-1598.

Hechler B, Leon C, Vial C, Vigne P, Frelin C, Cazenave JP and Gachet C (1998) The $P2Y_1$ receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. *Blood* **92**:152-159.

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D and Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* **409**:202-207.

Houston D, Ohno M, Nicholas RA, Jacobson KA, Harden TK (2006) [32P]2-iodo-N6-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate ([32P]MRS2500), a novel radioligand for quantification of native P2Y1 receptors. *Br J Pharmacol* **147**:459-67.

Ivanov AA, Fricks I, Harden TK and Jacobson KA (2007) Molecular dynamics simulation of the $P2Y_{14}$ receptor. Ligand docking and identification of a putative binding site of the distal hexose moiety. *Bioorg Med Chem Lett* **17**:761-766.

Jacobson KA, Ivanov AA, de Castro S, Harden TK and Ko H (2008) Development of selective agonists and antagonists of P2Y receptors. *Purinergic Signal*.

Jin J, Daniel JL and Kunapuli SP (1998) Molecular basis for ADP-induced platelet activation. II. The $P2Y_1$ receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* **273**:2030-2034.

Kellerman D, Evans R, Mathews D and Shaffer C (2002) Inhaled P2Y2 receptor agonists as a treatment for patients with Cystic Fibrosis lung disease. *Adv Drug Deliv Rev* **54**:1463-1474.

Kennedy C, Qi AD, Herold CL, Harden TK and Nicholas RA (2000) ATP, an agonist at the rat $P2Y_4$ receptor, is an antagonist at the human $P2Y_4$ receptor. *Mol Pharmacol* **57**:926-931.

Kim HS, Ohno M, Xu B, Kim HO, Choi Y, Ji XD, Maddileti S, Marquez VE, Harden TK and Jacobson KA (2003) 2-Substitution of adenine nucleotide analogues containing a bicyclo[3.1.0]hexane ring system locked in a northern conformation: enhanced potency as $P2Y_1$ receptor antagonists. *J Med Chem* **46**:4974-4987.

Kim HS, Ravi RG, Marquez VE, Maddileti S, Wihlborg AK, Erlinge D, Malmsjo M, Boyer JL, Harden TK and Jacobson KA (2002) Methanocarba modification of uracil and adenine nucleotides: high potency of Northern ring conformation at $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{11}$ but not $P2Y_6$ receptors. *J Med Chem* **45**:208-218.

Ko H, Das A, Carter R, Fricks I, Ivanov AA, Melman A, Kovac P, Hajduch J, Kirk KL, Harden TK and Jacobson KA (2009) Structure Activity Relationship of UDP-Glucose Analogues as Agonists of the $P2Y_{14}$ Receptor: Modified Terminal Sugar Moiety. *J Med Chem* **in press**.

Ko H, Fricks I, Ivanov AA, Harden TK and Jacobson KA (2007) Structure-activity relationship of uridine 5'-diphosphoglucose analogues as agonists of the human $P2Y_{14}$ receptor. *J Med Chem* **50**:2030-2039.

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.

Li AH, Chang L, Ji X, Melman N and Jacobson KA (1999) Functionalized congeners of 1,4-dihydropyridines as antagonist molecular probes for A3 adenosine receptors. *Bioconjug Chem* **10**:667-677.

Nakahata N and Harden TK (1987) Regulation of inositol trisphosphate accumulation by muscarinic cholinergic and H1-histamine receptors on human astrocytoma cells. Differential induction of desensitization by agonists. *Biochem J* **241**:337-344.

Nicholas RA, Watt WC, Lazarowski ER, Li Q and Harden K (1996) Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol Pharmacol* **50**:224-229.

Volonte C, Amadio S, D'Ambrosi N, Colpi M and Burnstock G (2006) P2 receptor web: complexity and fine-tuning. *Pharmacol Ther* **112**:264-280.

Waldo GL, Corbitt J, Boyer JL, Ravi G, Kim HS, Ji XD, Lacy J, Jacobson KA and Harden TK (2002) Quantitation of the $P2Y_1$ receptor with a high affinity radiolabeled antagonist. *Mol Pharmacol* **62**:1249-1257.

Chapter III. UDP is a competitive antagonist at the human $P2Y_{14}$ receptor

Abstract

G-protein coupled P2Y receptors (P2Y-R) are activated by adenine and uridine nucleotides. The $P2Y_{14}$ receptor ($P2Y_{14}$-R) is activated by at least four naturally occurring UDP-sugars, with UDP-glucose (UDP-Glc) being the most potent agonist. With the goal of identifying a competitive antagonist for the $P2Y_{14}$-R, UDP was examined for antagonist activity in COS-7 cells transiently expressing the human $P2Y_{14}$-R and a chimeric G protein that couples Gi-coupled receptors to stimulation of phosphoinositide hydrolysis. UDP antagonized the agonist action of UDP-Glc, and Schild analysis confirmed the antagonism was competitive (pK_B= 7.28). UDPβS also antagonized the h$P2Y_{14}$-R with an apparent affinity similar to that of UDP. In contrast, no antagonist activity was observed with ADP, CDP, or GDP, and other uracil analogues also failed to exhibit antagonist activity. Antagonist activity of UDP was not observed at other human P2Y receptors. In contrast to its antagonist action at the human $P2Y_{14}$-R, UDP was a potent agonist (EC_{50} = 0.35 μM) at the rat $P2Y_{14}$-R. These results identify the first competitive antagonist of the $P2Y_{14}$-R and demonstrate pharmacological differences between receptor orthologs.

Introduction

P2Y receptors are members of the superfamily of G-protein coupled receptors and are activated by adenine and uridine nucleotides and nucleotide-sugars. At least eight receptors comprise the P2Y-R family. $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are coupled to Gq and

activate phospholipase C, while $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors couple to Gi, leading to the inhibition of adenylyl cyclase and activation of ion channels (Burnstock, 2006). The $P2Y_{11}$-R uniquely couples both to Gq to activate phospholipase C and to Gs to stimulate adenylyl cyclase (Communi et al., 1997; Qi et al., 2001).

The human $P2Y_{14}$-R was identified as the eighth legitimate member of the P2Y receptor family (Chambers et al., 2000). UDP-glucose (UDP-Glc) was proposed to be the endogenous agonist for the $P2Y_{14}$-R, with UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine acting as less potent $P2Y_{14}$-R agonists. $P2Y_{14}$-R mRNA was detected in a broad range of human tissues including placenta, stomach, intestine, adipose, brain, lung, spleen and heart, and also in specialized cells such as circulating neutrophils (Chambers et al., 2000; Scrivens and Dickenson, 2006). The rat and mouse $P2Y_{14}$-R exhibit 80% and 83% amino acid identity to the human receptor, and both rodent orthologs are activated by known agonists with a similar profile to that of the human $P2Y_{14}$-R (Freeman et al., 2001).

Cellular UDP-Glc is released in a constitutive manner into the medium of a broad range of cell types. While most extracellular nucleotides are metabolized quickly, UDP-Glc accumulates in the medium of several cell lines (Lazarowski et al., 2003). The mechanisms of UDP-Glc release and extracellular metabolism remain unclear. Observations of UDP-Glc-promoted signaling were reported in multiple types of immune cells (Fumagalli et al., 2003; Skelton et al., 2003; Scrivens and Dickenson, 2005; Muller et al., 2005), suggesting that the $P2Y_{14}$-R may have a yet to be defined role in the regulation of immune system homeostasis.

Characterization of the $P2Y_{14}$-R has been slowed by the lack of a selective competitive antagonist. We have identified and developed novel, selective ligands for several P2Y receptors that have proven useful for pharmacological resolution of molecularly

defined P2Y-R in cells and tissues (Boyer et al., 1996; Jacobson et al., 2006; Houston et al., 2006; Houston et al., 2007). Accordingly, we are interested in identifying a selective antagonist for the $P2Y_{14}$-R.

Ault and Broach (2006) recently used a yeast model system in which various nucleotides and nucleotide-sugars were examined for their ability to stimulate growth of mutant $P2Y_{14}$-R-expressing yeast cells in studies focused on identification of mutant $P2Y_{14}$ receptors with differential agonist sensitivities. Studies performed using one of these mutant receptors revealed that UDP antagonized UDP-Glc-promoted receptor activation in a concentration-dependent manner (Ault and Broach, 2006). We hypothesized that UDP acts as a competitive antagonist at the wild-type $P2Y_{14}$-R, and therefore, used a transfected COS-7 cell system to investigate UDP activity at the human and rat $P2Y_{14}$-R. Here we show that UDP is a selective and competitive antagonist of the human $P2Y_{14}$-R. Thus, signals emanating from extracellular UDP apparently occur as a consequence of activation of the $P2Y_6$-R as well as through antagonism of the $P2Y_{14}$-R. Surprisingly, UDP is a potent full agonist at the rat $P2Y_{14}$-R.

Methods

Materials

UDP-Glc, UDP, ADP, CDP, GDP, and 2-methyl-thio-ADP were purchased from SigmaAldrich (St. Louis, MO). UP_3U was synthesized according to Methods detailed in Pendergast et al (2001). The source of UP_4U was as previously reported (Ivanov et al 2007). ATP and UTP were purchased from GE Healthcare (Piscataway, NJ). [^{32}P]PPi was synthesized as described previously (Lazarowski et al., 2003). UDPβS as well as a

mammalian expression vector for ENPP1 were generous gifts from Dr. José Boyer of Inspire Pharmaceuticals, Durham, NC.

Cell Culture and Transfection

COS-7 cells were grown on 12-well culture dishes and maintained in DMEM supplemented with 10% FBS and 4 mM L-glutamine at 37°C in a 10% CO_2 environment. Cells were transfected 48 h prior to assay with pcDNA3.1 expression vectors encoding either the human or rat $P2Y_{14}$ receptor with an N-terminal Hemaglutinin epitope. The expression vector for the $hP2Y_{14}$-R was obtained as previously reported (Lazarowski et al 2003). Transfections also included pcDNA3.1-$G\alpha_{q/i}$, a vector that directs expression of a chimera of Gαq containing the last five amino acids of Gαi. This chimeric G protein promotes activation of phospholipase C through Gαi-coupled receptors (Coward et al., 1999). The levels of basal inositol phosphates increased markedly in COS-7 cells upon expression of human or rat $P2Y_{14}$-R and $G\alpha_{q/i}$. Since we previously illustrated that UDP-sugars are basally released by various cell types (Lazarowski et al., 2003), in some experiments pcDNA3.1 expressing ENPP1 was co-transfected with the goal of hydrolyzing extracellular nucleotide sugars and potentially decreasing inositol phosphate accumulation in the absence of added $P2Y_{14}$-R agonists. FuGENE 6 (Roche Applied Science, Indianapolis, IN) was used as the transfection reagent following the manufacturer's protocol.

Inositol Phosphate Accumulation Assay

Cells were labeled 8-18 h prior to assay with 0.5-3 μCi/ well [^{3}H]-*myo*-inositol (American Radiolabeled Chemicals, St. Louis, MO) in inositol-free and serum-free DMEM. Assays were initiated with the addition of 10 mM LiCl with or without drugs, and incubations continued for 45 min at 37°C. Reactions were stopped by aspiration of medium and addition

of ice-cold 50 mM formic acid. After neutralization with 150 mM ammonium hydroxide, [^{3}H]inositol phosphates were isolated by Dowex column chromatography as described previously (Nakahata and Harden, 1987). Stable cell lines for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, or $P2Y_{11}$ receptors were generated in 1321N1 human astrocytoma cells as previously described (Nicholas et al., 1996; Kennedy et al., 2000). Experiments testing the potential activity of UDP at the $P2Y_2$-R and $P2Y_4$-R included hexokinase to eliminate contaminating UTP as described previously in Nicholas et al. (1996). Briefly, UDP was treated with 10 U/mL hexokinase in the presence of 22 mM glucose for 1 h at 37ºC, and 1 U/mL hexokinase was included in the assay buffer for the duration of the incubation. Variability in cpm of [^{3}H]inositol phosphate accumulation across experiments occurred due to differences in the amount of [^{3}H]inositol utilized for labeling and/or the duration of the prelabeling period.

Measurement of UDP-Glc in the cell medium

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

Quantification of $P2Y_{14}$-R expression

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

Rat $P2Y_{14}$-R subcloning

The rat homolog of the $P2Y_{14}$-R ($rP2Y_{14}$-R) was amplified from rat genomic DNA using Pfu polymerase with the following primers: (5'-GAGACGCGTCCGACAACACAACAACCACAGAAC-3') and (5'-AGACTCGAGTTACAAAGTATCTGTGCTTTCC-3'). The primers contained either a MluI (upstream primer) or a XhoI (downstream primer) restriction site, respectively (sites are underlined) to facilitate cloning. The amplification conditions were 94°C for 5 min; 35 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 90 s; and a final extension for 4 min at 72°C. The amplified rP2Y14-R fragment was digested with MluI and XhoI, purified, and ligated into a similarly digested, modified pcDNA3 expression vector, which fuses an HA-epitope to Asp-2 at the N-terminus of the receptor. An individual clone encoding the

receptor was sequenced and found to be identical to the published sequence (Freeman et al., 2001).

Data Analyses

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

Results

Agonist activity of UDP-Glc at the hP2Y$_{14}$-R.

To assay the functional activity of the hP2Y$_{14}$-R, we utilized COS-7 cells transiently co-expressing the hP2Y$_{14}$-R and a G$\alpha_{q/i}$ chimera. The G$\alpha_{q/i}$ chimera is a Gαq protein in which the last five amino acids at the carboxyl terminus have been substituted with those of Gαi. This chimeric Gα subunit is activated by GPCRs that couple to the Gαi family of G-proteins and signal through downstream Gαq effectors such as phospholipase C (Coward et al., 1999). Expression of the hP2Y$_{14}$-R or the G$\alpha_{q/i}$ chimera alone in COS-7 cells resulted in levels of [^{3}H]inositol phosphate accumulation similar to that observed in cells expressing

empty vector alone. [^{3}H]Inositol phosphate accumulation was not changed by the addition of 100 μM UDP-Glc to cells expressing empty vector, the hP2Y$_{14}$-R, or G$\alpha_{q/i}$ (Fig. 1). Co-expression of the hP2Y$_{14}$-R and G$\alpha_{q/i}$ resulted in increased [^{3}H]inositol phosphate accumulation in the presence of buffer alone as we previously reported (Lazarowski et al., 2003). Addition of 100 μM UDP-Glc to cells co-expressing the hP2Y$_{14}$-R and G$\alpha_{q/i}$ resulted in a two-fold increase in inositol phosphate accumulation, consistent with agonist-promoted activation of phospholipase C (Fig. 8).

To investigate the nature of the hP2Y$_{14}$-R-dependent [^{3}H]inositol phosphate accumulation in the absence of added agonist, we co-expressed ENPP1 with the hP2Y$_{14}$-R and G$\alpha_{q/i}$ in COS-7 cells with the goal of removing any released nucleotide/nucleotide-sugar potentially present in the medium. UDP-Glc levels (3.6 ± 1.9 nM) in the bulk medium from COS-7 cells expressing hP2Y$_{14}$-R and G$\alpha_{q/i}$ were similar to that of control cells (3.3 ± 0.3 nM). In contrast, co-expression of ENPP1 in cells also expressing hP2Y$_{14}$-R and G$\alpha_{q/i}$ resulted in an approximately 78 % reduction in UDP-Glc levels (0.8 ± 0.5 nM) compared to control. Although expression of ENPP1 alone had no effect on basal [^{3}H]inositol phosphate accumulation, expression of ENPP1 with hP2Y$_{14}$-R and G$\alpha_{q/i}$ resulted in an approximately 40% decrease ($p < 0.01$) in basal [^{3}H]inositol phosphate levels compared to cells expressing receptor and G protein alone (Fig. 9A). Expression of ENPP1 had no effect on surface expression of the hP2Y$_{14}$-R as quantified with an immunoassay (data not shown; see Methods), and did not notably change the concentration effect curve for added UDP-Glc (Fig. 9B). Thus, we conclude that the hP2Y$_{14}$-R-dependent elevation of [^{3}H]inositol phosphate levels in the absence of added agonist occurs largely because of autocrine/paracrine release of P2Y$_{14}$-R agonist. However, these results do not entirely rule

out the possibility that the overexpressed $P2Y_{14}$-R exhibits constitutive activity in this test system.

Antagonist Effect of UDP at the $hP2Y_{14}$-R.

Four UDP-sugars were identified as agonists at the $hP2Y_{14}$-R, and neither UTP nor UDP exhibited agonist activity (Chambers et al., 2000). To determine whether UDP is an antagonist at the wild-type $hP2Y_{14}$-R, we generated a series of concentration-effect curves for UDP-Glc-promoted stimulation of phospholipase C in the presence of increasing concentrations of UDP (Fig. 10A). UDP caused a parallel rightward shift of the UDP-Glc concentration-effect curve, and Schild analysis (Fig. 10B) confirmed that the antagonism produced by UDP was competitive (slope = 1.15 ± 0.06, n = 3). The pK_B of UDP for antagonism of the $hP2Y_{14}$-R was 7.28 ± 0.04.

We also assessed whether UDP exhibited antagonist activity at other P2Y-R stably expressed in 1321N1 human astrocytoma cells. $P2Y_1$-R was maximally activated by 1 μM 2MeSADP (Fig. 11A), $P2Y_2$-R and $P2Y_4$-R were each activated by 3 μM UTP (Fig. 11B, C), and $P2Y_{11}$-R was activated by 100 μM ATP (Fig. 11D). Although 10 μM UDP completely blocked a near-maximal concentration of UDP-Glc at the $hP2Y_{14}$-R (Fig. 10), UDP had no effect at any other P2Y receptors tested. Thus, we conclude that UDP is a selective antagonist at the $hP2Y_{14}$-R.

To determine whether the antagonist effect of UDP at the $hP2Y_{14}$-R is specific to the uracil structure, we also tested other nucleotides and nucleotide derivatives as antagonists at the $hP2Y_{14}$-R. In contrast to the action of UDP, other nucleoside diphosphates including ADP, CDP, and GDP, at concentrations of 10 μM or 100 μM did not inhibit UDP-Glc (3 μM)-promoted [^{3}H]inositol phosphate formation (Fig. 12). We also tested whether other

uridine-based molecules would antagonize activation of the hP2Y$_{14}$-R by UDP-Glc. Neither UTP, UP$_3$U, nor UP$_4$U inhibited UDP-Glc activation of the receptor, although each of these nucleotides, when tested alone, produced a stimulatory effect in untransfected COS-7 cells (data not shown). The discovery of antagonist activity of UDP at the hP2Y$_{14}$-R was also supported by the observation that the UDP analogue, UDPβS, inhibited activation of this receptor by UDP-Glc (Fig. 13). The IC$_{50}$ value observed for UDPβS was similar to that determined for UDP under the same assay conditions. From these results, we conclude that UDP appears to be unique among naturally-occurring nucleotides in its capacity to inhibit UDP-Glc-dependent activation of the hP2Y$_{14}$-R.

Effects of UDP at the rP2Y$_{14}$-R.

Since pharmacological studies often are carried out with rat or mouse tissues, it is important to assess whether receptor orthologs exhibit pharmacological selectivity similar to those of human P2Y-R. Based on the precedent that ATP acts as an antagonist at the human P2Y$_4$-R but is an agonist at the rat P2Y$_4$-R (Bogdanov et al. 1998; Kennedy et al. 2000), we compared the action of UDP at the rP2Y$_{14}$-R with its action at the hP2Y$_{14}$-R. The rat P2Y$_{14}$-R, which exhibits approximately 80% amino acid sequence identity to the hP2Y$_{14}$-R, and almost 90% identity in the transmembrane regions alone, was reported to display a similar UDP-sugar selectivity to that of the hP2Y$_{14}$-R (Freeman et al., 2001), but the actions of other uridine nucleotides on the rP2Y$_{14}$-R have not been reported.

Expression of either the rP2Y$_{14}$-R or Gα$_{q/i}$ in COS-7 cells had no effect on [^{3}H]inositol phosphate accumulation as compared to untransfected cells, but co-expression of receptor and Gα$_{q/i}$ resulted in markedly increased basal accumulation (Fig. 14A). Consistent with other reports (Freeman et al., 2001), UDP-Glc was a potent agonist at the rP2Y$_{14}$-R

(Fig. 14B). Whereas UDP had no effect on inositol phosphate accumulation in wild-type COS-7 cells, in cells expressing $G\alpha_{q/i}$ alone, or in cells expressing the rP2Y$_{14}$-R alone (data not shown), concentration of UDP-dependent increases in formation of [^{3}H]inositol phosphates occurred in COS-7 cells co-expressing the rat P2Y$_{14}$-R with $G\alpha_{q/i}$ (Fig 14B). The maximal stimulatory effect observed with UDP was similar to that observed with UDP-Glc as were the EC50 values of UDP (0.35 μM ± 0.17) and UDP-Glc (EC_{50} = 0.28 μM ± 0.05). No additivity was observed between UDP and UDP-Glc on rP2Y$_{14}$-R-promoted [^{3}H]inositol phosphate formation (Fig. 14C).

Discussion

In this study, we show that UDP is a competitive antagonist at the human P2Y$_{14}$-R, and this action is receptor-selective since UDP does not inhibit agonist-promoted activation of other hP2Y receptors. Moreover, the activity of UDP at the P2Y$_{14}$-R is species-dependent since we observed that UDP is a potent, and apparently full, agonist at the rat P2Y$_{14}$-R.

Chambers et al. (2000) reported in their initial study of the hP2Y$_{14}$-R that UDP has no agonist activity, and we observed similar results in the studies reported here. Using a reporter system in yeast, Ault and Broach (2006) generated a mutant hP2Y$_{14}$-R displaying a mutation in intracellular loop 1 and various mutations in several of the transmembrane regions. This mutant, selected for its ability to support growth of yeast at lower concentrations of UDP-Glc than the wild-type receptor, exhibited an enhanced UDP-Glc-stimulated response that was inhibited by UDP, and a K_B in the micromolar range was reported. The >20-fold higher potency of UDP observed in our studies likely reflects large differences in the assay systems employed. For example, whereas incubations with nucleotide were for minutes in the current study they were for hours in assays measuring

$P2Y_{14}$-R mediated regulation of growth of yeast. Our results illustrate that UDP is a potent competitive antagonist of the wild-type $hP2Y_{14}$-R.

Demonstration of antagonist action at the $hP2Y_{14}$-R suggests that UDP may have broader physiological importance as an extracellular signaling molecule than has been previously appreciated. Both UDP and UDP-glucose are known to be released from cells, although the mechanisms of their release remain unclear. UDP is the most potent and selective agonist of the $hP2Y_6$-R (Lazarowski and Harden, 1994; Communi et al., 1996), and physiological responses attributed to UDP-initiated $P2Y_6$-R-promoted signaling include modulation of IL-8 production in monocytes (Warny et al., 2001) and human mature dendritic cells (Idzko et al., 2004). Additionally, UDP was observed to induce a positive inotropic effect in mouse cardiomyocytes (Wihlborg et al. 2006) and to promote ion transport in human placental cytotrophoblast cells (Roberts et al., 2006). Our data indicate that potential contributions of the $P2Y_{14}$-R to responses associated with UDP must be considered. While reported distribution of $P2Y_6$-R mRNA overlaps with that of $P2Y_{14}$-R mRNA in many cells and tissues, such as lung, heart, placenta, and neutrophils (Communi et al., 1996; Moore et al., 2001), it remains unclear whether the two receptor types are co-expressed in the same cells or in different cells that share the same extracellular space.

The actions of extracellular neurotransmitters and hormones are highly regulated by their release, metabolism, and reuptake. The possibility that direct antagonism of GPCR activation occurs by extracellular signaling molecules has been suggested by observations that ATP is a competitive antagonist of the $hP2Y_{12}$-R (Cusack and Hourani, 1982; Bodor et al., 2003) and of the $hP2Y_4$-R (Bogdanov et al., 1998; Kennedy et al., 2000). The physiological relevance of UDP antagonism at the $hP2Y_{14}$-R will be important to investigate,

as will the idea that UDP simultaneously activates the hP2Y$_6$-R while inhibiting the hP2Y$_{14}$-R.

Our finding that UDP has agonist activity at the rP2Y$_{14}$-R was surprising. Study of the rat and human receptors under identical conditions rules out trivial explanations of this observation. These data do not unambiguously rule out the possibility that UDP is a partial agonist at the human receptor under some conditions. However, the agonist versus antagonist action of the nucleotide at the rat versus human P2Y$_{14}$-R has been observed over a broad range of expression levels of these two receptors. Our activity data suggest that the binding affinity of UDP for the human and rat receptors in fact are quite similar, but development of a radioligand binding assay will be necessary to fully assess this assertion. The fact that the maximal agonist activity of UDP was similar to that of UDP-Glc over a broad range of P2Y$_{14}$-R expression levels (data not shown) suggests that the intrinsic efficacy of UDP at the rat P2Y$_{14}$-R is similar to that of UDP-Glc.

The differential activity of UDP observed between the rat and human orthologs of the P2Y$_{14}$-R shares similarities to the actions of ATP at the P2Y$_4$-R. Kennedy et al. (2000) compared the ligand selectivities of the rat and human P2Y$_4$ receptors under conditions that minimize effects of released nucleotides and of extracellular bioconversion of nucleotides and observed that ATP is an agonist at the rat P2Y$_4$-R and a competitive antagonist at the human P2Y$_4$-R. Residues in the second extracellular loop of the P2Y$_4$ receptor are the primary determinants for the agonist versus antagonist activity of ATP between the two species orthologs (Herold et al., 2004). Like the P2Y$_4$-R, the P2Y$_{14}$-R shares approximately 80% amino acid sequence identity between the rat and human orthologs, with 90% identity when analysis is restricted to transmembrane regions only. Recent work by Ko et al. (2007)

defines the human $P2Y_{14}$ receptor through structure-activity studies in conjunction with molecular modeling studies. Residues in the second extracellular loop of the $hP2Y_{14}$-R are predicted to interact with the diphosphate moiety and hydroxyl groups on the hexose ring of UDP-Glc. Comparative modeling of the rat $P2Y_{14}$-R and receptor mutagenesis directed from these predictions may identify key domains responsible for agonist efficacy at the $hP2Y_{14}$-R.

The mouse $P2Y_{14}$-R has been cloned and is reported to be activated by the same UDP-sugar agonists as the human and rat receptors (Freeman et al., 2001). The rat and mouse $P2Y_{14}$-R share 89% overall amino acid sequence identity and are essentially identical in their transmembrane spanning domains and the second extracellular loop. Thus, we anticipate that the agonist action of UDP observed with the rat receptor will be similarly observed at the mouse $P2Y_{14}$-R.

The finding that UDP acts as a competitive antagonist at the $hP2Y_{14}$-R provides an excellent template for rational synthesis of antagonist analogues that exhibit high affinity at the $hP2Y_{14}$-R. Our structure-activity studies of UDP analogues at the $hP2Y_6$-R (Besada et al., 2006) also provide potential avenues for development of $P2Y_{14}$-R antagonists that do not act as ligands for the $P2Y_6$-R. Synthesis of a hydrolysis-resistant competitive antagonist for the $P2Y_{14}$-R is an obvious goal, as is a high affinity radiolabeled antagonist.

UDP-glucose, UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine previously were identified as full or partial agonists at human and rodent $P2Y_{14}$-R (Chambers et al., 2000; Freeman et al., 2001). The pharmacological selectivity for the $P2Y_{14}$-R is now broadened with the finding that UDP also acts at this receptor. The identification of UDP as a competitive antagonist for the $hP2Y_{14}$-R provides new insight into the physiological

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

Acknowledgments

We appreciate the excellent technical assistance of Sonia de Castro for the synthesis of UP_3U.

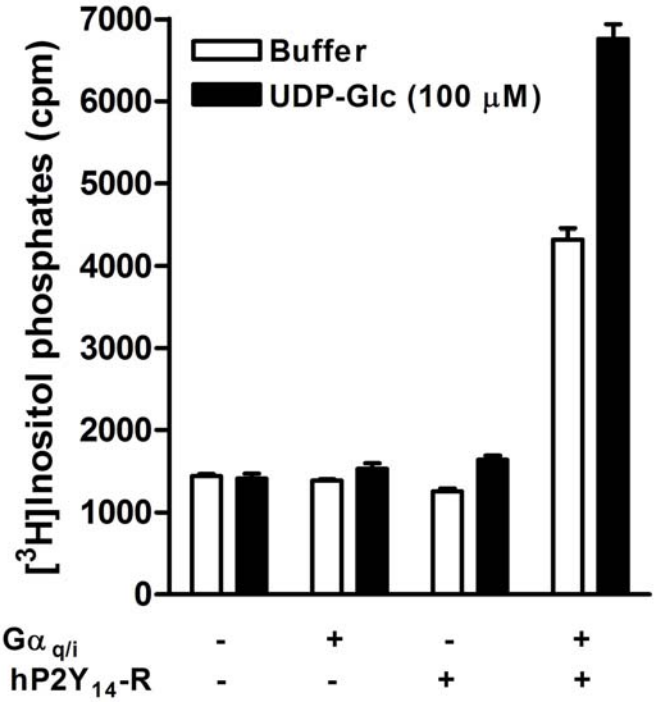


Figure 8. **hP2Y$_{14}$-R- and G$\alpha_{q/i}$-dependent increases in [^{3}H]inositol phosphate accumulation.** COS-7 cells were transfected with empty vector or expression vectors for hP2Y$_{14}$-R and/or G$\alpha_{q/i}$ as described in Methods. Cells were labeled with [^{3}H]inositol for 18 h prior to assay. LiCl (10 mM) buffer was added to the cells to inhibit inositol monophosphatase, and the cells were simultaneously incubated in the absence or presence of 10 μM UDP-Glc for 45 min. [^{3}H]Inositol phosphates were isolated as described in Methods. Data shown are means ± S.E. calculated from triplicate samples and are representative of results obtained in three independent experiments.

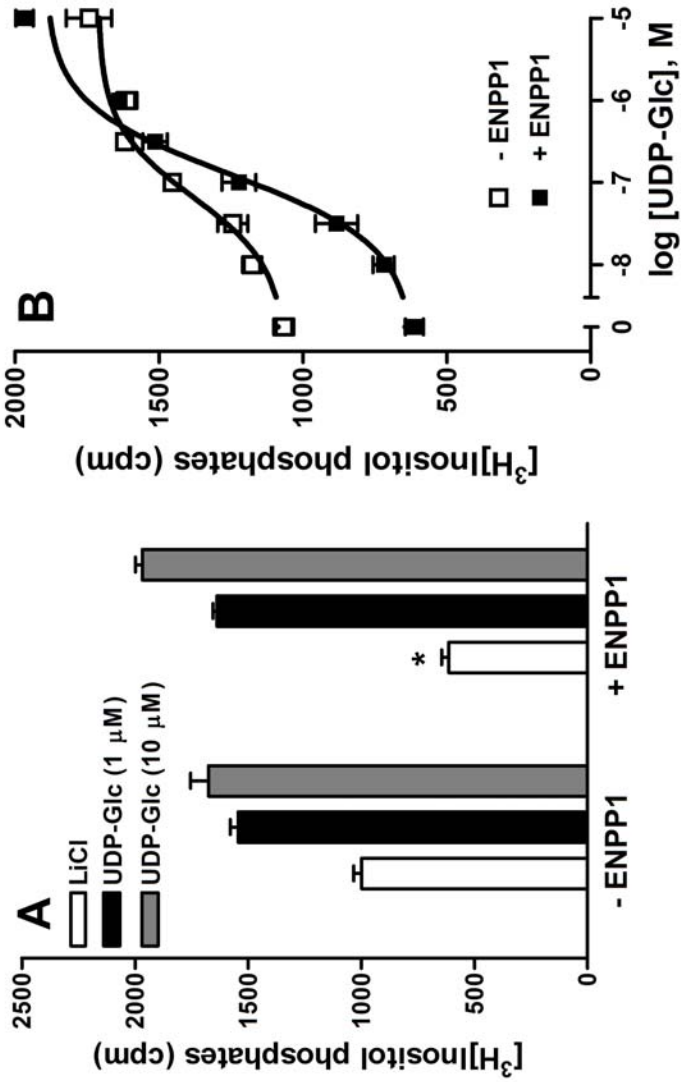


Figure 9. **Co-expression of ENPP1 reduces the basal activation of the hP2Y$_{14}$-R.** COS-7 cells were co-transfected with expression vectors for hP2Y$_{14}$-R and G$\alpha_{q/i}$ with (+) or without (-) an expression vector for ENPP1. A) [^{3}H]Inositol-labeled cells were incubated with 10 mM LiCl in the absence or presence of UDP-Glc. * $p<0.01$ B) [^{3}H]Inositol-labeled cells were incubated with 10 mM LiCl in the absence or presence of UDP-Glc at the indicated concentrations. Data shown are means ± S.E. calculated from triplicate determinations.

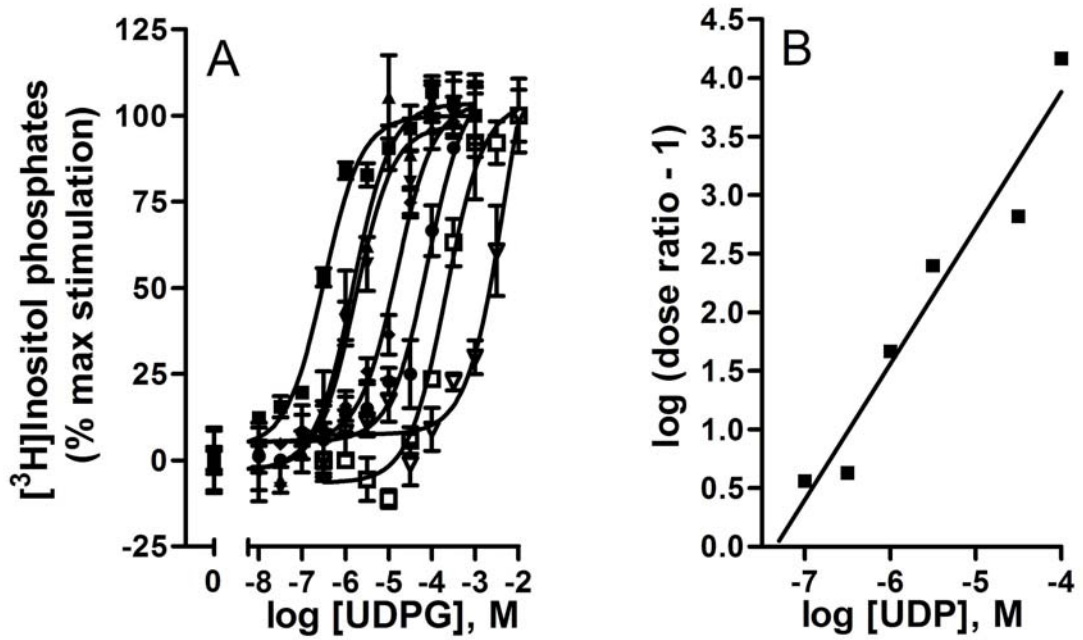


Figure 10. **UDP is a competitive antagonist at the hP2Y$_{14}$-R.** A) [^{3}H]Inositol-labeled COS-7 cells co-expressing hP2Y$_{14}$-R and G$\alpha_{q/i}$ were incubated with LiCl (10 mM) and increasing concentrations of UDP-glucose in the absence or presence of the indicated concentrations of UDP: ■, Buffer; ▲, 0.1 μM; ▼, 0.3 μM; ♦, 1 μM; ●, 3 μM; □, 30 μM; ▽, 100 μM. Data shown are means ± S.E. calculated from triplicate samples and are representative of results obtained in three independent experiments. B) EC50 values from the concentration-effect curves in (A) were used for Schild regression analysis. The data shown are results from a representative experiment repeated three times to yield a mean pK_B of 7.28 ± 0.04 and a slope of 1.15 ± 0.06.

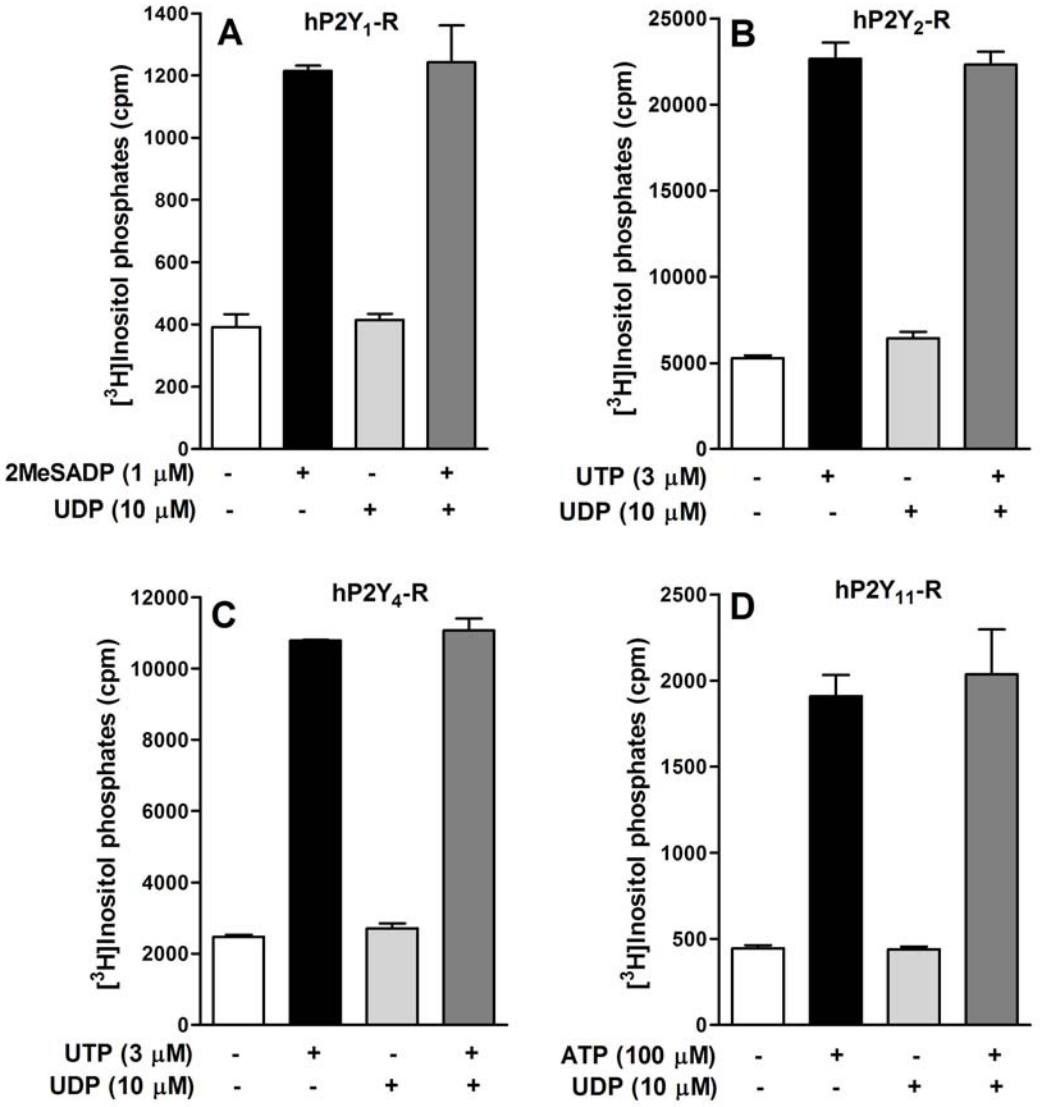


Figure 11. **UDP is a selective antagonist at the hP2Y$_{14}$-R.** [^{3}H]Inositol-labeled 1321N1 human astrocytoma cells stably expressing either the A) human P2Y$_1$-R, B) human P2Y$_2$-R, C) human P2Y$_4$-R, or D) human P2Y$_{11}$-R were incubated for 30 min with 10 mM LiCl with the cognate agonist (indicated), or 10 μM UDP, or both agonist and UDP, and inositol phosphate accumulation was quantified as described in Methods. Data shown are means ± S.E. calculated from triplicate samples and are representative results obtained in three or more independent experiments.

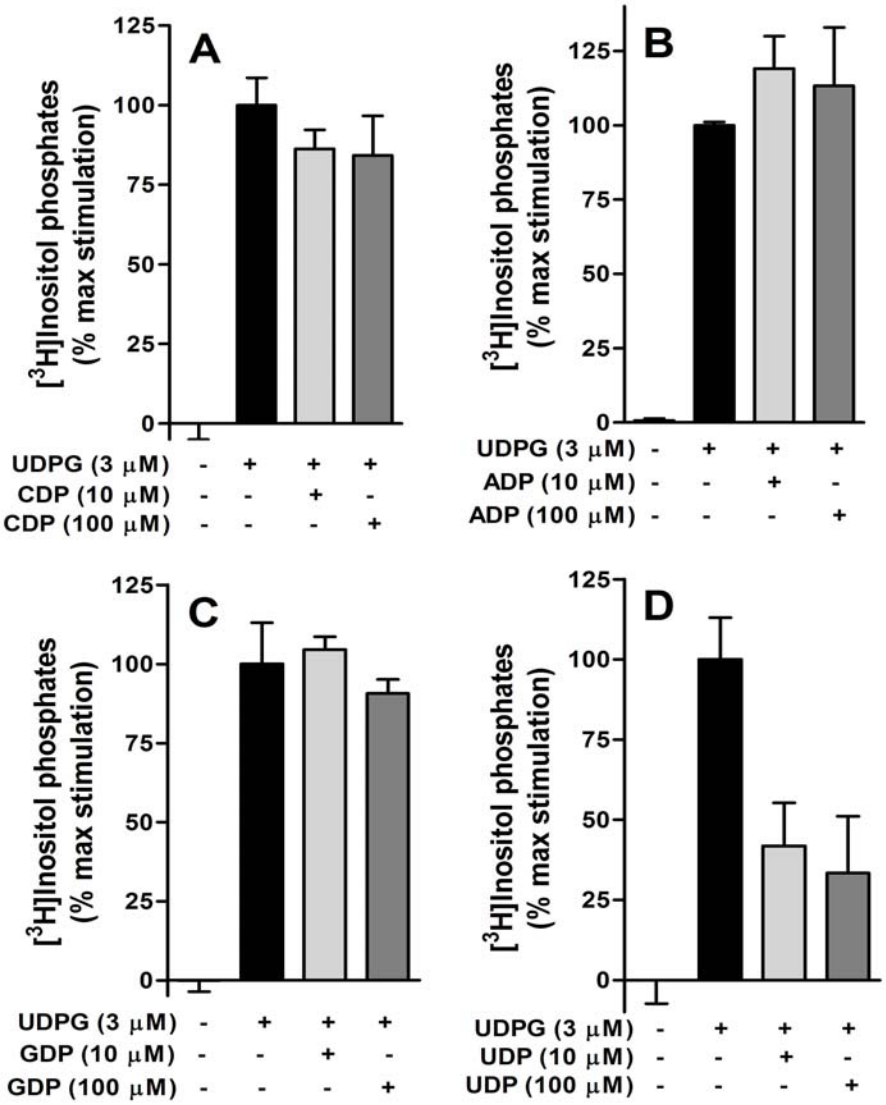


Figure 12. UDP is unique among nucleotide diphosphates for its antagonist effect at the hP2Y$_{14}$-R. A) CDP, B) ADP, C) GDP, or D) UDP (10 μM or 100 μM) and LiCl (10 mM) were applied simultaneously with 3 μM UDP-Glc to [^{3}H]inositol-labeled COS-7 cells transiently expressing hP2Y$_{14}$-R and G$\alpha_{q/i}$. Data were normalized to values from maximal activation of hP2Y$_{14}$-R by UDP-Glc alone, and the LiCl alone value was subtracted from each data point. The data shown in (A), (C), and (D) are results from a representative experiment repeated three times. The data shown in (B) is the average of results from four experiments.

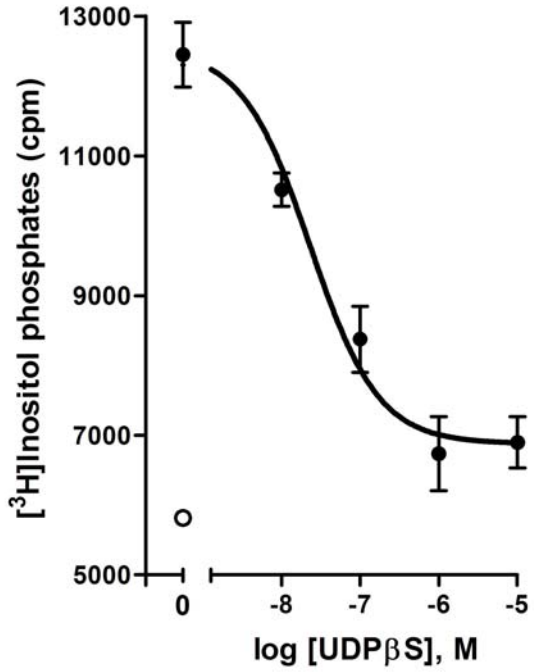


Figure 13. **Antagonist effect of UDPβS at the hP2Y$_{14}$-R.** [^{3}H]inositol-labeled COS-7 cells transiently expressing hP2Y$_{14}$-R and G$\alpha_{q/i}$ were incubated with 10 mM LiCl in the absence (○) or presence (●) of 1 μM UDP-Glc in the presence of the indicated concentrations of UDPβS for 30 min. The data shown are means ± S.E. of triplicate determinations, and the results are representative of those obtained in three experiments.

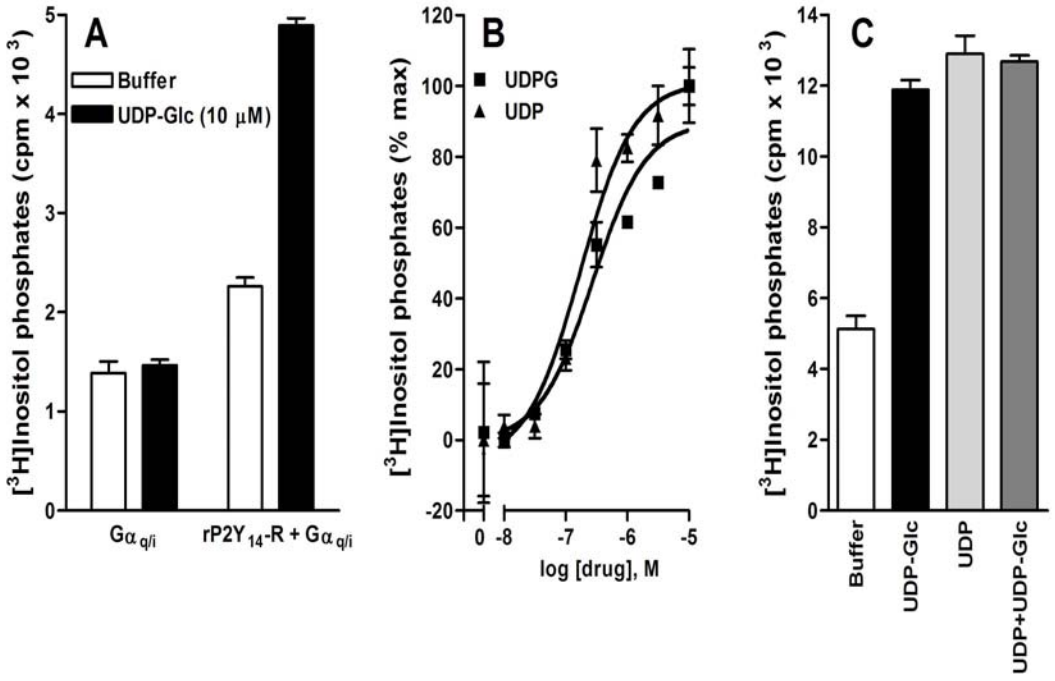


Figure 14. **Agonist effect of UDP-Glc and UDP at the rP2Y$_{14}$-R.** A) COS-7 cells transfected with an expression vector for G$\alpha_{q/i}$ and with empty vector and/ or rP2Y$_{14}$-R were [^{3}H]inositol-labeled and incubated with 10 mM LiCl in the absence or presence of 10 μM UDP-Glc. B) [^{3}H]Inositol-labeled COS-7 cells transiently expressing the rP2Y$_{14}$-R and G$\alpha_{q/i}$ were incubated with 10 mM LiCl and UDP-Glc or UDP at the indicated concentrations, and [^{3}H]inositol phosphate accumulation was quantified as described in Methods. Data shown are means ± S.E. calculated from triplicate samples and are results from a representative experiment repeated three times, yielding an EC50 of 0.28 μM ± 0.05 for UDP-Glc, and 0.35 μM ± 0.17 for UDP. Data were normalized to the maximal activation of rP2Y$_{14}$-R by UDP-Glc. C) Cells incubated with UDP-Glc (10 μM) + UDP (100 μM) exhibited no difference in [^{3}H]inositol phosphate accumulation compared to accumulation in the presence of either agonist alone. Data shown are means ± S.E. calculated from triplicate samples and are representative of results obtained in three independent experiments.

References

Arunlakshana and Schild (1959) Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.* **14**:48-58.

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, Mathe C, Gagneron J, Gosselin G, Maddileti S, Harden TK, and Jacobson KA (2006) Structure-activity relationships of uridine 5'-diphosphate analogues at the human P2Y6 receptor. *J. Med. Chem.* **49**:5532-5543.

Bodor ET, Waldo GL, Hooks SB, Corbitt J, Boyer JL, and Harden TK (2003) Purification and functional reconstitution of the human $P2Y_{12}$ receptor. *Mol. Pharmacol.* **64**:1210-1216.

Bogdanov YD, Wildman SS, Clements MP, King BF, and Burnstock G (1998) Molecular cloning and characterization of rat $P2Y_4$ nucleotide receptor. *Br. J. Pharmacol.* **124**:428-430.

Boyer JL, Siddiqi S, Fischer B, Romero-Avila T, Jacobson KA, and Harden TK (1996) Identification of potent P2Y-purinocepter agonists that are derivatives of adenosine 5'-monophosphate. *Br. J. Pharmacol.* **118**:1959-1964.

Burnstock G (2006) Purinergic signaling. *Br. J. Pharmacol.* **147**:S172-S187.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S, and Livi GP (2000) A G protein-coupled receptor for UDP-glucose. *J. Biol. Chem.* **275**:10767-10771.

Communi D, Parmentier M, and Boynaems JM (1996) Cloning, functional expression and tissue distribution of the human $P2Y_6$ receptor. *Biochem. Biophys. Res. Commun.* **222**:303-308.

Communi D, Govaerts C, Parmentier M, and Boynaems JM (1997) Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J. Biol. Chem.* **272**:31969-31973.

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

Cusack NJ and Hourani SM (1982) Competitive inhibition by adenosine 5'-triphosphate of

the actions on human platelets of 2-chloroadenosine 5'-diphosphate, 2-azidoadenosine 5'-diphosphate and 2-methylthioadenosine 5'-diphosphate. *Br. J. Pharmacol.* **77**:329-333.

Freeman K, Tsui P, Moore D, Emson PC, Vawter L, Naheed S, Lane P, Bawagan H, Herrity N, Murphy K, Sarau HM, Ames RS, Wilson S, Livi GP, and Chambers JK (2001) Cloning, pharmacology, and tissue distribution of G-protein-coupled receptor GPR105 (KIAA0001) rodent orthologs. *Genomics* **78**:124-128.

Fumagalli M, Brambilla R, D'Ambrosi N, Volonte 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**:218-223.

Herold CL, Qi AD, Harden TK, and Nicholas RA (2004) Agonist versus antagonist action of ATP at the $P2Y_4$ receptor is determined by the second extracellular loop. *J. Biol. Chem.* **279**:11456-11464.

Houston D, Ohno M, Nicholas RA, Jacobson KA, and Harden TK (2006) [^{32}P]2-iodo-N6-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate ([^{32}P]MRS2500), a novel radioligand for quantification of native $P2Y_1$ receptors. *Br. J. Pharmacol.* **147**:459-467.

Houston D, Costanzi S, Jacobson KA and Harden TK (2007) Development of Selective High Affinity Antagonists, Agonists, and Radioligands for the P2Y1 Receptor. *Combinatorial Chemistry & High-Throughput Screening*, in press.

Idzko M, Panther E, Sorichter S, Herouy Y, Berod L, Geissler M, Mockenhaupt M, Elsner P, Girolomoni G, and Norgauer J (2004) Characterization of the biological activities of uridine diphosphates in human dendritic cells: Influence on chemotaxis and CXCL8 release. *J. Cell Physiol.* **201**:286-293.

Ivanov AA, Ko H, Cosyn L, Maddileti S, Besada P, Fricks I, Costanzi S, Harden TK, Van Calenbergh S, and Jacobson KA (2007) Molecular Modeling of the Human $P2Y_2$ Receptor and Design of a Selective Agonist, 2'-Amino-2'-deoxy-2-thiouridine 5'-Triphosphate. *J. Med. Chem.,* **50**:1166 -1176.

Jacobson KA, Costanzi S, Ivanov AA, Tchilibon S, Besada P, Gao ZG, Maddileti S, and Harden TK (2006) Structure activity and molecular modeling analyses of ribose- and base-modified uridine 5'-triphosphate analogues at the human $P2Y_2$ and $P2Y_4$ receptors. *Biochem. Pharmacol.* **71**:540-549.

Kennedy C, Qi AD, Herold CL, Harden TK, and Nicholas RA (2000) ATP, an agonist at the rat $P2Y_4$ receptor, is an antagonist at the human $P2Y_4$ receptor. *Mol. Pharmacol.* **57**:926-931.

Ko H, Fricks I, Ivanov AA, Harden TK, and Jacobson KA (2007) Structure activity of uridine 5'-diphosphoglucose (UDP-glucose) analogues as agonists of the human $P2Y_{14}$ receptor. *J. Med. Chem.* **50**:2030-2039.

Lazarowski ER and Harden TK (1994) Identification of a uridine nucleotide-selective G-protein-linked receptor that activates phospholipase C. *J. Biol. Chem.* **269**:11830-11836.

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.

Lazarowski ER (2006) Regulated release of nucleotides and UDP sugars from astrocytoma cells. *Novartis. Found. Symp.* **276**:73-84.

Moore DJ, Chambers JK, Wahlin JP, Tan KB, Moore GB, Jenkins O, Emson PC, and Murdock PR (2001) Expression pattern of human P2Y receptor subtypes: a quantitative reverse transcription-polymerase chain reaction study. *Biochim. Biophys. Acta.* **1521**:107-119.

Muller T, Bayer H, Myrtek D, Ferrari D, Sorichter S, Ziegenhagen MW, Zissel G, Virchow JC, Jr., Luttmann W, Norgauer J, Di Virgilio F, and Idzko M (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.

Nakahata N and Harden TK (1987) Regulation of inositol trisphosphate accumulation by muscarinic cholinergic and H1-histamine receptors on human astrocytoma cells. Differential induction of desensitization by agonists. *Biochem J.* **241**: 337-344.

Nicholas RA, Watt WC, Lazarowski ER, Li Q, and Harden TK (1996) Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol. Pharmacol.* **50**:224-229.

Pendergast W, Yerxa BR, Douglass JG 3rd, Shaver SR, Dougherty RW, Redick CC, Sims IF, and Rideout JL (2001) Synthesis and P2Y receptor activity of a series of uridine dinucleoside 5'-polyphosphates. *Bioorg Med Chem Lett*. **11**:157-160.

Qi AD, Kennedy C, Harden TK, and Nicholas RA (2001) Differential coupling of the human $P2Y_{11}$ receptor to phospholipase C and adenylyl cyclase. *Br. J. Pharmacol.* **132**:318-326.

Roberts VH, Greenwood SL, Elliott AC, Sibley CP, and Waters LH (2006) Purinergic receptors in human placenta: evidence for functionally active P2X4, P2X7, $P2Y_2$, and $P2Y_6$. *Am J Physiol Regul Integr Comp Physiol*. **290**: R1374-1386.

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.

Warny M, Aboudola S, Robson SC, Sevigny J, Communi D, Soltoff SP, and Kelly CP (2001) $P2Y_6$ nucleotide receptor mediates monocytes interleukin-8 production in response to UDP or lipopolysaccharide. *J. Biol. Chem.* **276**: 26051-26056.

Wihlborg AK, Balogh J, Wang L, Borna C, Dou Y, Joshi BV, Lazarowski E, Jacobson KA, Arner A, and Erlinge D (2006) Positive inotropic effects by uridine triphosphate (UTP) and uridine diphosphates (UDP) via $P2Y_2$ and $P2Y_6$ receptors on cardiomyocytes and release of UTP in man during myocardial infarction. *Circ. Res.* **98**: 970-97.

Chapter IV. Gi-dependent cell signaling responses of the human $P2Y_{14}$-receptor in model cell systems

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 $P2Y_{14}$ receptor ($P2Y_{14}$-R). The $P2Y_{14}$-R was stably expressed in HEK293 and C6 rat glioma cells by retroviral infection. Nucleotide sugar-dependent $P2Y_{14}$-R activation was examined by measuring inhibition of forskolin-stimulated cyclic AMP accumulation. The effect of $P2Y_{14}$-R activation on mitogen activated protein kinase (MAPK) signaling also was studied in $P2Y_{14}$-R-HEK293 cells and in differentiated HL-60 human myeloid leukemia cells. UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-N-acetylglucosamine promoted inhibition of forskolin-stimulated cyclic AMP accumulation in $P2Y_{14}$-R-HEK293 and $P2Y_{14}$-R-C6 cells, and this signaling effect was abolished by pretreatment of cells with pertussis toxin. Inhibition of cyclic AMP formation by nucleotide sugars also was observed in direct assays of adenylyl cyclase activity in membranes prepared from $P2Y_{14}$-R-C6 cells. UDP-glucose promoted concentration-dependent and pertussis toxin-sensitive extracellular regulated kinase (ERK) 1/2 phosphorylation in $P2Y_{14}$-R-HEK293 cells. $P2Y_{14}$-R mRNA was not observed in wild type HL-60 cells, but was readily detected in DMSO-differentiated cells. Consistent with this observation, no effect of UDP-glucose was observed in wild type HL-60 cells, but UDP-glucose-promoted pertussis-sensitive activation of ERK1/2 occurred after differentiation. These results illustrate that the human $P2Y_{14}$-R signals through Gi to inhibit adenylyl

cyclase, and $P2Y_{14}$-R activation also leads to ERK1/2 activation. This work also identifies two stable $P2Y_{14}$-R-expressing cell lines and differentiated HL-60 cells as model systems for the study of $P2Y_{14}$-R-dependent signal transduction**.**

Introduction

The $P2Y_{14}$-R is a seven-transmembrane-spanning G-protein coupled receptor that is activated by UDP-glucose (UDP-Glc) and other UDP-sugars (Chambers *et al.*, 2000). $P2Y_{14}$-R mRNA is expressed in stomach, intestine, placental and adipose tissues, lung, heart, and throughout the brain, as well as 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 $P2Y_{14}$-R has been implicated in several immune cell functions. Moore and colleagues (2003) reported that $P2Y_{14}$-R mRNA is upregulated in several brain regions after immunological challenge of mice with lipopolysaccharide. Additionally, UDP-Glc was reported to promote chemotaxis of bone marrow-derived hematopoietic stem cells (Lee *et al.*, 2003).

The $P2Y_{14}$-R is a member of a subgroup of P2Y receptors, which includes the $P2Y_{12}$ and $P2Y_{13}$ receptors thought primarily to activate heterotrimeric G proteins of the Gi family, but a comprehensive understanding of the signal transduction pathways activated by the $P2Y_{14}$-R is not available. Nonetheless, several reports are consistent with the idea that the $P2Y_{14}$-R couples to Gi. For example, Chambers and his colleagues illustrated in their initial study of the cloned human $P2Y_{14}$-R that UDP-glucose promotes pertussis toxin-sensitive binding of radiolabeled GTPγS to membranes prepared from HEK293 cells expressing this receptor. Modest UDP-glucose-promoted inhibition of cyclic AMP accumulation also has been reported for murine T-lymphocytes (Scrivens and Dickenson, 2005), human neutrophils

(Scrivens and Dickenson, 2006), and C6 glioma cells (Krzeminski *et al.*, 2008), although the effects were not shown unambiguously to involve the $P2Y_{14}$-R. Other pharmacological studies of the $P2Y_{14}$-R have relied on coexpression with the promiscuous G protein, Gα16 (Chambers *et al.*, 2000), or with a chimeric Gq (Moore et al., 2003; Fricks et al., 2008) engineered to couple Gi-activating receptors to activation of phosphoinositide hydrolysis and Ca^{2+} mobilization (Coward et al., 1999).

Studies of the $P2Y_{14}$-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, non-hydrolyzable, 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 $P2Y_{14}$-R activation, and with this goal in mind, we generated two different cell lines that stably express the human $P2Y_{14}$-R. Robust $P2Y_{14}$-R-dependent inhibition of adenylyl cyclase was observed in both cell lines, and $P2Y_{14}$-R-dependent MAP kinase signaling was studied in $P2Y_{14}$-R-HEK293 cells. We also discovered that expression of native $P2Y_{14}$-R is induced during differentiation of HL-60 myeloid leukemia cells, and that UDP-glucose promotes activation of MAP kinase signaling in these cells. The model cell systems reported here should provide useful platforms for investigation of the $P2Y_{14}$-R at the cellular and biochemical level.

Methods

Cell Culture

HEK293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37ºC in a 10% CO_2 environment. C6 rat glioma cells were cultured in DMEM supplemented with 5% FBS in a 5% CO_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).

$P2Y_{14}$-R Expression

Human $P2Y_{14}$-R cDNA was amplified and ligated into the retroviral expression vector pLXSN as described previously (Wolff *et al.*, 2005). Retrovirus encoding the $P2Y_{14}$-R was produced in PA317 cells according to the method of Johnson and colleagues (Johnson *et al.*, 1998) and was used to infect HEK293 cells or C6 glioma cells. Geneticin-resistant cells were selected for two weeks in medium containing 0.4 mg/ml G418. Clonal HEK293 cells stably expressing the $hP2Y_{14}$-R 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). Briefly, $P2Y_{14}$-R-C6 rat glioma cells were grown on 150 mm dishes until confluent. Cells were washed gently with PBS and then lysed with ice-cold 1 mM Tris, pH 7.4. Cells were harvested by scraping dishes and homogenized with a glass homogenizer for ten strokes. Lysates were centrifuged at 40,000 x g for 10 min. Membranes were resuspended in 10 mM Tris, pH 7.4, containing 1 mM EDTA and centrifuged again at 40,000 x g for 10 min. Washed membranes were resuspended in assay buffer (25 mM HEPES, pH 7.4, 5 mM MgCl, 150 mM NaCl, 1 mM EDTA) and used immediately.

Cyclic AMP Accumulation

Cells were grown in 24-well plates and incubated with 1 μCi [^{3}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 500 μM 3-isobutyl-1-methyl-xanthine (IBMX), with or without drugs, and incubation continued for 12 min at 37ºC. Incubations were terminated by aspiration of medium and addition of 450 μL ice-cold 5% trichloroacetic acid. [^{3}H]Cyclic AMP was isolated by sequential Dowex and alumina chromatography (Salomon *et al.*, 1974) and quantified by liquid scintillation counting.

Adenylyl Cyclase Activity

Quantification of adenylyl cyclase activity was carried out according to the procedure described previously (Harden *et al.*, 1982). Briefly, assay tubes on ice contained drug or vehicle and a reaction mix of assay buffer containing, at final assay concentrations, 0.01 mM [α-^{32}P]ATP (10-15 cpm/pmol), 0.5 mM [^{3}H]cyclic AMP (10,000 cpm/assay), 8 mM creatine phosphate, creatine phosphokinase (6 U/assay), 0.01 mM GTP, 0.5 mM IBMX, 25 mM HEPES (pH 7.5), 5 mM $MgSO_4$, 2 mM EDTA, and 150 mM NaCl. Assays were initiated by the addition of 100 μg of membrane protein, and the incubations were carried out for 12 min at 30ºC. The reaction was terminated with addition of 0.85 mL of ice-cold 5% trichloroacetic acid. [^{32}P]Cyclic AMP was isolated by sequential Dowex and alumina chromatography and quantified by liquid scintillation counting. Recovery of [^{3}H]cyclic AMP over columns averaged 50-60 %.

MAP Kinase Activation Assays

HEK293 cells were grown on 12-well plates until 70-90% confluent. Cells were serum-starved 24 h prior to assay. Drugs were added to cells for the indicated times, and the assay was terminated by aspiration of medium. The cells were washed once with PBS, and Laemmli buffer containing 60 μM dithiothreitol (DTT) was added to each well. The resultant cell lysates were passed through a 27G needle ten times, heated to 95ºC for five

min, and proteins resolved by electrophoresis on a 12.5% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, blocked with 5% BSA, washed with TBST (20 mM Tris, pH 7.4, 120 mM NaCl, 0.1% Tween20), and then incubated with antibody for phospho-ERK1/2, phospho-p38, or phospho-JNK, according to the manufacturer's directions. After washing with TBST, 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, then incubated with chemiluminescent substrate (Pico West system, Thermo Fisher Scientific, Waltham, MA) and exposed to film. Membranes were stripped with 200 mM glycine, pH 2.6, for 1 h at 25ºC, then re-probed with a primary antibody against total MAP kinase to verify equal loading of lanes. HL-60 cells were serum-starved 24 h prior to the assay, and resuspended in Hanks Buffered Salt Solution for the assay at a density of $5x10^6$ cells/mL, 0.2 mL/assay. Drugs were added for the indicated times, and the cells were lysed by adding one volume of Laemmli buffer containing 60 μM DTT to the cells. Lysates were analyzed as described above.

Data Analysis

EC_{50} values were determined using Prism software (GraphPad, San Diego, CA) and are presented as mean ± SE. Statistical significance was determined by Analysis of Variance (ANOVA), 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 SigmaAldrich (St. Louis, MO). UDP-glucose, UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine were all from FLUKA,

purchased through SigmaAldrich. HPLC analysis of the UDP-Glc used in the current experiments revealed >98% purity. ATP was purchased from GE Healthcare (Piscataway, NJ). Pertussis toxin was purchased from List Biologicals (Campbell, CA). [^{3}H]Adenine and [^{3}H]cyclic AMP were purchased from American Radiolabeled Chemicals (St. Louis, MO). [α-^{32}P]ATP was purchased from PerkinElmer (Waltham, MA). Antibodies for P-ERK1/2, ERK1/2, P-p38, p38, P-JNK, and JNK were purchased from Cell Signaling Technologies (Beverly, MA). All cell culture medium and serum was from Gibco (Invitrogen, Carlsbad, CA). PAR agonist peptide SLIGKV was a generous gift from Dr. Joann Trejo. Anisomysin and sorbitol were kind gifts from Dr. Gary Johnson.

Results

UDP-sugars promote inhibition of forskolin-stimulated cyclic AMP formation in cells stably expressing the human P2Y$_{14}$-R

To examine potential regulation of adenylyl cyclase activity downstream of the human P2Y$_{14}$-R, we stably expressed this receptor in HEK293 cells. UDP-Glc promoted concentration-dependent inhibition of forskolin-stimulated accumulation of cyclic AMP in P2Y$_{14}$-R-expressing HEK293 cells. An EC_{50} value of 82 ± 11 nM (n = 3) was observed for UDP-Glc, and the maximal inhibition of forskolin-stimulated cyclic AMP accumulation ranged from 60-80% (Fig. 15). In contrast, no effect of UDP-Glc was observed on basal or forskolin-stimulated cyclic AMP accumulation in HEK293 cells infected with vector alone. Pre-incubation of P2Y$_{14}$-HEK293 cells with pertussis toxin resulted in complete loss of UDP-Glc-dependent inhibition of cyclic AMP accumulation, indicating that the P2Y$_{14}$-R signals through Gα-subunits of the Gi family.

While UDP-Glc is reported to be a full agonist at the $P2Y_{14}$-R, several studies of the $P2Y_{14}$-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 $P2Y_{14}$-R in $P2Y_{14}$-HEK293 cells. UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine all promoted inhibition of cyclic AMP 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, while UDP-N-acetylglucosamine exhibited an approximately 10-fold higher EC_{50} value (Fig. 16 and Table 4).

We concluded it important to stably express the $P2Y_{14}$-R in several cell backgrounds. Previous studies by our 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 recombinant pLXSN virus harboring the $hP2Y_{14}$-R coding sequence. Again, whereas UDP-Glc had no effect on cyclic AMP accumulation in C6 cells expressing empty vector alone, robust inhibitory effects were observed with UDP-Glc in $P2Y_{14}$-R-expressing C6 cells. An EC_{50} value of 107 ± 68 nM was determined and up to 90% inhibition of forskolin-stimulated accumulation of cyclic AMP occurred in the presence of a maximally effective concentration of UDP-Glc. The action of UDP-Glc in $P2Y_{14}$-R-C6 cells was completely blocked by pre-treatment of the cells with pertussis toxin (Fig. 17). The concentration-dependent inhibition of cyclic AMP formation with other UDP-sugars also was established, and the EC_{50} values are reported in Table 4.

Effects of UDP-sugars on adenylyl cyclase activity in membranes from $P2Y_{14}$-R-expressing C6 cells

Based on the robust $P2Y_{14}$-R-dependent inhibition of cyclic AMP accumulation observed in C6 glioma cells, we also isolated plasma membrane-enriched fractions to determine whether UDP-Glc- and $P2Y_{14}$-R-dependent inhibition of adenylyl cyclase activity could be observed in a cell-free system. While no effect of UDP-Glc was observed in membranes from control cells, approximately 50% inhibition of forskolin-stimulated adenylyl cyclase activity was observed in the presence of 10 μM UDP-Glc in membranes isolated from $P2Y_{14}$-R-C6 glioma cells (Fig. 18). This UDP-Glc-promoted response did not occur in membranes from $P2Y_{14}$-R-C6 cells pretreated with pertussis toxin.

MAP kinase activation by UDP-glucose in $P2Y_{14}$-R-HEK293 cells

Many important biological processes are regulated by MAP kinase signaling pathways. To assess the capacity of the $P2Y_{14}$-R to activate MAP kinase signaling cascades, $P2Y_{14}$-R-HEK293 cells were treated with UDP-Glc and cell lysates were analyzed for phosphorylated ERK1/2 by Western blotting. Whereas no effect was observed in mock-infected cells, UDP-Glc-dependent ERK1/2 phosphorylation occurred in $P2Y_{14}$-R-HEK293 cells (Fig. 19A).

The time course for UDP-Glc activation of ERK1/2 was compared to that occurring as a consequence of activation of the protease activated receptor-2 (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 effects of UDP-Glc on ERK1/2 phosphorylation were concentration-dependent, and the EC_{50} (30 nM) observed was similar to that obtained in

studies of inhibition of cyclic AMP accumulation in these cells (Fig 19C). As was observed in the studies of adenylyl cyclase activity, $P2Y_{14}$-R-regulated ERK1/2 phosphorylation was not observed in cells preincubated with pertussis toxin (Fig. 19B).

The capacity of the $P2Y_{14}$-R to activate p38 and Jun-N terminal kinase (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 19D). In contrast to a robust activation observed in the presence of anisomycin, we also observed no effects of UDP-Glc on JNK phosphorylation (Fig. 19D).

Functional $P2Y_{14}$-R are endogenously expressed in differentiated HL-60 cells

With the goal of identifying a cell line that natively expresses the $P2Y_{14}$-R, we tested by RT-PCR several candidate cell lines previously reported to express $P2Y_{14}$-R mRNA, as well as 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 $P2Y_{14}$-R mRNA was detected in undifferentiated HL-60 cells, differentiation of these cells with 1.3% DMSO to a neutrophil-like cell resulted in a marked increase in expression of $P2Y_{14}$-R mRNA (data not shown).

We also investigated the cell signaling responses discussed above to determine whether functional $P2Y_{14}$-R could be observed in differentiated HL-60 cells. In multiple experiments, 100 μM UDP-Glc exhibited no effect on cyclic AMP accumulation promoted by either forskolin, amthamine, an 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 cyclic AMP accumulation in these cells.

Potential activation of the MAP kinase signaling pathway also was studied in HL-60 cells. Whereas 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. 20A). 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. 20B). Neither UDP-Glc- nor fMLP-dependent ERK1/2 activation was observed in cells pre-incubated with pertussis toxin (Fig 20C), 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 and colleagues who illustrated both basal and mechanically-induced release of UDP-Glc from multiple mammalian cell types (Lazarowski *et al.*, 2003).

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-glucose, UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine all were previously reported 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.*, 2000; Freeman *et al.*, 2001). Our results measuring responses of a native signaling pathway also indicate that these four UDP-sugars are full agonists and exhibit relatively similar potencies. It remains to be elucidated which one or more of these molecules is the endogenous agonist(s) for the receptor. Indeed, it is likely that multiple UDP-sugars are physiological agonists for the $P2Y_{14}$-R, such that the activating ligand may differ according to tissue or cell type.

Our findings that neither UDP-Glc nor fMLP had an effect on stimulated cyclic AMP accumulation in differentiated HL-60 cells indicates that activated Gi does not couple to inhibition of adenylyl cyclase in these cells. Perhaps the isoforms of adenylyl cyclase expressed in these cells are not subject to inhibition by Gi. HL-60 cells gain a neutrophil phenotype upon differentiation, and therefore are likely to reflect receptor-promoted signaling responses that are characteristic of neutrophils. Receptor-promoted inhibition of adenylyl cyclase has not been unequivocally demonstrated in neutrophils, and we are unaware of studies clearly defining the signaling pathways downstream of Gi-coupled

receptors in this tissue. A small inhibition of cyclic AMP accumulation was reported to occur upon incubation of neutrophils with UDP-Glc (Scrivens and Dickenson, 2006). However, since none of the other $P2Y_{14}$-R agonists inhibited forskolin-promoted cyclic AMP formation in neutrophils, it is unclear whether this effect was in fact dependent on either UDP-Glc or the $P2Y_{14}$-R.

Gi-coupled GPCR predictably activate MAP kinase signaling, and we illustrate here that UDP-Glc-dependent activation of ERK1/2 occurs in $P2Y_{14}$-R-HEK293 cells. Although $P2Y_{14}$-R were not detected in undifferentiated HL-60 cells, message for the $P2Y_{14}$-R was observed upon differentiation of HL-60 cells, and occurrence of UDP-Glc-promoted MAP kinase signaling indicated the presence of functional $P2Y_{14}$-R. HL-60 cells have been used as a model of neutrophil-like cells that undergo differentiation-associated morphological and functional changes including up-regulation of chemoattractant receptors and the capacity to chemotax (Hauert *et al.*, 2002). Differentiated HL-60 cells exhibit capacities to generate superoxide, ingest particles, and degranulate with efficiencies similar to that of neutrophils (Newburger *et al.*, 1979). In addition, they have distinct technical advantages over primary cells, such as viability and transfectability. Differentiated HL-60 cells also have been used as a model system for studies of FPR, and consistent with previous observations (Rane et al., 1997; Christophe et al., 2002; Paruch et al., 2006), we observed fMLP-promoted ERK1/2 activation in differentiated HL-60 cells. These findings establish differentiated HL-60 cells as a model system for investigation of biological functions of the $P2Y_{14}$-R.

The duration of $P2Y_{14}$-R-promoted ERK1/2 phosphorylation in HEK293 cells and HL-60 cells was prolonged compared with that of other G protein coupled receptors. PAR2, which promoted very transient ERK1/2 activation in HEK293 cells, is known to couple to Gi,

as well as to G12/13 and Gq (Fyfe *et al.*, 2005). The fMLP receptor, FPR, has been reported to couple to Gi, but the mechanism linking FPR to ERK1/2 phosphorylation is not clear (Selvatici et al., 2006; Huet et al., 2007; Kam 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 Gα-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. Observation of receptor-specific differences in the time course of stimulation of ERK1/2 phosphorylation has led to the suggestion that short term (e.g. 5 min) versus long-term receptor-dependent activation (e.g. 30-60 min) may promote distinct cellular functions. For example, short-term ERK1/2 activation may be important for cell migration whereas sustained ERK1/2 activation may play a more critical role in cell proliferation (Luttrell, 2005; May and Hill, 2008). 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 as well as its potential role in neutrophil biology.

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Table 4. **EC_{50} values for P2Y14-R agonists in P2Y14-HEK293 and P2Y14-C6 cells.** [3H]Adenine-labeled cells were treated with IBMX (200 μM), forskolin (30 μM), and varying concentrations of either UDP-glucose, UDP-galactose, UDP-glucuronic acid, or UDP-N-acetylglucosamine for 15 min. [3H]Cyclic AMP was quantified as described in Methods. EC_{50} values were determined with GraphPad Prism software and are presented as mean ± SE of results from three experiments.

Agonist	**$P2Y_{14}$-HEK293** **EC_{50} ± SE, nM**	**$P2Y_{14}$-C6** **EC_{50} ± SE, nM**
UDP-glucose	**82 ± 11**	**92 ± 51**
UDP-galactose	**96 ± 29**	**240 ± 53**
UDP-glucuronic acid	**60 ± 7**	**108 ± 66**
UDP-N-acetylglucosamine	**919 ± 205**	**225 ± 68**

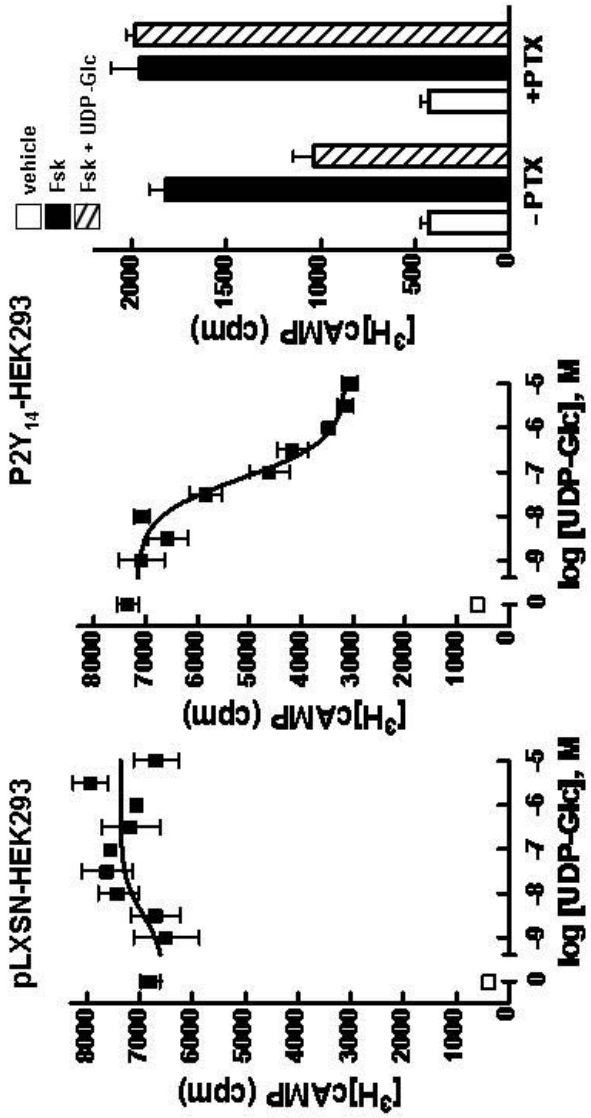

Figure 15. **Concentration-dependent and pertussis toxin-sensitive inhibition of cyclic AMP accumulation promoted by UDP-Glc in $P2Y_{14}$-HEK293 cells.** Empty vector-infected (left panel) or $P2Y_{14}$-R-expressing (middle panel) cells were labeled with [^{3}H]adenine 18 h prior to assay. Cells were incubated with 200 μM IBMX in the absence (□) or presence of 30 μM forskolin and the indicated concentrations of UDP-Glc (■) for 12 min prior to quantification of [^{3}H]cyclic AMP accumulation. $P2Y_{14}$-R-expressing (right panel) cells were preincubated with 100 ng/mL pertussis toxin for 4 h,and [^{3}H]cyclic AMP accumulation was measured in the presence of 200 μM IBMX alone (open bars), 200 μM IBMX + 30 μM forskolin (filled bars), or 200 μM IBMX + 30 μM forskolin + 10 μM UDP-Glc (hatched bars). The data shown are presented as mean ± SE and are representative of results from three independent experiments.

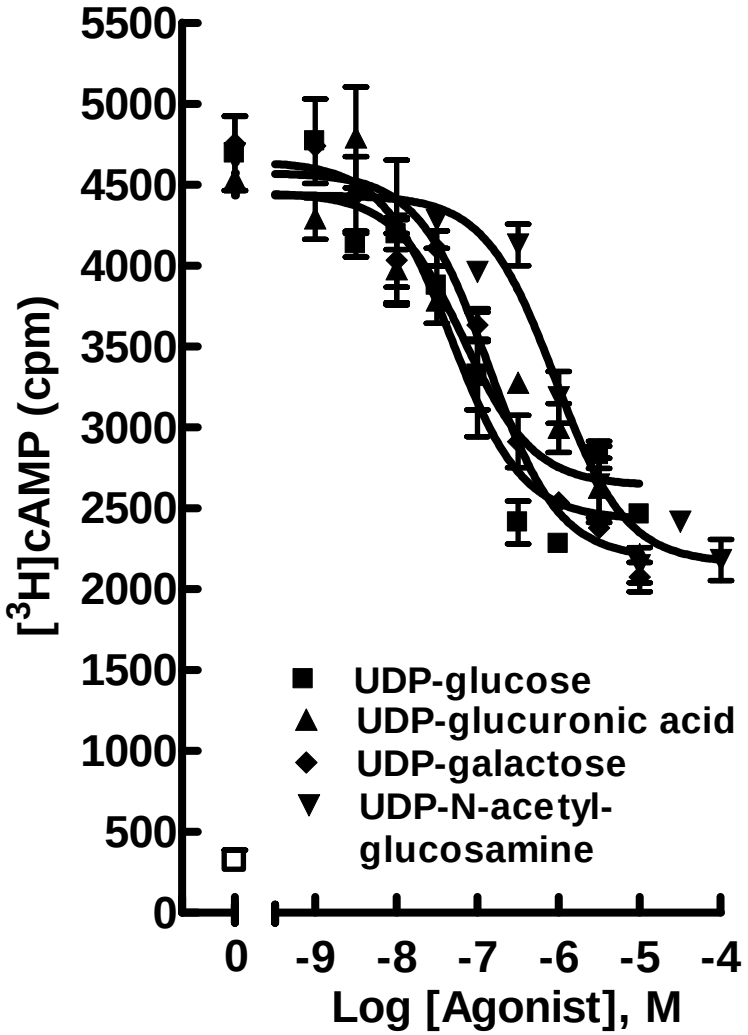

Figure 16. UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine inhibit accumulation of cyclic AMP in $P2Y_{14}$-HEK293 cells. [^{3}H]Adenine-labeled $P2Y_{14}$-HEK293 cells were incubated with 200 μM IBMX alone (□) or IBMX with 30 μM forskolin and varying concentrations of UDP-glucose (■), UDP-galactose (♦), UDP-glucuronic acid (▲), or UDP-N-acetylglucosamine (▼). Data shown are mean ± SE and are representative of results of three independent experiments.

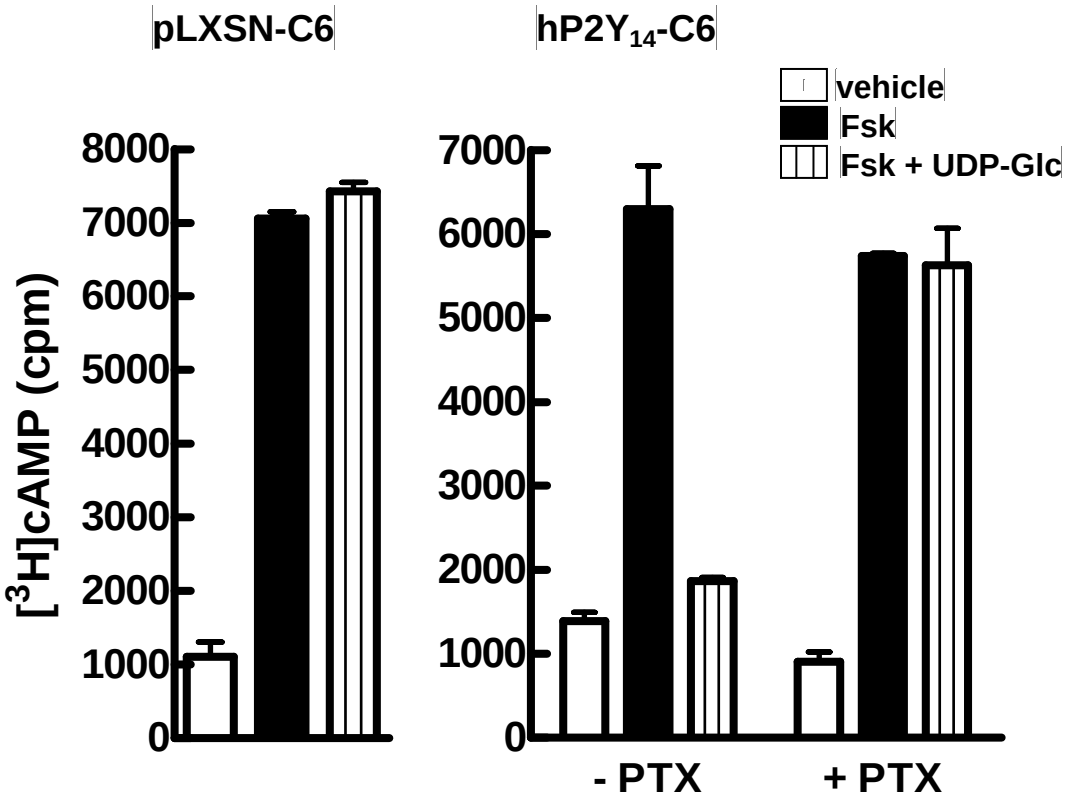

Figure 17. **UDP-Glc promotes pertussis toxin-sensitive inhibition of cyclic AMP accumulation in $P2Y_{14}$-C6 cells.** Empty vector-infected (left panel) C6 rat glioma cells were prelabeled with [^{3}H]adenine and then incubated in the presence of 200 μM IBMX alone (open bar), 200 μM IBMX + 30 μM forskolin (filled bar), or 200 μM IBMX + 30 μM forskolin + 10 μM UDP-Glc (striped bar). $P2Y_{14}$-C6 cells (right panel) were incubated in the absence or presence of 100 ng/mL pertussis toxin prior to quantification of [^{3}H]cyclic AMP accumulation in the presence of 200 μM IBMX alone (open bars), 200 μM IBMX + 30 μM forskolin (filled bars), or 200 μM IBMX + 30 μM forskolin + 10 μM UDP-Glc (striped bars). Data shown are presented as mean ± SE and are representative of results from three independent experiments.

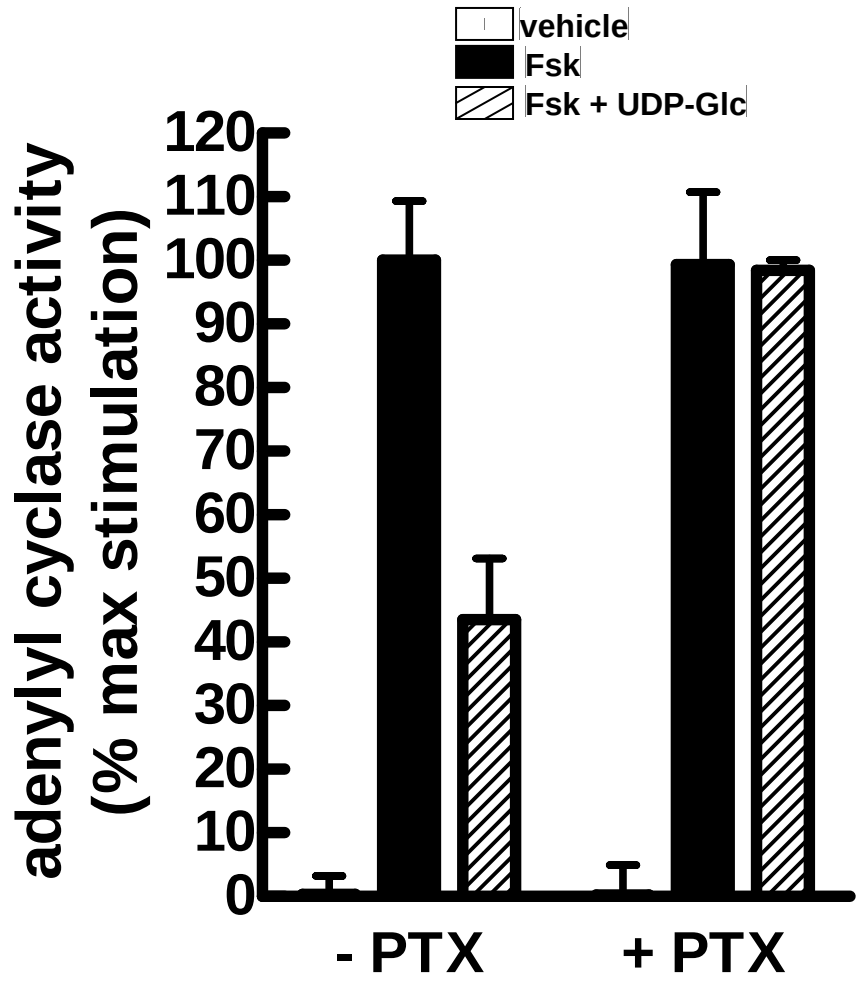

Figure 18. **UDP-Glc-dependent inhibition of adenylyl cyclase by UDP-Glc in membranes from $P2Y_{14}$-C6 cells.** Membranes were isolated from $P2Y_{14}$-C6 cells or from cells pretreated with 100 ng/mL pertussis toxin overnight. Membranes were incubated with 200 μM IBMX in the absence (open bars) or presence (filled bars) of 30 μM forskolin, or with 200 μM IBMX + 30 μM forskolin + 10 μM UDP-Glc as described in Methods. [^{32}P]Cyclic AMP was isolated by sequential Dowex-alumina chromatography. Data shown are the mean ± SE of three independent experiments, plotted as a percentage of maximal forskolin-stimulated enzyme activity.

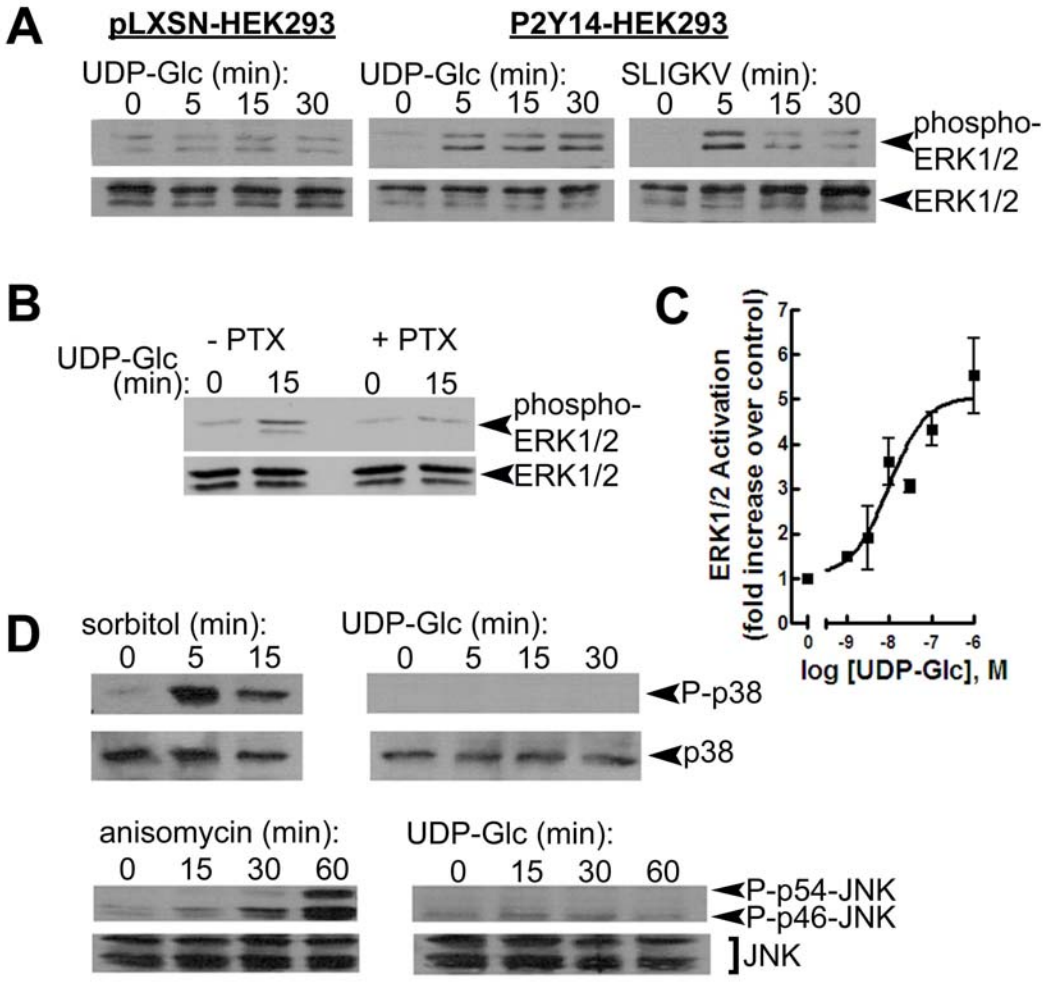

Figure 19. **UDP-Glc-promoted activation of MAP kinase signaling in $P2Y_{14}$-HEK293 cells.** A) Empty vector or P2Y14-R-expressing HEK293 cells were serum-starved for 18 h and then incubated with 100 μM UDP-Glc or 100 μM SLIGKV for the times indicated. Samples were probed for phospho-ERK1/2 and ERK1/2 as described in Methods. 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. 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 (Frederick, MD) and graphed using GraphPad Prism software (San Diego, CA). 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 ± SE of four independent experiments. D) $P2Y_{14}$-HEK293 cells were treated with either 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 in Methods.

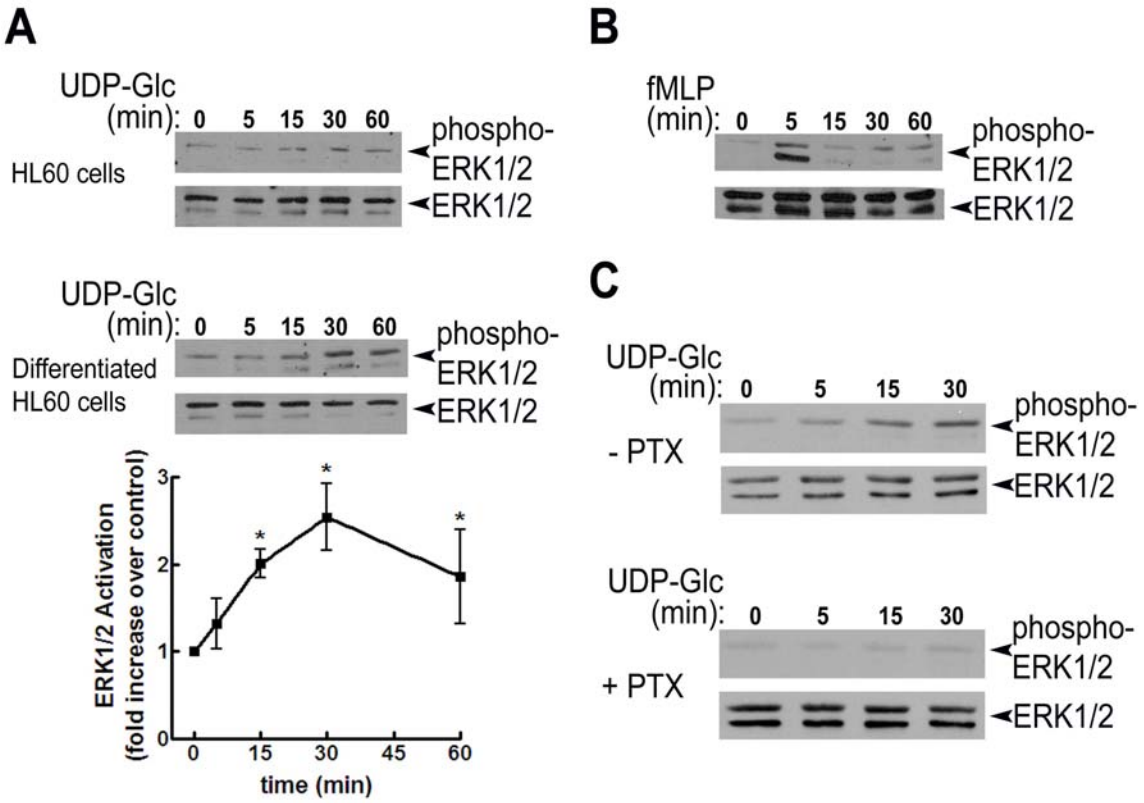

Figure 20. **UDP-Glc-promoted activation of MAP kinase activation in differentiated HL-60 cells.** A) Wild-type or differentiated (see Methods) HL-60 cells were serum-starved for 18 h prior to addition of 100 μM UDP-Glc and incubation continued for the indicated times. Western blots for phospho-ERK1/2 and ERK1/2 were generated as described in Methods. Phospho-ERK1/2 was quantified using Scion Image software (Frederick, MD) and graphed using GraphPad Prism software (San Diego, CA). 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 ± SE of six independent experiments (*$p < 0.02$). B) 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 in Methods. C) Differentiated HL-60 cells were treated in the absence (-PTX) or presence (+PTX) of 200 ng/mL pertussis toxin for 4 h prior to 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 in Methods.

References

Amadesi S, Nie J, Vergnolle N, Cottrell GS, Grady EF, Trevisani M, Manni C, Geppetti P, McRoberts JA, Ennes H, Davis JB, Mayer EA and Bunnett NW (2004) Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. *J Neurosci* **24**:4300-4312.

Boulay F, Tardif M, Brouchon L and Vignais P (1990) The human N-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. *Biochemistry* **29**:11123-11133.

Castillo CA, Albasanz JL, Fernandez M and Martin M (2007) Endogenous expression of adenosine A1, A2 and A3 receptors in rat C6 glioma cells. *Neurochem Res* **32**:1056-1070.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S and Livi GP (2000) A G protein-coupled receptor for UDP-glucose. *J Biol Chem* **275**:10767-10771.

Christophe T, Karlsson A, Rabiet MJ, Boulay F and Dahlgren C (2002) Phagocyte activation by Trp-Lys-Tyr-Met-Val-Met, acting through FPRL1/LXA4R, is not affected by lipoxin A4. *Scand J Immunol* **56**:470-476.

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

Dai Y, Wang S, Tominaga M, Yamamoto S, Fukuoka T, Higashi T, Kobayashi K, Obata K, Yamanaka H and Noguchi K (2007) Sensitization of TRPA1 by PAR2 contributes to the sensation of inflammatory pain. *J Clin Invest* **117**:1979-1987.

Esther CR, Jr., Sesma JI, Dohlman HG, Ault AD, Clas ML, Lazarowski ER and Boucher RC (2008) Similarities between UDP-glucose and adenine nucleotide release in yeast: involvement of the secretory pathway. *Biochemistry* **47**:9269-9278.

Freeman K, Tsui P, Moore D, Emson PC, Vawter L, Naheed S, Lane P, Bawagan H, Herrity N, Murphy K, Sarau HM, Ames RS, Wilson S, Livi GP and Chambers JK (2001) Cloning, pharmacology, and tissue distribution of G-protein-coupled receptor GPR105 (KIAA0001) rodent orthologs. *Genomics* **78**:124-128.

Fricks IP, Maddileti S, Carter R, 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.

Fyfe M, Bergstrom M, Aspengren S and Peterson A (2005) PAR-2 activation in intestinal epithelial cells potentiates interleukin-1beta-induced chemokine secretion via MAP kinase signaling pathways. *Cytokine* **31**:358-367.

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.

Hauert AB, Martinelli S, Marone C and Niggli V (2002) Differentiated HL-60 cells are a valid model system for the analysis of human neutrophil migration and chemotaxis. *Int J Biochem Cell Biol* **34**:838-854.

Huet E, Boulay F, Barral S and Rabiet MJ (2007) The role of beta-arrestins in the formyl peptide receptor-like 1 internalization and signaling. *Cell Signal* **19**:1939-1948.

Johnson LG, Mewshaw JP, Ni H, Friedmann T, Boucher RC and Olsen JC (1998) Effect of host modification and age on airway epithelial gene transfer mediated by a murine leukemia virus-derived vector. *J Virol* **72**:8861-8872.

Kam AY, Liu AM and Wong YH (2007) Formyl peptide-receptor like-1 requires lipid raft and extracellular signal-regulated protein kinase to activate inhibitor-kappa B kinase in human U87 astrocytoma cells. *J Neurochem* **103**:1553-1566.

Klinker JF, Wenzel-Seifert K and Seifert R (1996) G-protein-coupled receptors in HL-60 human leukemia cells. *Gen Pharmacol* **27**:33-54.

Kreda SM, Okada SF, van Heusden CA, O'Neal W, Gabriel S, Abdullah L, Davis CW, Boucher RC and Lazarowski ER (2007) Coordinated release of nucleotides and mucin from human airway epithelial Calu-3 cells. *J Physiol* **584**:245-259.

Kreda SM, Seminario-Vidal L, Heusden C and Lazarowski ER (2008) Thrombin-promoted release of UDP-glucose from human astrocytoma cells. *Br J Pharmacol* **153**:1528-1537.

Krzeminski P, Pomorski P and Baranska J (2008) The P2Y14 receptor activity in glioma C6 cells. *Eur J Pharmacol* **594**:49-54.

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.

Lee BC, Cheng T, Adams GB, Attar EC, Miura N, Lee SB, Saito Y, Olszak I, Dombkowski D, Olson DP, Hancock J, Choi PS, Haber DA, Luster AD and Scadden DT (2003) P2Y-like receptor, GPR105 ($P2Y_{14}$), identifies and mediates chemotaxis of bone-marrow hematopoietic stem cells. *Genes Dev* **17**:1592-1604.

Luttrell LM (2005) Composition and function of G protein-coupled receptor signalsomes controlling mitogen-activated protein kinase activity. *J Mol Neurosci* **26**:253-264.

May LT and Hill SJ (2008) ERK phosphorylation: spatial and temporal regulation by G protein-coupled receptors. *Int J Biochem Cell Biol* **40**:2013-2017.

Moore DJ, Murdock PR, Watson JM, Faull RL, Waldvogel HJ, Szekeres PG, Wilson S, Freeman KB and Emson PC (2003) GPR105, a novel Gi/o-coupled UDP-glucose receptor expressed on brain glia and peripheral immune cells, is regulated by immunologic challenge: possible role in neuroimmune function. *Brain Res Mol Brain Res* **118**:10-23.

Newburger PE, Chovaniec ME, Greenberger JS and Cohen HJ (1979) Functional changes in human leukemic cell line HL-60. A model for myeloid differentiation. *J Cell Biol* **82**:315-322.

Paruch S, El-Benna J, Djerdjouri B, Marullo S and Perianin A (2006) A role of p44/42 mitogen-activated protein kinases in formyl-peptide receptor-mediated phospholipase D activity and oxidant production. *FASEB J* **20**:142-144.

Rane MJ, Carrithers SL, Arthur JM, Klein JB and McLeish KR (1997) Formyl peptide receptors are coupled to multiple mitogen-activated protein kinase cascades by distinct signal transduction pathways: role in activation of reduced nicotinamide adenine dinucleotide oxidase. *J Immunol* **159**:5070-5078.

Salomon Y, Londos C and Rodbell M (1974) A highly sensitive adenylate cyclase assay. *Anal Biochem* **58**:541-548.

Schachter JB, Boyer JL, Li Q, Nicholas RA and Harden TK (1997) Fidelity in functional coupling of the rat P2Y1 receptor to phospholipase C. *Br J Pharmacol* **122**:1021-1024.

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.

Selvatici R, Falzarano S, Mollica A and Spisani S (2006) Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils. *Eur J Pharmacol* **534**:1-11.

Servant G, Weiner OD, Herzmark P, Balla T, Sedat JW and Bourne HR (2000) Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* **287**:1037-1040.

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.

Smith MM and Harden TK (1985) Muscarinic cholinergic receptor-mediated attenuation of adenylate cyclase activity in rat heart membranes. *J Cyclic Nucleotide Protein Phosphor Res* **10**:197-210.

Thomas EA, Matli JR, Hu JL, Carson MJ and Sutcliffe JG (2000) Pertussis toxin treatment prevents 5-HT(5a) receptor-mediated inhibition of cyclic AMP accumulation in rat C6 glioma cells. *J Neurosci Res* **61**:75-81.

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**:C624-632.

Chapter V. Conclusions

Ligand development studies have met with considerable success for the $P2Y_1$-R and $P2Y_{12}$-R, and we have applied a structure-activity relationship approach to develop ligands for the $P2Y_{14}$-R. Additionally, molecular modeling studies have been useful in guiding these studies. Our studies on the $P2Y_{14}$-R are focused on developing high affinity, selective, and non-hydrolyzable agonists and antagonists. These studies, reviewed in Chapter 2, have yielded several novel agonists at the $P2Y_{14}$-R, including 2-thio-UDP-Glc, an agonist that exhibits six-fold greater potency than UDP-Glc (Ko et al., 2007). Molecular modeling studies have aided our conceptualization of the $P2Y_{14}$-R tertiary structure (Ivanov et al., 2007), and guided a methodical analysis of the parameters of the ligand pharmacophore. Identification of the hexose moiety of UDP-Glc as a fertile region for modification leads to a newly focused approach for future development of ligands. Ongoing studies are aimed toward producing pharmacological tools that will be useful in further characterization of the $P2Y_{14}$-R. A high affinity, selective radioligand would be useful for binding assays, while a fluorophore-conjugated ligand could be useful for monitoring receptor expression and localization with microscopy.

As a result of our ligand development studies, it is clear that most UDP-sugars activate the $P2Y_{14}$-R (Ko et al., 2009). Release of UDP-Glc from cells has been established (Lazarowski et al., 2003; Kreda et al., 2007; Kreda et al., 2008) and it is likely that other UDP-sugars are released from cells as well. Our finding that other UDP-sugars, in addition to the originally identified four, are agonists at the $P2Y_{14}$-R

necessitates our re-thinking the profile of cognate agonists for the $P2Y_{14}$-R and specifically, we must consider the possibility that the $P2Y_{14}$-R has many cognate agonists. Most UDP-sugars are equipotent at the $P2Y_{14}$-R and so could theoretically activate the receptor in place of UDP-Glc. Depending on the extracellular environment at the site of $P2Y_{14}$-R expression, one or more UDP-sugars may activate the $P2Y_{14}$-R endogenously.

The $P2Y_{14}$-R was classified as a $P2Y_{12}$-like receptor based on sequence homology, and has been suspected to couple to the Gi family of G proteins, as does the $P2Y_{12}$-R. With the work presented in Chapter 4, we unambiguously established that the $P2Y_{14}$-R couples to inhibition of adenylyl cyclase stimulation, and to ERK1/2 phosphorylation, in an agonist-dependent and pertussis toxin-sensitive manner (Fricks et al., submitted). While the inhibition of adenylyl cyclase activity is likely to occur through Gi activation, the signaling from Gi-coupled receptors to activate MAPK is not well delineated, although previous evidence suggests that it may be dependent on Gβγ. Both $G\alpha_i$ subunits and Gβγ dimers released from activated Gi have been reported to activate ERK1/2 through several distinct mechanisms, however the mechanism by which $P2Y_{14}$-R couples to ERK1/2 phosphorylation is unknown.

Two mechanisms for Gi-dependent activation of ERK1/2 have been proposed. $G\alpha_{i2}$ was reported to interact directly with Rap-1-GAP, suggesting that activated $G\alpha_{i2}$ would lead to inactive Rap-1 (Pace et al., 1995; Mochizuki et al., 1999). Because active Rap-1 sequesters C-Raf away from Ras, alleviation of the Rap/Raf interaction would allow Raf to interact with Ras and consequently activate MAPK signaling downstream. Another model of Gi stimulation of ERK1/2 implicates signaling downstream of adenylyl cyclase.

Upon inhibition of adenylyl cyclase, PKA activation concomitantly decreases, resulting in alleviation of PKA inhibition of C-Raf (Tang and Gilman, 1992; Radhika and Dhanasekaran, 2001). Raf is then free to interact with Ras and activate the MAPK cascade.

Evidence for Gβγ-dependent activation of MAPK has been reported for multiple Gi-coupled GPCRs, including the M2 muscarinic receptor (Lopez-Ilasaca et al., 1997). The β1-adrenergic receptor also is reported to activate MAPK through a Gβγ-dependent mechanism that is partially sensitive to pertussis toxin (Galandrin et al., 2008). Each of the proposed signaling pathways converges on Ras, although the intermediates appear to be different depending on the receptor. Recombinant α2A-adrenergic receptors in HEK293 cells and COS-7 cells have been suggested to use a pathway involving PLCβ and the intracellular calcium-activated Pyk2 kinase, which would activate Src, bringing together the Ras-activating complex Shc and SOS (Della Rocca et al., 1997). A second, distinct pathway involving phosphatidyl-inositol-3-kinase (PI3K) is implicated in activation of ERK1/2 by LPA and thrombin receptors. This model predicts that PI3K activates a not yet identified tyrosine kinase, which would, in turn, promote formation of the Ras-activating complex dynamin II/ Grb2/ SOS (Kranenburg et al., 1997; Kranenburg et al., 1999; Wunderlich et al., 1999). The potential for the $P2Y_{14}$-R to use one or more of these signaling pathways to activate ERK1/2 could be explored with the use of the Gβγ-sequestering carboxyl tail of β-adrenergic receptor kinase (β-ARK) (Inglese et al., 1994) to determine whether the βγ dimer is necessary for MAPK signaling. Further studies could utilize pharmacological inhibitors of protein intermediates to identify which proteins are involved in the $P2Y_{14}$-R-dependent activation of MAPK.

Alternatively to signals downstream of the G protein, the involvement of β-arrestins in agonist-dependent transduction to MAPK cascades has been reported for some GPCRs. In addition to their role in mediating endocytosis of ligand-bound receptor, β-arrestins also act as scaffolding proteins that link some GPCRs, such as the β_2-adrenergic receptor, to MAPK cascades (Luttrell et al., 1999). In the proposed model, β-arrestin scaffolds the ligand-bound receptor in a complex with the tyrosine kinase Src, subsequently activating Ras-mediated MAPK cascades. The β-arrestins have also been proposed to act as a scaffold protein for the complex associating Raf, MEK1, and ERK (DeFea et al., 2000; Luttrell et al., 2001). While the β-arrestin mechanism of GPCR-induced MAPK signaling has been described for few GPCRs, the array of possible pathways to determine the mechanism of $P2Y_{14}$-R-dependent MAPK activation will require extensive investigation.

In addition to the tissue specific $G\alpha_t$, $G\alpha_z$, and $G\alpha_{gust}$, the Gi family of proteins includes $G\alpha i_1$, $G\alpha i_2$, $G\alpha i_3$, and Gαo. Which Gi proteins may be preferred by the $P2Y_{14}$-R will be a topic of future study. Indeed which Gi isoform couples to $P2Y_{14}$-R may be dependent on the cell or tissue type, and relative expression of each of the Gi proteins. Functional studies in cells lacking each of the Gα subunits, such as with siRNA or from knockout mouse-derived cells, may provide some insight as to which of the Gi proteins the $P2Y_{14}$-R may couple.

The possibility that the $P2Y_{14}$-R couples to other Gi or Gβγ signaling pathways such as ion channels, phospholipases, protein kinases, and receptor tyrosine kinases is a viable hypothesis, and awaits further investigation. There are many proteins reported to interact

with Gβγ, and the potential for $P2Y_{14}$-R to activate signaling pathways through Gβγ has not been explored.

Another observation reported in Chapter 4 was that the natively expressed $P2Y_{14}$-R does not couple to inhibition of adenylyl cyclase in differentiated HL-60 cells. Differentiated HL-60 cells are a cell model system frequently used to study neutrophil biology, and inhibition of adenylyl cyclase by GPCRs has not been unequivocally demonstrated in neutrophils nor in HL-60 cells, despite the abundant expression of Gi in both cell types. Moreover, the Gi-coupled chemoattractant receptor FPR coupled to activation of adenylyl cyclase in neutrophils (Mahadeo et al., 2007). Adenylyl cyclases I, V, and VI are the primary isoforms capable of inhibition by Gi (Watts and Neve, 2005). Neutrophils have been reported to express only adenylyl cyclases III, IV, VII and IX (Mahadeo et al., 2007), and therefore one would not expect to observe inhibition of adenylyl cyclase in neutrophils. It is likely that Gi-coupled receptors such as the $P2Y_{14}$-R have a unique signaling profile in this specialized cell type, and while we have established that the $P2Y_{14}$-R couples to ERK1/2 phosphorylation in differentiated HL-60 cells, it is possible that other signaling processes also are activated by the $P2Y_{14}$-R. More detailed signal transduction studies using differentiated HL-60 cells will delineate the signaling pathways activated by the $P2Y_{14}$-R, and may reflect a potential function for the $P2Y_{14}$-R in neutrophils.

In addition to studies of signal transduction, the HL-60 cell model also will be a useful means for studies of $P2Y_{14}$-R-regulated biology. Expression of $P2Y_{14}$-R mRNA has been demonstrated in neutrophils, but HL-60 cells have technical advantages over neutrophils. Many of the GPCRs that are expressed on neutrophils are Gi-coupled

chemokine receptors that regulate immune responses such as chemotaxis. Studies of UDP-Glc-dependent cellular processes in differentiated HL-60 cells may lead to elucidation of a $P2Y_{14}$-R function in immune cells.

The cell models developed for studies of the $P2Y_{14}$-R will circumvent problems with the previously used cell system in which the $P2Y_{14}$-R was coupled to the chimeric Gq/i. In previous studies, receptor-dependent signaling was observed in the absence of agonist. This phenomenon appeared to be somewhat dependent on agonist in the medium on the cultured cells because treatment of the cells with UDP-sugar hydrolyzing enzymes, such as E-NPP or UDP-Glc pyrophosphorylase reduced such activity. However, a residual component of the activation in the absence of agonist could not be explained by the presence of agonist in the medium. In the cell lines stably expressing the $P2Y_{14}$-R, there is no evidence of receptor activity in the absence of added agonist. Upon measuring accumulation of cAMP, treatment of the cells with pertussis toxin would be expected to significantly increase the forskolin-stimulated level of cAMP if the receptor were constitutively active, because pertussis toxin would prevent Gi activation. Results from such experiments indicate that the level of forskolin stimulation is similar in cells treated with pertussis toxin to those without.

Another fallacy of the system using Gq/i became clear with recent studies of UDP. In Chapter 3, I described the identification of UDP as a competitive antagonist at the $P2Y_{14}$-R (Fricks et al., 2008), and additional investigations in which the basal activity of the $P2Y_{14}$-R was reduced with co-expression of E-NPP1 revealed that UDP is in fact, a partial agonist in this experimental system. With the development of a robust cellular assay for assessing $P2Y_{14}$-R signaling through its cognate G protein, Gi, as described in

Chapter 4, we have begun to characterize $P2Y_{14}$-R function via its native signaling processes. In contrast to the results in COS-7 cells in which the $P2Y_{14}$-R and Gq/i were co-expressed, when UDP was studied in a cellular system in which the $P2Y_{14}$-R coupled to its native G protein, UDP exhibited the efficacy of a full agonist, indicating that the Gq/i chimera may not be the ideal system for characterizing native $P2Y_{14}$-R function.

In multiple types of mammalian cells stably expressing the $P2Y_{14}$-R, UDP exhibited agonist activity in assays measuring inhibition of forskolin-stimulated adenylyl cyclase. This was a surprising finding given that no other lab has reported agonist action of UDP at the $P2Y_{14}$-R despite screens of nucleotides for agonism in many different experimental systems. Results showing that the effect of UDP is $P2Y_{14}$-R-dependent and pertussis toxin-sensitive will be detailed in a paper soon to be submitted for publication (Carter, Fricks, et al.).

The reason for the seemingly differential action of UDP at the $P2Y_{14}$-R in the two experimental systems is not immediately clear. The simplest explanation is that the activation state of the receptor is slightly perturbed when coupled to Gq/i, such that it is capable of binding UDP. However, activation of the G protein is not maximal. Indeed UDP appears to have some partial agonist activity in the transfected system, but it also acts to block binding of UDP-Glc, and so appears to be a competitive antagonist.

Interestingly, in parallel studies of the rat $P2Y_{14}$-R coexpressed with $G_{q/i}$ in COS-7 cells, UDP was a full agonist. The rat and human $P2Y_{14}$-R share 80% amino acid sequence homology, and by extrapolation, also would be expected to have similar structural features. Despite their similarities, the prospect of the rat $P2Y_{14}$-R coupling to

Gq/i with a different orientation than the human $P2Y_{14}$-R, and thus, permitting activation by UDP, is a distinct possibility.

Studies of the β1 Adrenergic receptor suggest that different ligands can promote different conformation states of the receptor and in so doing, engage distinct signaling pathways (Galandrin et al., 2008). In effect, it is likely that for some GPCRs, the receptor conformation attained with a particular ligand dictates the receptor's G protein selectivity, or possibly alters the efficiencies of receptor coupling to various G proteins. Juxtaposed with the idea of ligand-induced receptor conformations, the availability of particular G proteins as the determining factor for relative agonist selectivity of a receptor is an alternative explanation for the differential action of UDP on the $P2Y_{14}$-R in separate cellular systems.

While the Gq/i chimera has been used in studies of many Gi-coupled GPCR, the structure of the $P2Y_{14}$-R may not be amenable to signaling through this engineered protein. It is possible that the $P2Y_{14}$-R is in a partially activated state when co-expressed with Gq/i, and the activation not attributable to agonist in the medium is due to receptor that is in a partially active conformation. An inverse agonist for the $P2Y_{14}$-R would allow us to examine this possibility experimentally, but currently none is available.

An altered receptor conformation would also explain the differences observed in UDP activity between the Gq/i system and the stable cell lines. If the agonist binding pocket were oriented in such a way in the Gq/i system that UDP-Glc was accommodated but UDP was not, then it is easy to understand how UDP may appear as a partial agonist/antagonist. When the $P2Y_{14}$-R coupled to its native G protein, the receptor

binding pocket was potentially in a conformation that would permit UDP binding as an agonist and subsequently, UDP activation at the $P2Y_{14}$-R.

An alternative explanation implicates receptor expression as responsible for the dichotomous action of UDP in different cellular systems. When increasing amounts of expression plasmid encoding the $P2Y_{14}$-R are co-transfected with Gq/i, the apparent efficacy of UDP also increases to a level indicative of a partial agonist. While we would expect that retroviral infection of cells would express receptor at levels close to that of an endogenous receptor, overexpression that introduced receptor reserve into the stable cell system would explain UDP's action as an apparent full agonist. Without a radioligand binding assay, however, we do not have the ability to quantify receptor in order to investigate such possibilities.

With the finding that UDP is a $P2Y_{14}$-R agonist, the relationship of $P2Y_{14}$-R to the $P2Y_6$-R must be addressed. Just as the $P2Y_1$-R and the $P2Y_{12}$-R are co-expressed on platelets and have a cooperative function when activated by their shared agonist, ADP, to activate two distinct cellular signaling pathways, it is possible that the $P2Y_6$-R and the $P2Y_{14}$-R have a similarly important physiological role when co-expressed. Selectively acting antagonists for each of these receptors may provide clues as to whether the $P2Y_6$-R and $P2Y_{14}$-R are functionally linked to any pathophysiological processes. In cellular systems, the two receptors are commonly found expressed together. Assays of UDP-dependent activity in cells in which one of the receptors was knocked down by siRNA would aid our understanding of $P2Y_{14}$-R versus $P2Y_6$-R signaling. Although the Gq and Gi pathways appear to be distinct, it is becoming more apparent that signaling networks cooperate to regulate some biological functions, and therapeutic development will be

forced to acknowledge the multiple regulatory mechanisms involved with treating any one disorder. Just as with the $P2Y_1$-R and $P2Y_{12}$-R, we may find that the $P2Y_6$-R and $P2Y_{14}$-R signaling pathways converge to modulate some critical aspect of physiology.

Although much focus has been on the $P2Y_{14}$-R as a potential immune cell modulator, the $P2Y_{14}$-R likely has multiple specialized functions depending on the tissue type. Just as the $P2Y_{12}$-R is both a modulator of platelet aggregation and is involved in microglial migration (Haynes et al., 2006), so too may the $P2Y_{14}$-R have distinct roles in the various tissues in which it is expressed. In addition to immune cells, high expression levels for $P2Y_{14}$-R mRNA have been reported in adipose tissue, placenta, brain and stomach.

Recently, an effect of UDP-Glc on smooth muscle contractility was observed in the forestomach of mouse (Bassil et al., 2009). In the same study, a knockout mouse in which the $P2Y_{14}$-R gene was deleted was generated. Adult $P2Y_{14}$-$R^{-/-}$ mice were compared to wild type mice and UDP-Glc did not induce contractions in the stomachs of $P2Y_{14}$-R KO mice, suggesting that the effect of UDP-Glc was $P2Y_{14}$-R-dependent. Thus, $P2Y_{14}$-R expressed in stomach may have a role in gastric motility.

While a normal phenotype was reported for the knockout mouse, no investigation of immune system physiology was described. It would seem from this report of the knockout mouse that the $P2Y_{14}$-R does not have a critical role in development or viability of mice, and may either have a redundant function in mouse, or function as part of a response mechanism, which will only be revealed after some primary insult such as bacterial infection or tissue damage. Demonstration of $P2Y_{14}$-R mRNA upregulation in rat brain after immunologic challenge (Moore et al., 2003) supports the notion that the $P2Y_{14}$-R may be important in an organism's response to injury or disease state.

The physiological relevance of the $P2Y_{14}$-R remains elusive, but the work presented here advances us toward the common goal of understanding the function of the $P2Y_{14}$-R. The coupling of the $P2Y_{14}$-R to two signal transduction pathways, inhibition of adenylyl cyclase and activation of ERK1/2, was established. Progress was made toward developing pharmacological tools that will advance our understanding of $P2Y_{14}$-R function. The cell models that were developed will be crucial for investigations of signal transduction and cell biological functions attributable to the $P2Y_{14}$-R. As we have no hint of a physiological function for the $P2Y_{14}$-R from the knockout mouse, we will continue our progress toward characterization of the $P2Y_{14}$-R even as we continue to develop better tools to make such efforts more accurate, efficient, and relevant.

References

Bassil AK, Bourdu S, Townson KA, Wheeldon A, Jarvie EM, Zebda N, Abuin A, Grau E, Livi GP, Punter L, Latcham J, Grimes AM, Hurp DP, Downham KM, Sanger GJ, Winchester WJ, Morrison AD and Moore GB (2009) UDP-glucose Modulates Gastric Function Through P2Y14 Receptor-Dependent and -Independent Mechanisms. *Am J Physiol Gastrointest Liver Physiol*.

DeFea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Dery O and Bunnett NW (2000) The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta -arrestin-dependent scaffolding complex. *Proc Natl Acad Sci U S A* **97**:11086-11091.

Della Rocca GJ, van Biesen T, Daaka Y, Luttrell DK, Luttrell LM, Lefkowitz RJ (1997) Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptor. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. *J Biol Chem.* **272**:19125-32.

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

Fricks IP, Maddileti S, Carter R, 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.

Galandrin S, Oligny-Longpre G, Bonin H, Ogawa K, Gales C and Bouvier M (2008) Conformational rearrangements and signaling cascades involved in ligand-biased mitogen-activated protein kinase signaling through the beta1-adrenergic receptor. *Mol Pharmacol* **74**:162-172.

Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB and Julius D (2006) The $P2Y_{12}$ receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci* **9**:1512-1519.

Inglese J, Luttrell LM, Iniguez-Lluhi JA, Touhara K, Koch WJ and Lefkowitz RJ (1994) Functionally active targeting domain of the beta-adrenergic receptor kinase: an inhibitor of G beta gamma-mediated stimulation of type II adenylyl cyclase. *Proc Natl Acad Sci U S A* **91**:3637-3641.

Ivanov AA, Fricks I, Harden TK and Jacobson KA (2007) Molecular dynamics simulation of the $P2Y_{14}$ receptor. Ligand docking and identification of a putative binding site of the distal hexose moiety. *Bioorg Med Chem Lett* **17**:761-766.

Ko H, Das A, Carter R, Fricks I, Ivanov AA, Melman A, Kovac P, Hajduch J, Kirk KL, Harden TK and Jacobson KA (2009) Structure Activity Relationship of UDP-

Glucose Analogues as Agonists of the $P2Y_{14}$ Receptor: Modified Terminal Sugar Moiety. *J Med Chem* **in press**.

Ko H, Fricks I, Ivanov AA, Harden TK and Jacobson KA (2007) Structure-activity relationship of uridine 5'-diphosphoglucose analogues as agonists of the human $P2Y_{14}$ receptor. *J Med Chem* **50**:2030-2039.

Kranenburg O, Verlaan I, Hordijk PL and Moolenaar WH (1997) Gi-mediated activation of the Ras/MAP kinase pathway involves a 100 kDa tyrosine-phosphorylated Grb2 SH3 binding protein, but not Src nor Shc. *Embo J* **16**:3097-3105.

Kranenburg O, Verlaan I and Moolenaar WH (1999) Gi-mediated tyrosine phosphorylation of Grb2 (growth-factor-receptor-bound protein 2)-bound dynamin-II by lysophosphatidic acid. *Biochem J* **339 (Pt 1)**:11-14.

Kreda SM, Okada SF, van Heusden CA, O'Neal W, Gabriel S, Abdullah L, Davis CW, Boucher RC, and Lazarowski ER (2007) Coordinated release of nucleotides and mucin from human airway epithelial Calu-3 cells. *J. Physiol.* **584**: 245-59.

Kreda SM, Seminario-Vidal L, Heusden C and Lazarowski ER (2008) Thrombin-promoted release of UDP-glucose from human astrocytoma cells. *Br J Pharmacol* **153**:1528-1537.

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.

Lopez-Ilasaca M, Crespo P, Pellici PG, Gutkind JS and Wetzker R (1997) Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. *Science* **275**:394-397.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG and Lefkowitz RJ (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283**:655-661.

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL and Lefkowitz RJ (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci U S A* **98**:2449-2454.

Mahadeo DC, Janka-Junttila M, Smoot RL, Roselova P and Parent CA (2007) A chemoattractant-mediated Gi-coupled pathway activates adenylyl cyclase in human neutrophils. *Mol Biol Cell* **18**:512-522.

Mochizuki N, Ohba Y, Kiyokawa E, Kurata T, Murakami T, Ozaki T, Kitabatake A, Nagashima K and Matsuda M (1999) Activation of the ERK/MAPK pathway by an isoform of rap1GAP associated with G alpha(i). *Nature* **400**:891-894.

Moore DJ, Murdock PR, Watson JM, Faull RL, Waldvogel HJ, Szekeres PG, Wilson S, Freeman KB and Emson PC (2003) GPR105, a novel Gi/o-coupled UDP-glucose receptor expressed on brain glia and peripheral immune cells, is regulated by immunologic challenge: possible role in neuroimmune function. *Brain Res Mol Brain Res* **118**:10-23.

Pace AM, Faure M and Bourne HR (1995) Gi2-mediated activation of the MAP kinase cascade. *Mol Biol Cell* **6**:1685-1695.

Radhika V and Dhanasekaran N (2001) Transforming G proteins. *Oncogene* **20**:1607-1614.

Tang WJ and Gilman AG (1992) Adenylyl cyclases. *Cell* **70**:869-872.

Watts VJ, Neve KA (2005) Sensitization of adenylate cyclase by Galpha i/o-coupled receptors. *Pharmacol Ther.* **106**:405-21.

Wunderlich L, Farago A and Buday L (1999) Characterization of interactions of Nck with Sos and dynamin. *Cell Signal* **11**:25-29.