Agonist-Promoted Regulation of the P2Y$_1$ Receptor: Quantification of Native and Recombinant Receptors with the Novel Radioligand [^{32}P]MRS2500

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A dissertation presented to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology.

Chapel Hill
2007

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ABSTRACT

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Agonist-Promoted Regulation of the P2Y$_1$ Receptor: Quantification of Native and
Recombinant Receptors with the Novel Radioligand [^{32}P]MRS2500
(Under the direction of Dr. T. Kendall Harden)

The P2Y family of G-protein coupled receptors are activated by adenine and uridine
di- and triphosphate nucleotides and nucleotide sugars and are implicated in a wide range of
important human physiologies. Difficulty studying these receptors and in their successful
manipulation as therapeutic targets has historically derived from a lack of available
pharmacological tools that discriminate among members of the P2Y receptor family. The
studies described here focus on the P2Y$_1$ receptor, a key mediator of ADP-induced platelet
aggregation. Based on the structure of the recently synthesized, high-affinity P2Y$_1$ receptor-
selective antagonist, 2-iodo-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′,5′-
bisphosphate (MRS2500), we undertook the development of a high-specific radioactivity
radioligand for the P2Y$_1$ receptor, suitable for the study of endogenous receptors in
mammalian tissues and cell lines. Using an enzymatic phosphorylation reaction, we
successfully generated [^{32}P]MRS2500 with a specific activity of 9120 Ci/mmol. The
selectivity and affinity of [^{32}P]MRS2500 for the P2Y$_1$ receptor were confirmed in
radioligand binding assays with Sf9 insect cell membranes overexpressing recombinant P2Y$_1$
receptors. The utility of [^{32}P]MRS2500 for the study of endogenous P2Y$_1$ receptors was
examined using washed human platelets and membranes prepared from various tissues of the
adult rat.

We applied this high-specific radioactivity radioligand to observe surface expression of $P2Y_1$ receptor binding sites in human platelets and MDCK(II) epithelial cells following incubation with $P2Y_1$ receptor agonists. In human platelets, the rapid, agonist-promoted desensitization of the $P2Y_1$ receptor observed after incubation with the selective agonist (N)-methanocarba-2-methylthioadenosine-diphosphate (MRS2365) also occurs for the G_q-coupled 5-HT_{2A} receptor after incubation of platelets with 5-hydroxytryptamine (5-HT). The rapid, agonist-promoted desensitization of the $P2Y_1$ receptor of platelets was accompanied by a modest decrease (< 20%) in the number of surface [^{32}P]MRS2500 binding sites and only a partial recovery of $P2Y_1$ receptor responsiveness after removal of the selective agonist MRS2365. Platelets, therefore, appear to employ a unique mechanism for prolonged termination of $P2Y_1$ receptor signaling in which desensitized receptors are maintained at the cell surface, unable to respond to subsequent agonist stimulation. In intact MDCK(II) cells overexpressing recombinant $P2Y_1$ receptors, incubation with 2MeSADP was followed by a 50% loss of surface [^{32}P]MRS2500 binding sites that was agonist-concentration dependent and required the formation of clathrin-coated pits. Mutagenesis studies indicated that this rapid, agonist-promoted loss of surface binding sites requires two serine residues, Ser352 and Ser354, in the receptor carboxyl terminus. The findings presented here indicate that $P2Y_1$ receptor cell surface expression is regulated in an agonist-dependent manner that differs in at least two cell types and suggests an important role for phosphorylation in agonist-dependent desensitization and internalization of the $P2Y_1$ receptor.

For my big sister, Dy, for giving so much more than I ever could have asked, and my mother for telling me that the only reason to do this was because I wanted to

ACKNOWLEDGEMENTS

source of indispensable knowledge and advice. Special thanks also goes Dr. Michele Wing, Dr. Erik Bodor and Jason Seifert for being great friends and labmates.

I would like to thank my Delta State University family, Dr. Henry Outlaw, Dr. Ernest Brothers, and Ms. Georgene Clark for being mentors and friends and for making sure I had the tools and the opportunity to pursue this degree, and to the community of Indianola, MS and the Bell Grove Baptist Church family for their support and commitment to my success.

Finally I would like to express my sincerest love and gratitude to family, Dy, Jaye, and Mom, for their unconditional love, kindness, generosity, and support and just for being family, and to Brian, for being there every single day to help me through.

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α,β-meATP	α,β-Methylene-adenosine 5′-triphosphate
β,γ-meATP	β,γ-Methylene-adenosine 5′-triphosphate
2-MeSADP	2-Methylthioadenosine 5′-diphosphate
2-MeSATP	2-Methylythioadenosine 5′-triphosphate
5-BrUTP	5-Bromo-uridine-5′-triphosphate
5-HT	5-Hydroxytryptamine
A2P5P	Adenosine 2′,5′-bisphosphate
A3P5P	Adenosine 3′,5′-bisphosphate
A3P5PS	Adenosine 3′ phosphate, 5′ phosphosulfate
ADP	Adenosine 5`-diphosphate
ADPβS	Adenosine 5′-(γ-thio)diphosphate
AKAP	A-kinase anchoring protein
Ala or A	Alanine
AMP	Adenosine monophosphate
AP2	Adaptor protein complex 2
Ap$_4$A	Diadenosine tetraphosphate
Arg or R	Arginine
Asn or N	Asparagine
Asp or D	Aspartic acid
ATP	Adenosine 5′-triphosphate
ATPγS	Adenosine 5′-(γ-thio)triphosphate
BzATP	3′-Benzoylbenzoyl adenosine 5′-triphosphate

C-terminus (tail)	Carboxyl terminus (tail)
cAMP	Adenosine $3',5'$-cyclic monophosphate
(c)DNA	(Complementary) deoxyribonucleic acid
CHO-K1	Chinese hamster ovary cells
CNS	Central nervous system
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
E-NDPK	Ecto-nucleotide diphosphokinase
E-NPP	Ecto-nucleotide pyrophosphorylase
E-NTPDase	Ecto-nucleotide triphosphate diphosphohydrolase
EL	Extracellular loop
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FDA	Food & Drug Administration
GABA	γ-amino-butyric acid
GDP	Guanosine $5'$-diphosphate
GEF	Guanine-nucleotide exchange factor
GFP	Green fluorescent protein
Gln or Q	Glutamine
Gly or G	Glycine
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinase
GTP	Guanosine $5'$-triphosphate

HA	Haemagluttinin
HEK293	Human embryonic kidney cell line
His or H	Histidine
HPLC	High pressure liquid chromatography
IBD	Irritable bowel disease
IL	Intracellular loop
Ile or I	Isoleucine
IP_3	Inositol (1,4,5)trisphosphate
Lys or K	Lysine
Leu or L	Leucine
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney cell line
mRNA	Messenger ribonucleic acid
MRS2179	N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate
MRS2279	2-chloro-N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate
MRS2500	2-iodo-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate
MRS2365	(*N*)-methanocarba-2-methlythioadenosine 5′-diphosphate
MRS2608	2-iodo-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′-monophosphate
NHERF	Na^+/H^+ exchanger regulatory factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDZ	Postsynaptic density protein/*Drosophilia melanogaster* discs large protein/Zona occludens 1 protein homology
PI3K	Phosphoinositide 3-kinase
PICK	PDZ-containing protein that interacts with C Kinase
PIP_2	Phosphatidylinositol (4,5)bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PPADS	Pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid
SAR	Structure-activity relationships
SDS	Sodium dodecyl sulfate
Ser or S	Serine
SNAP	Soluble N-ethyl maleimide-sensitive factor attachment protein
SNARE	(SNAP) receptor
Thr or T	Threonine
TM	Transmembrane region
TxA_2	Thromboxane A_2
Tyr or Y	Tyrosine
UDP	Uridine 5′-diphosphate
UP_4U	Diuridine tetraphosphate
UTP	Uridine 5′-triphosphate
UTPγS	Uridine 5′-(γ-thio)triphosphate
vWF	von Willebrand factor

CHAPTER 1

INTRODUCTION

1.1 G-Protein Coupled Receptor Signaling

G-protein coupled receptors (GPCRs) are heptahelical transmembrane proteins that transmit signals from extracellular hormones, peptides, neurotransmitters and other ligands to the intracellular environment, serving as primary responders for innumerable cellular events. An overwhelming majority of compounds that alter human biology, both therapeutic and illicit, target G-protein coupled receptor signaling pathways; as such, ligand development, second messenger signaling and regulation of GPCRs are topics of extensive research. P2Y receptors are a unique class of ubiquitously expressed GPCRs that perpetuate signaling initiated by extracellular nucleotides. The studies described herein address the development of a novel pharmacological tool and its application to the study of the $P2Y_1$ receptor, a drug target for human cardiovascular disease.

1.1.1 Second Messenger Signaling

GPCRs transduce signals initiated by various extracellular components through their interaction with guanine-nucleotide binding proteins (G-proteins). In an unstimulated system, the receptor is associated with a heterotrimer consisting of a guanosine diphosphate (GDP)-bound Gα subunit and associated β and γ subunits. Upon activation, the receptor acts as a guanine-nucleotide exchange factor (GEF) for the Gα subunit, causing the Gα subunit to exchange GDP for guanosine triphosphate (GTP), and subsequent dissociation of the

heterotrimer from the receptor. The Gα and G$\beta\gamma$ subunits are each able to signal to downstream effectors thereby regulating the levels of second messengers responsible for various cellular outcomes (Figure 1.1).

Sixteen genes encoding 23 expressed gene products for Gα subunits have been cloned and identified to associate with GPCRs, and these can be divided into four major subfamilies based on sequence homology (McCudden *et al.*, 2005). The first Gα subunit to be identified was Gα_s (hereafter G$_s$), which stimulates adenylyl cyclase, leading to increased levels of the first recognized second messenger, cyclic adenosine 5′-monophosphate (cAMP) (Berthet *et al.*, 1957; Sutherland *et al.*, 1958). Related to Gα_s is the primary Gα subunit of the olfactory system Gα_{olf} (Buck, 2000). Subsequently, a Gα subunit responsible for decreasing intracellular cAMP by inhibiting adenylyl cyclase was identified, Gα_i. Three G$_i$ isoforms exist, G$_{i1-3}$ and are subclassified along with cyclase-inhibitory Gα_o, Gα_z and related Gα subunits of the gustatory and visual systems, Gα_{gust} and Gα_t (Arshavsky *et al.*, 2002; Margolskee, 2002). A third class of Gα subunits including Gα_q, Gα_{11}, Gα_{14} and Gα_{16} activates phospholipase C β (PLCβ), an enzyme that cleaves the minor membrane phospholipid phosphatidylinositol, 4,5-bisphosphate (PIP$_2$) to generate the second messengers inositol trisphosphate (IP$_3$) and diacylglycerol (DAG) (Rhee, 2001). The fourth class of Gα subunits consists of Gα_{12} and Gα_{13} which activate guanine-nucleotide exchange factors for the small G-protein RhoA, eliciting downstream signaling through RhoA-mediated pathways (Fukuhara *et al.*, 1999; Longenecker *et al.*, 2001).

Signaling can also occur through $\beta\gamma$ dimers. Five Gβ and twelve Gγ subunits have been identified and these can pair almost indiscriminately (Clapham *et al.*, 1997; Ray *et al.*, 1995). Exceptions include the inability of Gβ_2 to associate with Gγ_1 and obligate association

of Gβ_5 with G-gamma-like domains of GTPase-accelerating regulators of G-protein signaling (RGS) (Jones *et al.*, 2004; Schmidt *et al.*, 1992). Though originally considered to have limited function, dimers of G$\beta\gamma$ subunits have been shown to activate several signaling pathways, including G-protein-regulated inward-rectifier K$^+$ channels, neuronal N- and P/Q-type Ca^{2+} channels, mitogen-activated protein kinases, and phosphoinositide-3′ kinase-γ (PI3Kγ), in addition to Gα-independent activation of PLCβ and inhibition of specific isoforms of adenylyl cyclase (Boyer *et al.*, 1992; Crespo *et al.*, 1994; Delmas *et al.*, 2000; Kammermeier *et al.*, 2000; Logothetis *et al.*, 1987; Lu *et al.*, 2001; Maier *et al.*, 2000; Tang *et al.*, 1991; Yamauchi *et al.*, 1997).

GPCRs interact with a number of other proteins and these interactions help facilitate G-protein signaling as well as promote certain types of G-protein-independent signaling. The AKAP family of proteins, or A-kinase anchoring proteins, are a large group of over 50 proteins that act as scaffolds, linking GPCRs to other components of the G-protein signaling pathway. AKAPs have been identified to promote MAPK signaling, phosphorylation and desensitization of the β2 adrenergic receptor, and a unique AKAP has been identified that links G$_{12}$ to RhoA and acts as a RhoGEF upon stimulation with the GPCR agonist lysophosphatidic acid (Diviani *et al.*, 2001; Dodge *et al.*, 2000; Fraser *et al.*, 2000; Shih *et al.*, 1999). GPCRs may also interact with PDZ-domain-containing proteins such as NHERF-2, a Na$^+$/H$^+$ exchanger regulatory protein and PICK, PDZ-containing protein that interacts with C Kinase, through a PDZ-binding motif found in the C-terminus of several GPCRs (Hall *et al.*, 1998a; Hall *et al.*, 1998b; Sheng *et al.*, 2001). The significance of these PDZ interactions is still under investigation.

Though GPCRs are typically considered to evoke signaling through the binding of one molecule of ligand to one receptor, evidence for receptor dimerization is growing. Atomic force microscopy studies revealed that rhodopsin exists in dimeric form in native tissues (Liang *et al.*, 2003). Based on predicted contacts between paired receptors, the structure of GPCR binding proteins including arrestins and the $G\alpha\beta\gamma$ heterotrimer, impaired expression of GPCR truncation mutants and splice variants, and resolution of some higher order GPCR oligomers by SDS-PAGE electrophoresis, homoligomerization is predicted to occur for many, if not all, GPCRs (Coge *et al.*, 1999; Karpa *et al.*, 2000; Lee *et al.*, 2000b; Lee *et al.*, 2003; Nimchinsky *et al.*, 1997). Taste receptors are known to require heterodimerization to respond to certain stimuli (Nelson *et al.*, 2002; Nelson *et al.*, 2001). In the case of κ and δ opioid receptors, heterodimerization has significant effects on pharmacology and downstream signaling, and coexpression of GABA receptor $GABA_B$-R1 and $GABA_B$-R2 subtypes is required for surface expression and receptor function (Devi, 2001; Galvez *et al.*, 2001; Heldin, 1995; Jordan *et al.*, 1999; Salahpour *et al.*, 2000).

1.1.2 Signal Termination

Following agonist activation, many GPCRs undergo a series of processes designed to terminate agonist-promoted signaling and protect the cell from overstimulation. The primary events responsible for GPCR signal termination are desensitization, internalization and downregulation, and while some mechanisms are common among GPCRs for each of these processes, the diversity of receptors and functions lend to a similarly diverse array of mechanisms for discontinuing downstream signaling.

a. Desensitization

The most widely accepted model for GPCR signal termination is based on studies with the β2 adrenergic receptor and begins with a process called desensitization. The model is depicted in Figure 1.2. Agonist binding to the β2 adrenergic receptor results in increased levels of intracellular cAMP through activation of G_s proteins. Increased cAMP leads to rapid activation of the second messenger-activated kinase protein kinase A (PKA) which phosphorylates the receptor and directly uncouples it from G-protein. As a result of this process, the continued presence of agonist is ineffectual to promote downstream signaling (reviewed in Pierce *et al.*, 2002). Other second messenger-activated kinases are also capable of participating in desensitization. Protein kinase C (PKC) is activated by DAG generated from PLC-mediated lipid hydrolysis and has been shown to play a role in desensitizing GPCRs (Bhattacharyya *et al.*, 2002; Hardy *et al.*, 2005; Waugh *et al.*, 1999). Desensitization of the activated receptor is referred to as homologous desensitization. Activation of another receptor resulting in second-messenger kinase-mediated phosphorylation and desensitization is referred to as heterologous desensitization.

Homologous desensitization may also be achieved through a more common mechanism involving a class of proteins known as G-protein receptor kinases (GRKs). GRKs phosphorylate the intracellular domains of activated GPCRs, causing the translocation of adaptor proteins called arrestins to the plasma membrane. Arrestins bind with high affinity to phosphorylated receptors and sterically inhibit further association with G-protein (reviewed in Pierce *et al.*, 2002). Seven GRKs have been identified, including two specific for the visual system, GRK1 and GRK7. The remaining five GRKs are widely distributed and have broad specificity for GPCRs (Pitcher *et al.*, 1998). Four arrestin molecules have

been cloned; two are specific for the visual system and the remaining two are ubiquitously expressed and show distinct specificity for GPCRs (Krupnick *et al.*, 1998).

b. Internalization

GPCRs may also undergo a process called internalization (also called sequestration, endocytosis), in which they are removed from the plasma membrane in an agonist-dependent manner, thereby terminating further exposure to agonist and subsequent signaling. Internalized receptors may be targeted to early endosomes from which they recycle rapidly to the cell surface or to lysosomes for degradation. A loss in total receptor protein as a result of degradation after agonist treatment is referred to as downregulation. Internalization may accompany or be independent of desensitization and modes of internalization are typically characterized as either clathrin-dependent or clathrin-independent, based on whether receptors utilize clathrin-coated pits for endocytosis.

Clathrin-coated pits are a polymeric network of clathrin heavy- and light-chain molecules that use adaptor proteins to recruit specific cargo. The clathrin-coated pit then forms a vesicle by pinching off from the plasma membrane, a process facilitated by the GTPase dynamin. The most abundant adaptor protein in clathrin-coated pits is the AP2 complex, which recognizes tyrosine and dileucine motifs on proteins and targets them for internalization via clathrin-coated pits (Hinrichsen *et al.*, 2006; Robinson, 2004). Other monomeric adaptor proteins interact with subunits of the AP2 complex to target proteins for internalization in clathrin-coated pits. Arrestin, in addition to regulating GPCR desensitization, interacts with clathrin heavy-chain and the β2 subunit of AP2 to recruit transmembrane receptors into clathrin-coated pits for internalization. Arrestin exists in a

basally phosphorylated state, and dephosphorylation is required for arrestin-mediated internalization of the β2 adrenergic receptor (Moore *et al.*, 2007; Shenoy *et al.*, 2001).

Additionally, arrestin is modified by the addition of ubiquitin, a 76-amino acid protein typically required to target proteins for degradation in the 26S proteasome. Ubiquitination of arrestin is also required for internalization of the β2 adrenergic receptor. Other adaptor proteins such as epsin, that interact with AP2, contain ubiquitin-binding domains which specify ubiquitinated cargo for recruitment to clathrin-coated pits (Shih *et al.*, 2002).

Though many cell surface proteins utilize clathrin-coated pits for internalization, mechanisms not requiring the formation of clathrin-coated pits have been elucidated for internalization of cell surface proteins. Clathrin-independent endocytosis often involves special membrane microdomains called lipid rafts, which are enriched in cholesterol and sphingolipids (Brown *et al.*, 1998; Simons *et al.*, 2000). Lipid raft-mediated endocytosis is generally characterized as sensitive to cholesterol depletion and may or may not involve the action of dynamin (Sauvonnet *et al.*, 2005); lipid-raft mediated endocytosis can also converge with clathrin-mediated endocytosis by the delivery of cargo to early endosomes (Llorente *et al.*, 2000). A specialized form of lipid-raft mediated endocytosis may involve specialized substructures called caveolae. Caveolae are flask-shaped invaginations that cover the plasma membrane of some animal cells and contain proteins called caveolins (Drab *et al.*, 2001; Fra *et al.*, 1994). Internalization via caveolae is typically dynamin-dependent and cargo can be delivered to early endosomes as with clathrin-mediated endocytosis (Damm *et al.*, 2005; Oh *et al.*, 1998). Another method of clathrin-independent endocytosis is macropinocytosis, a process in which membrane ruffling causes the protrusion and fusion of

vesicles containing large areas of plasma membrane including specific lipid rafts and phosphoinositides and large amounts of fluid (Grimmer *et al.*, 2002; Manes *et al.*, 2003). The mechanisms of each of these non-clathrin forms of endocytosis are still controversial but evidence suggests many of the same adaptor proteins and targeting signals specify proteins for endocytosis via these structures.

1.2 Purinergic Signaling

1.2.1 Historical Perspective of Purinergic Signaling

Physiological responses induced by extracellular nucleosides and nucleotides were first observed in cardiac tissue in 1929, but the mechanism of action of extracellular nucleotides was unclear until the description of purinergic receptors almost fifty years later (Drury *et al.*, 1929). Several studies indicated a role for ATP and its metabolites in cardiovascular physiology, and pharmacological studies suggested differences in the activity of nucleotides based on the extent of phosphorylation. Nonetheless, ATP maintained a singular identity as an intracellular energy source until the pioneering work of Geoffrey Burnstock provided tangible evidence and a solid framework within which to establish a role for ATP as an extracellular signaling molecule. In the 1960's Burnstock observed non-adrenergic, non-cholinergic excitatory responses in the guinea pig taenia coli, and in 1972 proposed a controversial model to describe the role of ATP as a neurotransmitter (Burnstock, 1972; Burnstock *et al.*, 1964). The model suggested that ATP, like other transmitters, must be released from nerve terminals, act through cell surface receptors, and have a mechanism for uptake or depletion from the synapse. Holton and Holton had first observed ATP release from rabbit ear sensory nerves in 1953, and in 1978, based on pharmacological and second

messenger signaling data for adenosine, ADP and ATP, Burnstock first described two classes of purinergic receptors, P1 receptors responsible for the actions of adenosine and P2 receptors responsible for adenine nucleotides (Holton *et al.*, 1953). With the aid of advances in pharmacology, cell signaling and molecular biology, these ideas eventually evolved into the current understanding that ATP is stored as a cotransmitter at nerve terminals and acts through distinct receptors, ionotropic P2X and metabotropic P2Y receptors, to mediate its effects, and that plasma-membrane-bound nucleotide-hydrolyzing enzymes rapidly degrade extracellular nucleotides. ATP was thus confirmed as a neurotransmitter and the ubiquitous effects of ATP and its metabolites as extracellular signaling molecules began to surface (reviewed inRalevic *et al.*, 1998). A model illustrating our current knowledge of the complexities of nucleotide receptor signaling is presented in Figure 1.3.

1.2.2 Receptors for Extracellular Purines and Pyrimidines

a. Adenosine (P1) Receptors

Extracellular adenosine is important for a number of cellular and physiological processes including cytoprotective regulation of oxygen consumption, angiogenesis, anti-inflammatory responses and myocardial preconditioning during ischemia (Shneyvays *et al.*, 2004; Tracey *et al.*, 1998). Extracellular adenosine is accumulated by release through an equilibrative transporter, cell damage, or extracellular nucleotide hydrolysis (Fredholm *et al.*, 2001; Linden, 2005; McGaraughty *et al.*, 2005). Four subtypes of G-protein coupled receptors activated by extracellular adenosine have been cloned and characterized: A_1, A_{2A}, A_{2B}, and A_3 (Ralevic *et al.*, 1998). Adenosine receptors primarily couple to the modulation of adenylyl cyclase, negatively by A_1 and A_3 through G_i proteins and positively by A_{2A} and

A_{2B} through G_s proteins, though alternate signaling pathways have been observed (Mamedova *et al.*, 2006). Adenosine-induced bradycardia occurs through A_1 receptor pertussis-toxin sensitive activation of K^+ channels (Belardinelli *et al.*, 1995). A_{2A} receptor density is highest in striatum where the receptor largely couples through G_{olf}, a G_s-related G-protein (Kull *et al.*, 2000). The A_{2B} receptor can also couple to G_q to stimulate inositol phosphate formation and the A_3 receptor is also positively coupled to inositol phosphate formation and calcium mobilization which appears to be the mechanism of action for A_3-mediated neurodegeneration in brain ischemia (Abbracchio *et al.*, 1995; Brackett *et al.*, 1994; Daly *et al.*, 1983; Englert *et al.*, 2002; Feoktistov *et al.*, 1995; Fossetta *et al.*, 2003; Peakman *et al.*, 1994; Shneyvays *et al.*, 2005; Shneyvays *et al.*, 2004; Zhou *et al.*, 1992).

Caffeine, the most widely used drug in the world, is a potent antagonist at A_1, A_{2A} and A_{2B} adenosine receptors. Adenosine receptors are additionally potential therapeutic targets for a number of conditions including neurodegenerative, cardiovascular, immune, and sleep disorders, inflammation, and cancer. Unfortunately, the ubiquitous expression of adenosine receptors and species differences in ligand selectivity make the development of useful, selective drugs for adenosine receptors difficult. Ligand development has proceeded with the aid of homology modeling based on the structure of bovine rhodopsin and structure-activity relationships for nucleosides and non-nucleoside derivatives. Agonists for adenosine receptors have been designed using adenosine as a template. Antagonists are largely based on the structure of methylxanthines. Successful agonists of the A1 receptor are based on N^6-substitutions of adenosine and several have shown clinical promise for the treatment of tachycardia, pain and migraine, and obesity-induced insulin resistance (Bayes *et al.*, 2003; Dong *et al.*, 2001; Ellenbogen *et al.*, 2005; Gao *et al.*, 2003; Giffin *et al.*, 2003; Sawynok,

1998; Zablocki *et al.*, 2004; Zambrowicz *et al.*, 2003). Potent antagonists of the A_1 receptor, including 8-aryl and 8-cycloalkyl derivatives of the xanthines and select non-xanthine compounds, are effective at treating dementia and anxiety, and through diuretic effects can be used to treat fluid retention disorders including congestive heart failure (Auchampach *et al.*, 2004; Gottlieb *et al.*, 2002; Maemoto *et al.*, 2004; Martin *et al.*, 1996; Moro *et al.*, 2006; Wilcox *et al.*, 1999). Adenosine derivatives selective for the A_{2A} receptor are useful tools for cardiac imaging and potent anti-inflammatory agents and have been indicated for the treatment of asthma and arthritis while antagonists may provide a neuroprotective effect beneficial in the treatment of Parkinson's disease (Aoyama *et al.*, 2000; Ascherio *et al.*, 2001; Fozard *et al.*, 2002; Hendel *et al.*, 2005; Matasi *et al.*, 2005; Ohta *et al.*, 2001; Peng *et al.*, 2004; Ross *et al.*, 2000; Weiss *et al.*, 2003; Xu *et al.*, 2005). The A_{2B} receptor is the least studied subtype of the family; however, activation of the A_{2B} receptor has been shown to promote angiogenesis and vasodilation and selective agonists, antagonists and radioligands have been developed (Beukers *et al.*, 2004; Feoktistov *et al.*, 1995; Gessi *et al.*, 2005; Ji *et al.*, 2001; Linden, 2005; Stewart *et al.*, 2004; Szentmiklosi *et al.*, 1995; Volpini *et al.*, 2003). Several selective ligands for the A_3 receptor have been developed through 4-thio substitution of adenosine for agonists and screening of diverse compound libraries for antagonists, since caffeine and xanthines bind the A_3 receptor with very low affinity (Jeong *et al.*, 2003; Moro *et al.*, 2005; Moro *et al.*, 2006). A_3 receptor agonists may be effective in cancer treatment since A_3 receptor activation is linked to apoptosis at very high agonist concentrations and A_3 receptors are highly expressed in tumor cells (Fishman *et al.*, 2002; Lu *et al.*, 2003; Madi *et al.*, 2004). Species differences in A_3 receptor pharmacological profiles have presented a hurdle in further evaluations.

b. P2X Receptors

P2X receptors are ATP-activated cation channels that exist as homomeric or heteromeric assemblies of three subunits and primarily mediate fast excitatory neurotransmission and inflammation in central and peripheral tissues. Seven P2X subunits have been cloned, denoted $P2X_{1-7}$ and each subunit is comprised of two transmembrane domains connected by a large extracellular loop and generally short intracellular amino- and carboxyl-termini. Functional homomers of all subunits except $P2X_6$ have been characterized by heterologous expression or studies of native tissues (Burnstock, 2006). Functional heteromers of $P2X_{2/3}$, $P2X_{2/6}$, $P2X_{4/6}$ and $P2X_{1/5}$ subunits have been detected by co-immunoprecipitation from native tissues and overexpression in mammalian cell lines and *Xenopus* oocytes [reviewed in (Ralevic *et al.*, 1998)]. The $P2X_7$ subunit does not form heteromers and is otherwise unique in its relatively low affinity for ATP and its ability to act as a nonselective pore for molecules up to 1000 daltons in size (Nuttle *et al.*, 1994; Rassendren *et al.*, 1997).

P2X receptors are widely expressed with many tissues expressing multiple P2X subunits. Exceptions include restriction of $P2X_3$ expression to sensory ganglia and near-exclusive expression of $P2X_1$ in smooth muscle (Chen *et al.*, 1995; Lewis *et al.*, 1995; Valera *et al.*, 1994). Identification of the role of P2X receptors and the subunits involved is based on pharmacological data with a limited set of largely non-selective agonists and antagonists; however, clear roles for P2X receptors have been established in several important human physiologies including neuropathic pain, inflammation, incontinence and hypertension with the help of genetically-engineered animal models. α,β-meATP is a non-selective P2X receptor agonist with activity at $P2X_1$ and $P2X_2$ homomers and heteromers containing either

of these subunits. L-β,γ-meATP is uniquely selective for the P2X$_1$ receptor and along with synthetic nucleotide antagonists NF029 and NF279 has helped to define a role for the P2X$_1$ receptor in vasoconstriction, suggesting the usefulness of P2X$_1$ antagonists in the treatment of hypertension (Evans *et al.*, 1995; Haines *et al.*, 1999; Horiuchi *et al.*, 1999; Le *et al.*, 1998). A heteromeric P2X$_{2/3}$ channel is expressed on a subset of primary afferents responsible for nociception, and a battery of non-selective antagonists including suramin and PPADS have exhibited anti-nociceptive effects in behavioral and electrophysiological experiments (Dowd *et al.*, 1999; Driessen *et al.*, 1998; Driessen *et al.*, 1994; Sawynok *et al.*, 1997; Tsuda *et al.*, 1999; Zheng *et al.*, 2000). Although P2X$_3$-deficient mice did not display impaired acute pain responses, a nucleotide-based P2X$_3$-selective antagonist, A-317491, reduced chronic inflammatory and neuropathic pain in the rat, giving strong evidence for P2X$_3$ or P2X$_{2/3}$ receptors as targets in chronic pain therapy (Chen *et al.*, 1995; Cockayne *et al.*, 2000; Khakh *et al.*, 1995; Lewis *et al.*, 1995; McGaraughty *et al.*, 2005; Souslova *et al.*, 2000). BzATP is the most potent known agonist of the P2X$_7$ receptor, although it acts at several other P2X receptor subtypes (Bianchi *et al.*, 1999). Activation of P2X$_7$ receptors causes the release of pro-inflammatory cytokines and apoptosis of macrophages resulting in the killing of the enclosed mycobacterium, suggesting a role for the P2X$_7$ receptor in the treatment of chronic inflammation (Lammas *et al.*, 1997; Solle *et al.*, 2001).

c. P2Y Receptors

The first cloned GPCRs activated by extracellular nucleotides were the chick P2$_Y$ receptor activated by ATP (later P2Y$_1$) and the mouse P2$_U$ receptor activated by UTP (later P2Y$_2$) (Lustig *et al.*, 1993; Webb *et al.*, 1993). Subsequently, several nucleotide-activated GPCRs were cloned and ascribed to the P2Y family. A number of these were later found to

be species orthologs of previously identified P2Y receptors or were not activated by nucleotides, resulting in discontinuous numbering of members of the P2Y receptor family.

P2Y receptors are activated by a diverse set of endogenous and synthetic nucleotide di- and triphosphates and nucleotide sugars (Table 1.1). The cognate agonist for the $P2Y_1$ receptor is ADP and ATP is a partial agonist. 2-Methylthio derivatives of ATP and ADP are potent $P2Y_1$ receptor agonists. The $P2Y_2$ receptor is activated by endogenous ATP and UTP and by dinucleotide tetraphosphates. The endogenous agonist for the human $P2Y_4$ receptor is UTP; ATP is a cognate antagonist. Interestingly, ATP and UTP are both agonists at the rat $P2Y_4$ receptor. Synthetic $P2Y_4$ receptor agonists include UTPγS and 5-BrUTP. The $P2Y_6$ receptor is the only UDP-activated receptor; with the exception of UDPβS, other nucleotides are significantly less potent. The $P2Y_{11}$ receptor is the only receptor activated solely by ATP. ATPγS, BzATP and dATP are also agonists, though pharmacological selectivity differs based on G-protein coupling. ADP is the cognate agonist for the $P2Y_{12}$ and $P2Y_{13}$ receptors, although ADPβS is a full agonist only at $P2Y_{13}$. 2MeSADP and 2MeSATP also activate each of these receptors. The $P2Y_{14}$ receptor is the only P2Y receptor activated by nucleotide sugars. UDP-glucose is the most potent agonist and other agonists include UDP-galactose and UDP-N-acetylglucosamine.

P2Y receptors are intronless genes, with the exception of $P2Y_{11}$, with human receptors ranging in length from 328 to 377 amino acids. The eight currently recognized family members fit into two evolutionarily distinct classes: the $P2Y_1$ subfamily and the $P2Y_{12}$ subfamily (Costanzi *et al.*, 2004). $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ are G_q-coupled receptors bearing roughly 35-52% sequence identity (Ralevic *et al.*, 1998). The $P2Y_{11}$ receptor is unique in that it is only 28-30% identical to other $P2Y_1$ subfamily members, and it

is capable of coupling to the stimulation of adenylyl cylase through G_s (Communi *et al.*, 1997; Qi *et al.*, 2001) Members of the $P2Y_{12}$ subfamily – $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ – couple to adenylyl cyclase inhibition through G_i, and are 45-55% identical among themselves but only 20-25% identical to members of the $P2Y_1$ subfamily (Abbracchio *et al.*, 2003). P2Y receptors are found in most tissues of the body and physiological responses mediated by P2Y receptors range from anxiety to tear formation (Abbracchio *et al.*, 2006a). Tissue distribution data for each of the P2Y family members are listed in Table 1.1. Indeed, several P2Y receptors have been studied as targets for disease, including the $P2Y_{12}$ receptor, antagonists of which were successful antiplatelet drugs even before the receptor was cloned.

As with other classes of purinergic receptors, our knowledge of precise signaling and physiology associated with P2Y receptor activation relies on the development of selective, high-affinity ligands. Further complexity is added to the pursuit of ligand development by the fact that interconversion of nucleotides can alter receptor activation profiles in tissues expressing multiple receptor subtypes and that some P2Y receptors are antagonized by agonists of other P2Y receptors (Kennedy *et al.*, 2000). Ligand development for P2Y receptors has proceeded using homology modeling based on the structure of bovine rhodopsin and structure-activity relationships for nucleotide derivatives based on the cognate nucleotide agonist for each receptor as a template. For agonists, efforts are aimed at increasing affinity while retaining activity, and antagonists are developed by attempting to modify nucleotide structures to the minimal components for binding while eliminating receptor activation.   Ribose replacement and conformational constraint of nucleotide analogues revealed that $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ receptors bind nucleotide agonists with the ribose or a substituted methanocarba ring constrained in the Northern conformation while the

$P2Y_6$ receptor is activated exclusively by the Southern conformation of UDP and its analogues (Costanzi *et al.*, 2005; Kim *et al.*, 2002). These studies have led to the successful development of a selective agonist, and selective antagonists and radioligands for the $P2Y_1$ receptor, which are discussed later in this chapter, and a potent, selective agonist for the $P2Y_6$ receptor. With the exception of $P2Y_{12}$ receptor, few selective ligands are available for the $P2Y_{12}$ subfamily. Recent studies with UDP-glucose analogues have identified a 2-thio derivative that has potent agonist, activity and a screen of non-sugar nucleotides suggests that nucleotide diphosphates, particularly UDP, may act as competitive antagonists at the $P2Y_{14}$ receptor (Ko *et al.*, 2007; Fricks, manuscript in preparation).

The $P2Y_1$ and $P2Y_{12}$ receptors are essential for platelet aggregation; therapeutic application and potential of each of these subtypes is discussed in section 1.4 of this chapter. The $P2Y_2$ receptor, which can be activated by dinucleotides such as Up_4U, is expressed in airway epithelium and conjunctival epithelium and positively regulates chloride and water secretion in these cells. As a result, in airway epithelium, the $P2Y_2$ receptor is currently under investigation as a clinical target for the treatment of disorders involving accumulation of mucus in the airway, including cystic fibrosis, chronic bronchitis and chronic obstructive pulmonary disease (Olivier *et al.*, 1996; Rogers *et al.*, 2006). Dinucleotide agonists of the $P2Y_2$ receptor were effective in Phase III clinical trials for the treatment of dry eye and are being pursued for FDA approval (Nichols *et al.*, 2004). The $P2Y_6$ receptor is overexpressed in T cells of bowel of individuals with irritable bowel disease (IBD) and is involved in the release of pro-inflammatory cytokines from monocytes, suggesting a potential use for a $P2Y_6$ antagonist in the treatment of IBD (Somers *et al.*, 1998). Several P2Y receptor subtypes are expressed on osteoblasts and osteoclasts and on astrocytes, oligodendrocytes and glia and are

being studied for bone resorption or neurological diseases (Abbracchio *et al.*, 2006b; Brambilla *et al.*, 2001; Brambilla *et al.*, 1999; Brambilla *et al.*, 2000; Brambilla *et al.*, 2002; Chorna *et al.*, 2004; Gallagher *et al.*, 2003; Gallagher, 2004; Gartland *et al.*, 2003).

Studies regarding agonist-promoted regulation of P2Y receptors have been limited but suggest that these receptors desensitize and internalize by both canonical and non-canonical pathways. An HA-tagged $P2Y_2$ receptor expressed in human astrocytoma cells desensitized rapidly in response to agonist as measured by a loss of responsiveness to ATPγS after pretreatment with the agonist. This desensitization appeared to be homologous, since pretreatment with an agonist of the G_q-coupled muscarinic receptor agonist did not have a similar effect. A loss of cell surface immunoreactivity was also observed after agonist treatment, although with slower kinetics than desensitization, and this loss of cell surface immunoreactivity was completely reversible within 1 hour after agonist removal (Sromek *et al.*, 1998). Another report indicated that the $P2Y_2$ receptor desensitizes rapidly in U973 promonocytic cells as measured by calcium mobilization and that desensitization for more than 30 minutes is accompanied by a decrease in $P2Y_2$ receptor mRNA. Incubation with activators or inhibitors of PKC did not reveal a direct role for this enzyme in $P2Y_2$ receptor desensitization (Santiago-Perez *et al.*, 2001). Additionally, β-arrestin translocation was not observed upon activation of the endogenous $P2Y_2$ receptor in HEK293 cells nor was $P2Y_2$ receptor desensitization affected by antisense depletion of arrestin expression (Mundell *et al.*, 2000). Expression of HA-tagged $P2Y_4$ or $P2Y_6$ receptors in 1321N1 cells revealed interesting differences in agonist-promoted regulation of these two uridine-nucleotide activated receptors. Similar to $P2Y_2$, the $P2Y_4$ receptor desensitized rapidly and was reversibly translocated to an intracellular compartment upon agonist treatment.

Phosphorylation sites in the carboxyl terminus of the receptor were identified that were necessary for desensitization and internalization. In contrast, the $P2Y_6$ receptor did not desensitize in response to agonist, and irreversible loss of cell surface immunoreactivity was observed only after prolonged treatment with agonist (Brinson *et al.*, 2001).

1.2.3 Mechanisms of Nucleotide Release

Consistent with the Burnstock hypothesis that ATP functions as a neurotransmitter, ATP can be released from excitatory cells into the extracellular space where ATP and its metabolites activate purinergic receptors. However, further investigation has revealed that nucleotide release is not limited to excitatory cells nor is it limited to adenine nucleotides and that many resting cells release ATP to maintain an equilibrium between ATP accumulation and ATP hydrolysis (Donaldson *et al.*, 2000; Lazarowski *et al.*, 2000). While mechanisms of nucleotide release are still largely unclear, several potential pathways have been proposed.

a. Exocytotic Release

In certain cell types, such as neurons, platelets, chromaffin cells, mast cells and pancreatic acinar cells, ATP is packaged in granular compartments from which it is released in a regulated manner (Burnstock, 1997; Evans *et al.*, 1992; Sorensen *et al.*, 2001). For neurons, ATP release occurs similarly to or in combination with the release of neurotransmitter by a well-studied process involving vesicle fusion and release known as regulated exocytosis. A complex called the SNARE complex [soluble N-ethyl maleimide sensitive factor attachment protein (SNAP) receptor] is involved in which members of the SNARE family on the initiating cell, v-SNARE proteins, pair with proteins on the target cell,

t-SNARE proteins, to regulate specificity, docking and fusion of vesicles (Chapman *et al.*, 1994; Li *et al.*, 1996; Rettig *et al.*, 2002; Rickman *et al.*, 2003).

b. Mechanical Stimulation

Nucleotide release induced by mechanical stimulation was first reported during sustained exercise of the human forearm muscle and has also been observed as a result of hypotonic cell swelling, hydrostatic pressure, mechanical loading, and medium disturbance. Increased ATP release has been documented from perfused endothelial cells, epithelial cells of the distended murine bladder or human airway, and in immortalized cell lines, and the increased production of inositol phosphates after mechanical stimulation of P2Y-receptor expressing cells has been demonstrated (Cockayne *et al.*, 2000; Ferguson *et al.*, 1997; Filtz *et al.*, 1994; Parr *et al.*, 1994). Mechanical stimulation of 1321N1 astrocytoma cells results in the release of UTP and UDP-glucose in addition to ATP (Lazarowski *et al.*, 1997a; Lazarowski *et al.*, 2003). The mechanisms for release of nucleotides from nonexcitatory cells by mechanical stimulation or agonist induction, discussed below, are as yet unclear but may involve ATP-binding-cassette transporters, connexin hemichannels and plasmalemmal voltage-dependent ion channels (Bodin *et al.*, 2001).

c. Agonist-Dependent Nucleotide Release

A host of agonists for GPCRs including thrombin, ADP, UTP, serotonin, acetylcholine, and bradykinin have been shown to increase concentrations of extracellular ATP in endothelial cells.   While in the case of exogenously applied nucleotides, increased extracellular ATP results from phosphorylation of ADP by NDPK (see below) or competition for ATP hydrolysis, the cause of increased extracellular ATP in response to other agonists is

unclear, though calcium mobilization does not appear to be involved (Buxton *et al.*, 2001; Cotrina *et al.*, 1998; Ostrom *et al.*, 2000).

1.2.4 Extracellular Nucleotide Metabolism

As with other transmembrane receptors, purinergic receptor signal termination is not limited to intracellular pathways, but also results from mechanisms evolved to remove agonist from the extracellular space. In the case of purinergic receptors, ectoenzymes capable of hydrolyzing or interconverting nucleotides are expressed on the cell surface and rapidly metabolize micromolar concentrations of extracellular nucleotides. These ectoenzymes vary in their enzymatic activity and nucleotide preference and are capable not only of protecting cells from prolonged stimulation but also of regulating the levels of nucleotide available to activate or inhibit specific P2 receptor subtypes.

Ectoenzymes can be divided into four major classes: ecto-nucleotide triphosphate diphosphohydrolase (E-NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), alkaline phosphatases, and ecto-5′-nucleotidase. Additionally, ecto-nucleoside diphosphokinase (E-NDPK) interconverts adenine and uridine nucleotide di- and triphosphates (Zimmermann, 2000). Membrane bound ectoenzymes have catalytic regions facing the extracellular environment and rely on divalent cations for maximal activity. Some isoforms are also cleavable and soluble and may be referred to as exonucleotidases. The primary characteristics including membrane topology and nucleotide preference of each major family are discussed below.

a. Ecto-Nucleoside Triphosphate Diphosphohydrolases

Previously referred to as ecto-apyrase, ecto-NTPase and ecto-ATPase, members of the E-NTPDase family can hydrolyze nucleoside 5′-triphosphates and nucleoside 5′-diphosphates.   Isoforms are found in both vertebrate and invertebrate species and all members of the family contain five apyrase-conserved regions in their extracellular domains responsible for catalytic activity (Handa *et al.*, 1996; Schulte am Esch *et al.*, 1999; Vasconcelos *et al.*, 1996; Zimmermann *et al.*, 1999). E-NTPDases also bear the actin-HSP 70 hexokinase β- and γ-phosphate binding motif and appear to share molecular ancestry with members of the actin/HSP70/sugar kinase family (Asai *et al.*, 1995; Kegel *et al.*, 1997).

NTPDases 1-3 and NTPDase8 are plasma membrane-bound. Based on heterologous expression studies, NTPDase1 converts nucleoside 5′-triphosphates and diphosphates to monophosphates with near equal preference, while NTPDase2 preferentially breaks down nucleoside 5′-triphosphates to the diphosphate product (Kaczmarek *et al.*, 1996; Kegel *et al.*, 1997; Kirley, 1997; Mateo *et al.*, 1999). NTPDase3 and NTPDase8 exhibit strong preference for nucleoside 5′-triphosphates, though both are active on diphosphates as well (Smith *et al.*, 1998; Smith *et al.*, 1999; Bigonnesse *et al.*, 2004; Mulero *et al.*, 1999). Thus, NTPDase2, NTPDase3, and NTPDase8 are uniquely capable of depleting the agonist for triphosphate-activated P2 receptors and causing transient accumulation of agonist for diphosphate activated-nucleotide receptors. NTPDase4 and NTPDase6 are expressed on the Golgi; NTPDase4 hydrolyzes nucleoside 5′-di- and triphosphates with the greatest preference for UDP and no activity for ATP or ADP, while NTPDase6 activity is primarily limited to nucleoside 5′-diphosphates (Braun *et al.*, 2000; Wang *et al.*, 1998). NTPDase5 is a soluble, secreted enzyme that exhibits preference for UDP and other nucleoside 5′-diphosphates.

1) NTPDase1: NTP $\rightarrow$ NMP + 2Pi; NDP $\rightarrow$ NMP + Pi

2) NTPDase2: NTP $\rightarrow$ NDP + Pi

3) NTPDase3, NTPDase8: NTP $\rightarrow$ NMP + 2Pi; NDP $\rightarrow$ NMP + Pi

   (NTP:NDP 1:0.3)

4) NTPDase4: NTP $\rightarrow$ NMP + 2Pi; NDP $\rightarrow$ NMP + Pi (except ATP,ADP)

5) NTPDase5, NTPDase6: NDP $\rightarrow$ NMP + Pi

b. Ecto-Nucleoside Pyrophosphatases

The three members of the E-NPP family, NPP1, NPP2 and NPP3, also known as ecto-phosphodiesterase/pyrophosphatase are phylogenetically unrelated to the E-NTPDases and are predicted to bear a single transmembrane domain with an intracellular N-terminus and large extracellular C-terminus where catalytic activity is located. NPP2 exists as two splice variants, otherwise known as autotaxin and PD-1α. Soluble forms of NPP1 and autotaxin have been identified, presumably due to proteolytic cleavage N-terminal to a cysteine-rich stalk near the transmembrane domain (Belli *et al.*, 1993; Clair *et al.*, 1997b). Members of the E-NPP family also contain the somatomedin B-like domain of vitronectin and an extracellular EF-hand domain. NPP2 and NPP3 but not NPP1 contain an RGD integrin recognition motif (Belli *et al.*, 1994; Belli *et al.*, 1993; Clair *et al.*, 1997a; Goding *et al.*, 1998; Kawagoe *et al.*, 1995; Zimmermann, 2000). The function of these motifs is largely unknown, although the EF-hand domain is suggested to mediate binding of divalent cations and is required for enzymatic activity in NPP1.

E-NPPs have diverse enzymatic activities and broad substrate specificity. They hydrolyze not only nucleoside 5$'$-tri- and diphosphates, but are also capable of hydrolyzing dinucleotide polyphosphates to the separate nucleoside monophosphate and polyphosphate

forms (see equation below). E-NPPs also hydrolyze phosphodiester bonds of nucleic acids and pyrophosphate linkages of nucleotide sugars, with activity for both purine and pyrimidines (Goding *et al.*, 1998; Zimmermann, 2000). E-NPPs are thus capable of generating AMP from cAMP and NAD^+ and, in the case of NPP2, adenosine from AMP.

1) E-NPP: $ATP \rightarrow AMP + PPi$; $cAMP \rightarrow AMP$

2) E-NPP: $Ap_nA \rightarrow Ap_{(n-1)} + AMP$

3) NPP2 (autotaxin): $ATP \rightarrow ADP + Pi$; $ADP \rightarrow AMP + Pi$; $AMP \rightarrow$ Adenosine + Pi; $PPi \rightarrow Pi$
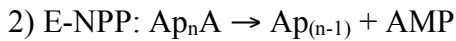
c. Alkaline Phosphatase

Not much is known about alkaline phosphatases; however, these enzymes are known to have catalytic activity for mono-, di-, and triphosphonucleosides, and are thus capable of hydrolyzing nucleoside triphosphates to their corresponding nucleosides as well as degradation of inorganic pyrophosphate to inorganic phosphate (Coleman, 1992). Alkaline phosphatases additionally require much higher nucleotide concentrations for activity with K_m values in the millimolar range.

$NTP \rightarrow NDP + Pi$; $NDP \rightarrow NMP + Pi$; $NMP \rightarrow$ Nucleoside + Pi; $PPi \rightarrow 2Pi$
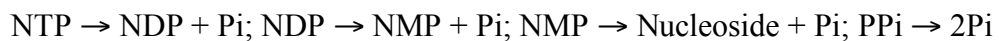
d. Ecto-5′-Nucleotidase

Ecto-5′-nucleotidase is the primary enzyme responsible for generating extracellular adenosine from AMP. A single gene, corresponding to the metalloenzyme CD73 has been found in vertebrates, though studies in invertebrates have been more numerous and include the 3-dimensional structure of *E. coli* ecto-5′-nucleotidase (Knofel *et al.*, 1999). The enzyme is expressed as a dimer and contains regulatory elements within the promoter region that control tissue distribution (Spychala *et al.*, 1999).

$$\text{NMP} \rightarrow \text{Nucleoside} + \text{Pi}$$

e. Ecto-Nucleotide Diphosphokinase

E-NDPK is a unique enzyme capable of interconverting nucleoside 5′di- and triphosphates such as ATP and UDP into ADP and UTP, respectively. E-NDPK activity has been found on astrocytoma and airway epithelial cells and is of critical consideration when interpreting activities of various P2Y receptors in such systems, especially since K_m values for E-NDPK fall in the range of 20-100 µM, concentrations at which agonists fully activate various P2Y receptors (Harden *et al.*, 1997; Lazarowski *et al.*, 1997b).

1.3 The P2Y₁ Receptor

1.3.1 General Features

A GPCR activated by ATP when expressed in *Xenopus* oocytes was first cloned from chick brain in 1993 and named P2Y₁ (Webb *et al.*, 1993). In 1994, the turkey homologue was cloned and identified to couple to activation of phospholipase C in turkey erythrocyte membranes (Filtz *et al.*, 1994). In 1996, the human receptor, a 373-amino acid polypeptide was cloned and second messenger activity and pharmacological selectivity were defined (Janssens *et al.*, 1996; Schachter *et al.*, 1996; Leon *et al.*, 1996). The endogenous agonist for the P2Y₁ receptor is ADP; 2MeSADP and 2MeSATP are also potent agonists and ATP is a low-potency partial agonist. Northern blot analysis and immunohistochemistry revealed almost ubiquitous tissue distribution for the P2Y₁ receptor with prominent expression in brain on neurons and astrocytes where it is coexpressed with the P2Y₂ and β2 adrenergic receptors (Janssens *et al.*, 1996; Leon *et al.*, 1996; Moore *et al.*, 2000; Zhu *et al.*, 2004; Zhu *et al.*, 2001).

The amino acid sequence of the $P2Y_1$ receptor is shown in Figure 1.4 with functional domains and residues reported to be involved in ligand binding and G-protein coupling highlighted. The $P2Y_1$ receptor contains a short extracellular amino terminus with multiple sites for N-linked glycosylation; treatment of detergent solubilized receptors with *N* glycosidase F resulted in a faster electrophoretically-migrating species, suggesting the presence of oligosaccharides in the untreated receptor (Waldo *et al.*, 2004). Extracellular loops 2 and 3 are connected by a disulfide bridge between two cysteine residues. The second intracellular loop contains an His-Arg-Tyr (HRY) motif, similar to the highly conserved Asp-Arg-Tyr (DRY) motif found in many G-protein coupled receptors that mediates G-protein coupling and receptor activation, though a significant role has not been found for this motif in the $P2Y_1$ receptor (Rovati *et al.*, 2006). Mutation of arginine residues 333 and 334 in the C-terminus to alanine did, however, result in a receptor incapable of activating G_q in CHO-K1 cells. The C-terminal tail also contains a four amino-acid PKC consensus sequence starting at Ser-336, SRAT, the terminal threonine of which appears to be required for desensitization (Fam *et al.*, 2003). A calmodulin binding motif is found between residues 332-343, and calmodulin-dependent protein kinase II was shown to be involved in receptor internalization but not desensitization (Arthur *et al.*, 2006; Tulapurkar *et al.*, 2006). The last four amino acids of the receptor are Asp-Thr-Ser-Lys (DTSL) and these were found to act as a PDZ ligand for the Na^+/H^+ exchanger NHERF-2. NHERF-2 was coimmunoprecipitated with the receptor C-tail and $PLC\beta_1$ from HEK293 cells and was found coexpressed with the $P2Y_1$ receptor in perivascular glial cells of the central nervous system. Coexpression of NHERF-2 with the $P2Y_1$ receptor resulted in increased latency of the Ca^{2+} response

25

compared to the $P2Y_1$ receptor alone, suggesting that NHERF-2 may act as a scaffold to stabilize the receptor signaling complex (Fam *et al.*, 2005).

Genetic deletion of the $P2Y_1$ receptor in mice results in significant hemostatic abnormalities (see section 1.4). Interestingly, although $P2Y_1$ knockout mice do not display renal abnormalities, a recent study reports that mice lacking global $P2Y_1$ expression were protected from renal disease progression in a model of crescentic nephrotoxic glomerulonephritis (Bailey *et al.*, 2004; Hohenstein *et al.*, 2007). Studies measuring receptor expression in the glomerulus of mice and rats were carried out on mRNA or using polyclonal antibodies to the receptor protein which exhibit very questionable selectivity; thus, results regarding the function of $P2Y_1$ in renal disease may reflect indirect changes in phenotype and will require investigation with more precise tools. Other effects of altering $P2Y_1$ function in animals include increased open arm exploration and increased food intake after cerebroventricular infusion of the $P2Y_1$ agonist ADPβS that was reversed by the selective antagonist MRS2179 (Kittner *et al.*, 2003; Kittner *et al.*, 2006). In each of these studies, effects of the $P2Y_1$ receptor were correlated to changes in the nitric oxide synthase pathway, suggesting a role for this intermediary. Taken together, these results indicate clear roles for the $P2Y_1$ receptor in mammalian physiology, in addition to its pronounced role in hemostasis, and underscore the need for broadly applicable, selective pharmacological reagents.

1.3.2 Ligand Development for the P2Y$_1$ Receptor

a. Adenosine Bisphosphates as Competitive Antagonists

Blockade of nucleotide receptor signaling was accomplished previously by the use of nonselective molecules such as reactive blue-2, suramin and PPADS which antagonized P1 and P2 receptors with different pharmacological selectivity but low to moderate potency, making them less than ideal for selectively identifying and blocking specific purinergic receptor subtypes. The identification of selective antagonists for P2 receptors began with the discovery of naturally occurring adenosine bisphosphates as competitive antagonists of the P2Y receptor of turkey erythrocyte membranes (tP2Y), a species ortholog of the human P2Y$_1$ receptor. Boyer, *et al* determined that adenosine bisphosphates bearing phosphate groups at the 5′ and 2′ or 3′ positions were selective partial agonists and competitive antagonists of the tP2Y receptor (Fig. 1.5). Moreover, at the human P2Y$_1$ receptor stably expressed in 1321N1 human astrocytoma cells, these compounds were simple competitive antagonists exhibiting no agonist activity with a pK_b value for A3P5P of 6.05 ± 0.01 (Boyer *et al.*, 1996).

b. Synthetic Nucleotide Analogues as Competitive Antagonists

The development of synthetic selective antagonists was pursued using structure-activity relationships for a series of adenine nucleotide derivatives to determine the minimal requirements for affinity and activity. Requisites for a biologically useful antagonist included higher potency and increased resistance to metabolic degradation by ectonucleotidases. Accordingly, substitutions on various positions of the adenine ring of adenosine bisphosphates were explored, followed by modifications and replacement of the ribose ring with various cyclic and acyclic structures and substitutions of the 3′ and 5′ phosphates with uncharged functional groups. These studies were complemented with

molecular modeling of the receptor to predict the energy and space determinants governing recognition of the various nucleotide analogues in the putative receptor binding site.

Substitutions at the 2, 6, and 8 positions of the adenine ring indicated that 2-substitutions were well tolerated at the tP2Y receptor as previously described, with preference for chloro and methylthio substitutions that conferred up to a 3-fold increase in antagonist potency while retaining partial agonist activity (Fischer *et al.*, 1993). In later studies, the successful substitution of halogens at the 2-position in combination with other structural modifications resulted in much greater increases in potency and several high affinity, pharmacologically useful compounds (Houston *et al.*, 2006; Kim *et al.*, 2003; Nandanan *et al.*, 1999; Nandanan *et al.*, 2000; Waldo *et al.*, 2002). Substitutions at the 8-position were largely disadvantageous, resulting in molecules that interacted poorly with the receptor.

Of particular importance was the finding that addition of a methyl group at the N^6 position eliminated partial agonist activity at the tP2Y receptor and increased the potency of adenosine bisphosphates as antagonists. The N^6-methyl substitution was sufficient to eliminate the partial agonist activity of a broad range of modified adenine nucleotide derivatives, generating a template on which to design pure competitive antagonists. Other substitutions at this position, including alkyl groups of longer chain length, a benzoyl group, or chloro or hydroxyl groups resulted in either decreased potency or complete loss of activity. Modification of the ribose ring indicated that the unphosphorylated hydroxyl group of A3P5P was not required for recognition at the tP2Y receptor and eliminated activity at A1 adenosine receptors. The N^6 methyl addition on the deoxyribose template of A3P5P resulted in a highly selective, competitive antagonist of high nanomolar apparent affinity.

c. MRS2179

N^6-methyl-2′deoxyadenosine-3′,5′-bisphosphate (MRS2179, Fig. 1.5) was the first widely used synthetic purinergic receptor antagonist of high potency and selectivity. Schild analysis of the ability of MRS2179 to inhibit 2MeSATP-promoted inositol phosphate accumulation in turkey erythrocyte membranes revealed pure competitive antagonist activity, as indicated by a parallel rightward shift of the 2MeSATP dose response curve. A pK_B of 6.99 ± 0.13 was calculated from these data (Boyer *et al.*, 1998). Similar results were obtained with the corresponding 2′,5′ bisphosphate. MRS2179 was used to inhibit the $P2Y_1$ receptor of human and rat platelets. MRS2179 inhibited ADP-mediated shape change, aggregation and Ca^{2+} release with a pK_B for the inhibition of the aggregation response of 6.55 ± 0.05 and with no effect on the ADP-mediated inhibition of adenylyl cyclase, indicating its use as a selective, potent, efficacious inhibitor of platelet aggregation via the $P2Y_1$ receptor (Baurand *et al.*, 2001). Intravenous infusion of MRS2179 in adult rats and mice resulted in decreased aggregation and increased bleeding time, consistent with blockade of ADP-mediated aggregation. MRS2179 was radiolabeled with ^{33}P and used to quantify $P2Y_1$ receptor binding sites in washed human platelets, indicating a density of 134 ± 8 binding sites per platelet (Baurand *et al.*, 2001). These results were encouraging for the development of $P2Y_1$-selective antithrombotic drugs.

The demonstration of MRS2179 as a highly selective competitive antagonist and subsequent commercial availability made possible the use of this molecule to identify or exclude a role for $P2Y_1$ receptor function in other tissues and physiological systems. A role for $P2Y_1$ receptor activity was indicated in several cellular processes most notably astrocyte

survival and slow excitatory neurotransmission in the central and peripheral nervous systems (Fang *et al.*, 2006; Gallego *et al.*, 2006; Kittner *et al.*, 2006; Mamedova *et al.*, 2006).

d. Acyclic Derivatives and Conformationally Constrained Nucleotide Analogues as Competitve P2Y$_1$ Receptor Antagonists

Fundamental to generating an even more useful selective antagonist was circumvention of the problem of metabolism by ectonucleotidases which primarily hydrolyze phosphates of the ribose ring of nucleotide substrates. Toward this end, several replacements of the ribose entity were explored, with heavy consideration of acyclic nucleotide analogues bearing symmetrically branched phosphorylated aliphatic chains or constrained carbocyclic rings (Kim *et al.*, 2000; Nandanan *et al.*, 2000). Of these modifications, two types emerged as having significant impacts on affinity while resisting nucleotide hydrolysis. Studies with acyclic bisphosphate nucleotides indicated that the ribose ring was not necessary for recognition by the tP2Y$_1$ receptor binding site, though it appeared necessary for receptor activation. Symmetrically branched, phosphorylated isopentyl, isobutyl, and isopropyl groups attached at the 9-position of the adenine ring were all recognized at the tP2Y receptor and bore no agonist activity with the isobutyl group retaining antagonist potency compared to its N^6-methyl precursor. Isopropyl and isopentyl groups were less potent (Fraser *et al.*, 2000; Kim *et al.*, 2002). Replacement of one of the phosphate groups with an uncharged ester greatly decreased potency in turkey erythrocytes and washed human platelets while replacement of both phosphate groups with uncharged esters greatly decreased potency at the tP2Y receptor while retaining inhibitory activity in washed human platelets. Interestingly, the uncharged bisphosphonates were capable of blocking ADP-mediated cAMP inhibition in platelets but not the Ca^{2+} increase, suggesting that these compounds were recognized by the

$P2Y_{12}$ but not the $P2Y_1$ receptor. Additionally, a bisphosphorylated cyclopropyl group attached at the 9-position of the N^6-methlyadenine ring was recognized by the tP2Y receptor albeit with lower potency than the riboside. These results taken together indicated that the bisphosphate nature of 9-substituted structures, and not the ribose ring, in addition to the N^6-methyl susbstitution, were the necessary requirements for $P2Y_1$ receptor antagonism, making a hydrolysis-resistant antagonist feasible.

Based on the dispensible nature of the ribose ring, further deviation from the nucleotide structure was pursued using phosphorylated carbocyclic rings. The importance of ring puckering was determined, based on previous observations by Marquez and colleagues suggesting a role for conformational preference in nucleotides as antiviral agents (Marquez *et al.*, 1996). Phosphorylated carbocyclic rings on adenine nucleotides constrained in either the Northern or the Southern conformation by a fused cyclopropane ring were examined for recognition at the $P2Y_1$ receptor. In the case of the weak agonist ATP, the Northern constrained derivative was 200-fold more potent than the unconstrained molecule and 250-fold more potent than the S-isomer, indicating a substantial role for sugar puckering in recognition at the $P2Y_1$ receptor. Using a molecular homology model of the $P2Y_1$ receptor based on the structure of bovine rhodopsin, ATP and MRS2179 were docked in the putative binding site and found to reside in the binding pocket preferably in the Northern conformation which was energetically favored by 20 kcal/mol over the Southern conformation. These results translated well to several nucleotide derivatives, with enhanced potency for *N*-constrained agonists and antagonists at the $P2Y_1$ receptor (Kim *et al.*, 2002; Nandanan *et al.*, 2000). This conformational preference applied to some, but not all of the G_q-coupled P2Y receptor subtypes, indicating similarities in the binding pockets of

nucleotide receptors that would later be further analyzed through a series of molecular modeling studies.

e. An Energetically Favorable Model for the P2Y₁ Receptor Binding Site

The molecular model of the P2Y$_1$ receptor was constructed using the electron density map of bovine rhodopsin as a template. Using molecular dynamics simulations, an energetically sound conformational hypothesis was generated identifying transmembrane domains, extracellular loops and a short amino terminus. Various ligands were docked into the model to establish a putative binding site and these studies were complemented with site-directed mutagenesis to confirm the requirement of specific residues for the coordination of agonists and antagonists in the binding site. A principle binding domain was established identifying residues necessary for coordinating various atoms of the nucleotide (Fig. 1.4). In this model, Gln307 and Ser314 of TM7 are in contact with the adenine ring while the phosphate groups of both polyphosphate agonists and bisphosphate antagonists reside in a positively charged pocket formed by Arg128 (TM3), Lys280(TM6), and Arg310(TM7). Based on site-directed mutagenesis studies, the physical properties of these residues appeared to be more important than their absolute conservation, since agonist activation was restored in S314T and R310K mutant receptors (Costanzi *et al.*, 2004).

In addition to the principle binding domain, two additional binding sites comprised by extracellular loops 2 and 3 (EL2, EL3), termed meta-binding sites, were identified and a three-step model for ligand binding was proposed, with association at the meta-binding sites necessarily preceding binding at the principle site. In meta-binding site I, Glu209 and Arg287 help coordinate the ribose ring and α and β phosphates of ATP, respectively. Meta-binding site II exists just beneath EL2 and shares amino acids Arg128, Lys280 and Gln307

with the principle binding site. Residues determined to be essential for ligand recognition are likely necessary for the formation of stable intermediates during the multistep ligand binding process (Moro *et al.*, 1998).

f. [^{3}H]MRS2279

The remarkable effect of conformational constraint coupled with the previously identified modifications that improved antagonist activity led to the development of 2-chloro-N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2279), a P2Y$_1$ antagonist with K_B value of 8 nM for the human P2Y$_1$ receptor stably expressed in astrocytoma cells. This molecule was the first non-nucleotide antagonist of a P2Y receptor, and based on its high affinity and stability was used to develop a high affinity radioligand, [^{3}H]MRS2279 (Fig. 1.5). Attaching [^{3}H]methylamine at the 6-position of the adenine ring yielded a radioligand of specific activity 89 Ci/mmol (Fig. 1.5). [^{3}H]MRS2279 bound to the human P2Y$_1$ receptor overexpressed in Sf9 insect cell membranes with a K_D of 7.05 nM, and various nucleotide antagonists inhibited [^{3}H]MRS2279 binding with pharmacological selectivity predicted from previous studies. [^{3}H]MRS2279 also bound specifically to the endogenous P2Y$_1$ receptor of rat brain and human platelets with affinity and selectivity similar to that of the recombinant receptor. Due to extensive metabolism of nucleotide agonists, agonist affinities were not determined in these systems, but were determined using [^{3}H]MRS2279 at the purified, recombinant P2Y$_1$ receptor, establishing direct agonist affinities for nucleotides at the P2Y$_1$ receptor for the first time. [^{3}H]MRS2279 also proved useful in developing the first radioligand binding assay suitable for screening P2Y$_1$ antagonists using Sf9 insect cell membranes as a model system. The development of a binding assay complemented previous and subsequent SAR and molecular modeling studies

well, making the predictions of activities of newly synthesized nucleotide analogues highly reliable (Kim *et al.*, 2003; Waldo *et al.*, 2002; Waldo *et al.*, 2004).

g. MRS2500

Substitutions at the 2-position of adenine had been previously successful, and were revisited on the (N)-methanocarba template. In competition binding assays, a 2-iodo derivative exhibited 10-fold higher affinity than MRS2279 for the P2Y$_1$ receptor overexpressed in Sf9 insect cell membranes (Fig. 1.5). The presence of an iodine and revised synthetic studies indicated that this molecule was a suitable template for developing a high specific radioactivity radioligand for the P2Y$_1$ receptor, and its subnanomolar affinity and non-nucleotide structure made it suitable for *in vivo* testing as an antithrombotic agent (Kim *et al.*, 2003).

Though MRS2179 effectively inhibited platelet aggregation through blockade of P2Y$_1$ receptors *in vivo*, MRS2500 proved more stable *in vivo* and its much higher affinity made it a much more attractive candidate for antiplatelet testing. In mice, MRS2500 potently inhibited collagen and epinephrine-induced thrombus formation and thrombus formation in response to laser-induced vascular injury. Additionally, the combination of MRS2500 and the irreversible P2Y$_{12}$ antagonist clopidogrel was more effective than either drug alone in a mouse model of arterial thrombosis, suggesting benefits of combination antiplatelet therapy targeting P2Y$_1$ and P2Y$_{12}$ receptors. MRS2500 alone also caused only a modest increase in bleeding time, suggesting advantages of reversible, competitive P2Y$_1$ antagonists with regard to safety (Hechler *et al.*, 2006).

h.   (N)-Methanocarba-2MeSADP, A P2Y$_1$ Receptor-Selective Agonist

Selective activation of P2Y$_1$ receptors was an elusive goal, particularly in platelets where P2Y$_1$ and P2Y$_{12}$ are activated by the endogenous agonist ADP.   However, the selective preference of the P2Y$_1$ receptor for Northern-constrained nucleotide analogues led to the finding that the nonselective platelet agonist 2MeSADP could be converted to a P2Y$_1$-selective agonist by replacement of the ribose ring with a cyclopentane ring locked in the Northern conformation, similar to MRS2279 and MRS2500 (Chhatriwala *et al.*, 2004).   The resulting compound (*N*)-methanocarba-2-methylthioadenosine-5′-diphosphate (MRS2365) activated P2Y$_1$ but not P2Y$_{12}$ and P2Y$_{13}$ receptors transiently expressed in Cos-7 cells and induced shape change without sustained aggregation in washed human platelets, indicative of a P2Y$_1$ receptor-specific response.   MRS2365 was also used to show that the refractoriness of platelets to ADP after initial exposure is a result of rapid, selective desensitization of the P2Y$_1$ receptor (Bourdon *et al.*, 2006).

1.3.3 Agonist-Promoted Regulation of the P2Y$_1$ Receptor

a. Homologous Desensitization and Regulation by Second Messenger Kinases

Desensitization of GPCRs by GRK phosphorylation and subsequent arrestin binding followed by internalization into clathrin-coated pits was established using the β2 adrenergic receptor as a model and is a well-characterized system for the regulation of GPCR signaling (Pierce *et al.*, 2002).   Data regarding desensitization of G$_q$-coupled GPCRs is limited, primarily due to lack of a reliable assay.   However, the unique combination of platelet physiology and a selective P2Y$_1$ agonist allowed for a recent detailed study of P2Y$_1$ receptor desensitization (Bourdon *et al.*, 2006).   Refractoriness of platelets to ADP after initial

exposure has been established; however, whether this lack of responsiveness was due to desensitization of the $P2Y_1$ receptor, the $P2Y_{12}$ receptor or both remained largely unresolved. Using the selective $P2Y_1$ agonist MRS2365, Bourdon and coworkers showed that the $P2Y_1$ receptor was fully desensitized in response to agonist with a t½ of 18 seconds, resulting in the inability of ADP to promote full platelet aggregation. Induction of G_q signaling through the 5-HT_{2A} receptor fully restored ADP-induced platelet activation, suggesting that desensitization is receptor-specific and not due to agonist-induced changes in downstream signaling. These studies clearly indicate that the $P2Y_1$ receptor in platelets undergoes homologous desensitization in response to agonist treatment. The rate of desensitization of the $P2Y_1$ receptor in human platelets was remarkably rapid, and these studies did not address whether this effect was specific to platelets or whether other G_q-coupled receptors in platelets desensitized with similar kinetics.

Toward understanding the mechanisms involved in desensitization of the $P2Y_1$ receptor, Mundell and Benovic investigated the effect of inhibiting arrestin expression on desensitization of the endogenous $P2Y_1$ receptor in HEK293 cells. Interestingly, they found that a decrease in the expression of either arrestin 2, arrestin 3 or both had no effect on the rate or magnitude of desensitization of $P2Y_1$ receptor signaling. Additionally, while activation of other GPCRs caused a recruitment of arrestin-2-GFP to the plasma membrane, selective activation of the $P2Y_1$ receptor with ADP did not result in a redistribution of arrestin-2-GFP. In another more recent study, Hardy and colleagues showed that expression of GRK-dominant negative mutants co-expressed with the $P2Y_1$ receptor in 1321N1 human astrocytoma cells had no effect on $P2Y_1$ receptor desensitization while selective inhibitors of PKC attenuated the loss of $P2Y_1$ receptor responsiveness that followed pretreatment of cells

with a $P2Y_1$ receptor agonist (Hardy *et al.*, 2005; Mundell *et al.*, 2000). Additionally, Fam and colleagues reported the role of PKC in a negative feedback loop responsible for regulating $P2Y_1$ calcium oscillation frequency in 1321N1 cells. They also reported the necessity of a threonine residue, T339, in a C-terminal PKC consensus sequence for this effect (Fam *et al.*, 2003). These data suggest that desensitization of the $P2Y_1$ receptor does not occur by the canonical GRK and arrestin-mediated pathway and that conventional and novel isoforms of the second messenger-activated kinase PKC may play an important role.

b. Agonist-Induced Internalization

Understanding the mechanisms of internalization of the $P2Y_1$ receptor has been difficult due to a lack of tools available to selectively label endogenously expressed cell surface receptors. A lack of reliable antibodies and high specific activity radioligands has limited our knowledge of $P2Y_1$ receptor endocytosis primarily to studies involving overexpressed, epitope-tagged or fluorescently tagged receptors. However, in 2004, Baurand and colleagues published a study examining internalization of $P2Y_1$ receptors in platelets using a polyclonal antibody to the endogenous $P2Y_1$ receptor. They observed a notably rapid rate of $P2Y_1$ receptor cycling with immunoreactivity appearing in intracellular compartments within 15 seconds and beginning to recycle back to the plasma membrane within 4 minutes. In the same study, they investigated the rate of internalization of GFP-tagged $P2Y_1$ receptors in 1321N1 human astrocytoma cells and found 50% of the total fluorescence in intracellular compartments after 15 minutes and 75% of the total fluorescence inside the cell after two hours (Baurand *et al.*, 2005). A more recent report by Mundell and coworkers utilized [^{3}H]2MeSADP, a nonspecific radioligand, to examine changes in surface levels of $P2Y_1$ and $P2Y_{12}$ receptors on fixed platelets. This study indicated that internalization of $P2Y_1$ receptor

occurs with slower kinetics and to a greater extent than the previous report, and that internalization, like desensitization, required the activity of novel and conventional isoforms of PKC (Mundell *et al.*, 2006a). Mundell *et al.* also reported PKC-dependent internalization of HA-tagged $P2Y_1$ receptors stably expressed in 1321N1 cells. A role for clathrin-coated pits has been described, and recent data suggests that subpopulations of clathrin-coated pits may exist, and receptor targeting to specific subsets of clathrin-coated pits may be dictated by the kinases and adaptor proteins involved in internalization, since the $P2Y_1$ receptor, which does not require arrestins or GRKs for internalization, sorts differently than the $P2Y_{12}$ or β2-adrenergic receptors which have been shown to require GRKs and arrestins for internalization (Mundell *et al.*, 2006b). These differences in rates of $P2Y_1$ receptor internalization in the two cell types are not surprising, since cell-type-specific trafficking mechanisms have been observed for other GPCRs including the m3 muscarinic receptor (Koenig *et al.*, 1996). Additionally, differences in methodology are likely to contribute to inconsistent observations. Such discrepancies underscore the need for selective, sensititve tools for quantification of native receptor binding sites as part of a comprehensive study of the mechanisms involved in the internalization of endogenous $P2Y_1$ receptors.

1.4 Nucleotide Receptors and Platelet Biology

The $P2Y_1$ receptor exhibits a broad tissue distribution, but its most notable role in human physiology comes from its localization on the surface of platelets - discoid, anucleated cells of the vasculature that play a primary role in the formation of blood clots.

1.4.1 Platelet Activation and Thrombus Formation

Upon entering a site of vascular injury, circulating platelets become activated in response to collagen and von Willebrand factor present in the subendothelial extracellular matrix. The interaction of these molecules with the platelet vWF and collagen receptors results in the inside-out activation of integrins, which mediate the adhesion of a platelet monolayer to the wall of the injured vessel (Jackson *et al.*, 2003; Nieswandt *et al.*, 2003; Ruggeri, 2002; Ruggeri, 2003). Activation of platelets results in the release of platelet dense granule contents and the localized formation of other potent mediators of platelet aggregation. These molecules include thromboxane A_2 (TxA$_2$), ADP, and thrombin, all of which act through G-protein coupled receptors to promote cytoskeletal rearrangement resulting in the transformation of the platelet from a discoid shape with a smooth outer surface to a spherical shape with protruding pseudopods. Shape change is accompanied by an increase in cytosolic calcium, the subsequent activation of $\alpha_{IIb}\beta_3$ integrin, and continued calcium-dependent granule secretion, forming a positive feedback loop that promotes successive platelet aggregation and the formation of a hemostatic plug (Offermanns, 2006).

1.4.2 Platelet Receptors for Adenine Nucleotides

ADP secreted from platelet dense granules acts through two G-protein coupled purinergic receptors, the P2Y$_1$ and P2Y$_{12}$ receptors, to mediate platelet aggregation. ATP is also released from activated platelets and acts through the P2X$_1$ receptor to induce a transient shape change from discoid to spherical shape without the formation of pseudopods (Rolf *et al.*, 2001; Rolf *et al.*, 2002). Both the P2Y$_1$ and P2Y$_{12}$ receptors are absolutely necessary for proper platelet aggregation and mice and humans lacking either receptor exhibit hemostatic

abnormalities including increased bleeding time, defective thrombus formation, and decreased sensitivity to other platelet agonists (Cattaneo, 2005; Fabre *et al.*, 1999; Foster *et al.*, 2001; Leon *et al.*, 1999a).

a. P2Y₁

In platelets, activation of the $P2Y_1$ receptor by ADP results in G_q-mediated stimulation of PLCβ and the subsequent production of the second messengers IP_3 and DAG. IP_3 binds to its receptor on the endoplasmic reticulum (ER), causing an increase in intracellular calcium while DAG activates PKC. Elevated cytosolic calcium is responsible for the activation of phospholipase A_2 and subsequent generation of the potent platelet agonist thromboxane, granule mobilization and secretion, and transient $\alpha_{IIb}\beta_3$ activation. Studies using $P2Y_1$-deficient mice indicate that the $P2Y_1$ receptor is necessary for ADP-promoted shape change and aggregation, since these animals demonstrate prolonged bleeding and decreased thrombus size, formation and stability compared to wild-type animals. Furthermore, platelets from these animals fail to undergo shape change, exhibit reduced calcium mobilization, and display impaired aggregation in response to collagen, thrombin and TxA_2 (Fabre *et al.*, 1999; Leon *et al.*, 1999a).

G_q is a necessary component of ADP-mediated platelet aggregation. In the absence of $P2Y_1$ activity, stimulation of G_q through the $5-HT_{2A}$ serotonin receptor is sufficient to restore ADP-mediated platelet shape change and activation (Bourdon *et al.*, 2006; Fabre *et al.*, 1999; Jin *et al.*, 1998). Additionally, the $P2Y_1$ receptor is selectively coupled to G_q in platelets since agonists of the TxA_2 and PAR receptors which couple to G_q and $G_{12/13}$ can promote shape change in platelets from G_q-deficient mice while ADP cannot (Offermanns, 2006).

The predominant isoform of PLCβ downstream of P2Y$_1$ activation in platelets appears to be PLCβ2 as evidenced by impairment of ADP-induced IP$_3$ formation and platelet aggregation in patients with decreased PLCβ2 expression and in PLCβ2/3-deficient mice (Lee *et al.*, 1995; Rhee *et al.*, 1992). IP$_3$ generated from the activation of PLCβ2 binds to IP$_3$ receptors on the ER, resulting in release of calcium from the ER. Diacylglycerol activates PKC which is required for platelet secretion, and independent of PKC, DAG can promote the exchange activity of CalDAG-GEF1 on Rap1, an intermediate step in the activation of α$_{IIb}$/β$_3$ (Bertoni *et al.*, 2002; Eto *et al.*, 2002). Until recently, the PKC isoforms involved in ADP-mediated signaling in platelets were unknown, but studies by Mundell et al suggest that novel and classical PKC isoforms regulate P2Y$_1$ signaling in platelets, potentially by a negative feedback mechanism resulting in rapid P2Y$_1$ receptor desensitization (Mundell *et al.*, 2006a).

b. P2Y$_{12}$

The P2Y$_{12}$ receptor signals through G$_i$ to promote sustained platelet aggregation independent of shape change and to enhance dense granule secretion and other effects of more potent platelet agonists. The presence of a distinct receptor negatively coupled to adenylyl cyclase in platelets was controversial until the development of selective P2Y$_1$ antagonists (Boyer *et al.*, 1996). Subsequently, the P2Y$_{12}$ receptor was cloned and identified to be the target of thienopyridine compounds capable of inhibiting platelet aggregation and thrombus formation *in vivo* (Hollopeter *et al.*, 2001). In the absence of P2Y$_{12}$ receptor activity, agonists of other G$_i$-coupled receptors including adrenaline can rescue ADP-induced platelet aggregation (Bourdon *et al.*, 2006; Leon *et al.*, 1999b). Similarly, impaired ADP-induced aggregation due to loss of P2Y$_1$ activity can be rescued with 5-HT which activates the G$_q$-coupled 5-HT$_{2A}$ receptor of platelets.

The P2Y$_{12}$ receptor couples selectively to Gα_{i2} to promote a decrease in intracellular cAMP and activation of the PI3K pathway via $\beta\gamma$ subunits. Signaling through PI3K is essential for potentiation of dense granule secretion and partial platelet aggregation induced by activation of the P2Y$_{12}$ receptor as evidenced by the use of nonselective PI3K inhibitors, wortmannin and LY 294002 (Kauffenstein *et al.*, 2001). Although there are multiple PI3K isoforms expressed in platelets, PI3K γ and PI3K β appear to be selectively activated downstream of the P2Y$_{12}$ receptor (Abrams *et al.*, 1996; Hirsch *et al.*, 2001; Jackson *et al.*, 2005; Lian *et al.*, 2005). PI3K γ-deficient mice display a defect in fibrinogen receptor activation and ADP-induced aggregation; these defects are similarly observed in the presence of selective P2Y$_{12}$ receptor antagonists and in G$_{i2}$-deficient mice (Hirsch *et al.*, 2001; Hollopeter *et al.*, 2001; Kim *et al.*, 2004). Selective inhibitors of PI3K β indicate that this isoform is specifically responsible for sustained aggregation in response to submaximal concentrations of thrombin and thromboxane receptor agonists (Jackson *et al.*, 2005).

A congenital defect in the P2Y$_{12}$ receptor has been observed in four human patients. In two patients, premature termination of translation is caused by a homozygous frameshift, while in another, a frameshift in a single allele results in deletion of two nucleotides. In the fourth, substitution of two amino acids in regulatory domains of the receptor gene yields a normal coding sequence and gene product of decreased expression but normal binding of the endogenous agonist (Cattaneo *et al.*, 2003; Hollopeter *et al.*, 2001). Platelets from these patients exhibit normal shape change but weak and rapidly reversible aggregation in response to ADP, and defects are observed in platelet aggregation induced by collagen, arachidonic acid and thromboxane A$_2$ but not thrombin; also, these patients have a history of mucosal

bleeding, increased bleeding time, easy bruising and/or excessive post-operative bleeding (Cattaneo *et al.*, 2000; Cattaneo *et al.*, 1992; Cattaneo *et al.*, 2003; Nurden *et al.*, 1995).

c. P2X$_1$

Activation of the ionotropic P2X$_1$ receptor primarily serves to induce platelet shape change and amplify platelet responses induced by low concentrations of other platelet agonists. ATP stored in dense granules is co-released with ADP and binds to the P2X$_1$ ion channel, causing the nonselective permeation of cations, followed by rapid desensitization of the channel. An increase in intracellular calcium is necessary for platelet shape change; however, activation of the P2X$_1$ receptor results in the transformation of platelets from discoid to spherical shape but does not cause the formation of filopodial extensions (Rolf *et al.*, 2001; Rolf *et al.*, 2002). Increased calcium concentration is known to activate myosin light-chain kinase which promotes cytoskeletal rearrangement. Additionally, depolarization of the platelet membrane is capable of enhancing calcium-dependent signaling downstream of GPCRs suggesting that P2X$_1$ receptor-dependent depolarization of the platelet membrane may also play a role in calcium-dependent signaling processes in response to P2X$_1$ activation (Mahaut-Smith *et al.*, 1999; Mason *et al.*, 2001). The P2X$_1$ channel is also permeant to sodium. No physiological role for increased intracellular sodium has been established; replacement of extracellular sodium with impermeant ions had no effect of the ability of the P2X$_1$ receptor to induce shape change (Clifford *et al.*, 1998).

Genetic deletion of P2X$_1$ in mice results in enhanced resistance to laser-induced or ferric-chloride induced thrombosis with no change in bleeding time, while overexpression of the P2X$_1$ receptor in mice causes an increase in collagen and epinephrine-induced thrombus formation, suggesting a significant physiological role for the P2X$_1$ receptor in potentiating

thrombotic events initiated by potent platelet agonists (Hechler *et al.*, 2003; Oury *et al.*, 2003).

1.4.3 P2Y Receptors as Current and Potential Therapeutic Targets in Thrombosis

Effective antiplatelet agents currently in clinical use target the $P2Y_{12}$ receptor; however, the essential role for the $P2Y_1$ receptor and the $P2X_1$ receptor in platelet function render these receptors powerful potential targets for antiplatelet therapy as well.

Ticlopidine (Ticlid®) and clopidogrel (Plavix ®) are clinically efficacious and widely prescribed thienopyridine compounds that selectively inhibit the $P2Y_{12}$ receptor. Both of these compounds are prodrugs, metabolized to their active forms by cytochrome P450 1a. The active metabolites selectively and irreversibly inhibit platelet aggregation by modification of two extracellular cysteine residues on the $P2Y_{12}$ receptor.   The need for metabolism of these compounds results in a delayed onset of action, with maximum inhibition of ADP-induced platelet aggregation occurring after 4-5 days. Because inhibition is irreversible, the effect is observed for approximately ten days, corresponding to the lifespan of a circulating platelet (Cattaneo, 2007).

Consistent with their effects on the $P2Y_{12}$ receptor, ticlopidine and clopidogrel are effective at inhibiting platelet aggregation by potent platelet agonists through their ability to suppress the amplification of response generated by ADP released from platelet dense granules. Clopidogrel is more potent and well-tolerated than ticlopidine and the delayed onset of action of clopidogrel can be obviated by increasing the loading dose by 4-8-fold. Side effects with both compounds include prolonged bleeding and rare cases of cytopenia, each of which is more pronounced with ticlopidine than clopidogrel. Other, more general,

drawbacks to these compounds include high interindividual variability and decreased activity when co-administered with statins, which appear to interfere from inhibition of cytochrome p450 activity (Cattaneo, 2007).

Prasugrel is a recently developed thienopyridine compound with the same mode of action as ticlopidine and clopidogrel, but with increased potency and lower interindividual variability. It is currently in Phase III clinical trials for patients with acute coronary syndromes undergoing percutaneous coronary intervention (Niitsu *et al.*, 2005).

Hydrolysis-resistant ATP analogues are under investigation as direct, reversible $P2Y_{12}$ antagonists. Intravenous infusion of cangrelor, a high affinity $P2Y_{12}$ antagonist, reversibly inhibits ADP-induced platelet aggregation in men and women without increased bleeding time (van Giezen *et al.*, 2005). AZD6140 is an orally available non-phosphate competitive $P2Y_{12}$ antagonist (Husted *et al.*, 2006; van Giezen *et al.*, 2005). In a single clinical trial with atherosclerotic outpatients currently receiving aspirin, addition of AZD6140 to therapy resulted in superior antiplatelet effects to the combination of clopidogrel and aspirin, with a rapid onset of action and only a moderate increase in bleeding time.

Several selective, high affinity $P2Y_1$ receptor antagonists have been developed as discussed previously. While MRS2179 yielded promising results in rodents *in vivo* and *ex vivo*, MRS2500 proved more stable *in vivo* and potently inhibited collagen and epinephrine induced thrombus formation and thrombus formation in response to laser-induced arterial injury in mice (Baurand *et al.*, 2001; Hechler *et al.*, 2006). Intravenous infusion of MRS2500 in mice caused only a modest increase in bleeding time, suggesting that $P2Y_1$ receptor antagonists may be advantageous in terms of safety. Additionally, the treatment of

P2Y$_1$-deficient animals with clopidogrel or of wild type animals with clopidogrel and MRS2500 resulted in increased efficacy compared to either drug alone, indicating that combination therapy targeting the P2Y$_1$ and P2Y$_{12}$ receptors may be a powerful antiplatelet strategy.

Few selective antagonists have been developed for the P2X$_1$ receptor, and whether or not it is a potential target in thrombosis has been controversial. However, the unique role of P2X$_1$ in inhibiting thrombus formation at sites of high shear rate suggest that, under certain circumstances, antagonists of the P2X$_1$ receptor may prove useful (Cattaneo, 2007). NF449, a suramin analogue, is a recently developed P2X$_1$ antagonist that is selective for P2X$_1$ over several P2X and P2Y receptors including P2Y$_1$. Selective inhibition of P2X$_1$ was observed *in vivo* with decreased platelet aggregation in a mouse model of systemic thromboembolism.

1.5 Specific Aims of the Current Research

Based on recent advances in ligand development for the P2Y$_1$ receptor and gaps in our knowledge regarding agonist-promoted regulation of this important biological sensor, the current research is designed to generate a high specific radioactivity radioligand for the P2Y$_1$ receptor, to apply this ligand to study agonist-promoted regulation of the P2Y$_1$ receptor in human platelets, and to identify the molecular determinants of agonist-dependent regulation of P2Y$_1$ receptors in mammalian epithelial cells.

The recently developed antagonist MRS2500 is the highest affinity P2Y$_1$ receptor antagonist available with a K_i value of 0.79 nM for inhibition of [^{3}H]MRS2279 binding in Sf9 insect cell membranes overexpressing the human P2Y$_1$ receptor (Kim *et al.*, 2003). The structure of this molecule, depicted in Figure 1.5, indicates the possibility of radiolabeling

this molecule to high specific radioactivity by placement of the radioisotope ^{125}I at the 2-position of the adenine ring or incorporation of the radioisotope ^{32}P in the 5′-phosphate group of the methanocarba ring. Preliminary data from our lab indicates that radiolabeling with ^{125}I requires a three-step synthesis reaction of prohibitively low percent yield to facilitate the routine synthesis of a radioligand with the short half life of ^{125}I (~60 days). Therefore, we have elected to use a single-step enzymatic synthesis reaction catalyzed by polynucleotide kinase to transfer the γ-phosphate of [γ^{32}P]ATP to a 3′-monophosphate precursor to generate [^{32}P]MRS2500. The synthesis of this radioligand and the development of a binding assay for [^{32}P]MRS2500 binding to the P2Y$_1$ receptor overexpressed in Sf9 insect cell membranes is described in Chapter 2. We have demonstrated the utlitity of this ligand for studying endogenous P2Y$_1$ receptors by applying it to assess the relative tissue distribution of P2Y$_1$ receptors in various peripheral tissues and brain regions of the adult male rat.

The role of the P2Y$_1$ receptor in platelet aggregation suggests that it is a powerful potential therapeutic target for conditions involving abnormal platelet function; however, information is limited regarding agonist-promoted regulation of P2Y$_1$ receptors in platelets. Previous studies from our lab have demonstrated that remarkably rapid desensitization of the P2Y$_1$ receptor occurs after treatment of platelets with the P2Y$_1$ receptor-selective agonist MRS2365 (Bourdon *et al.*, 2006). In Chapter 3, we apply a combination of the P2Y$_1$ receptor-selective ligands MRS2365 and [^{32}P]MRS2500 to further explore agonist-promoted regulation of the P2Y$_1$ receptor of human platelets. By examining the desensitization of the G$_q$-coupled 5-HT$_{2A}$ receptor of platelets, we determine whether the remarkably rapid rate of desensitization observed for the platelet P2Y$_1$ receptor is unique to this receptor. Furthermore, we assess the rate of recovery of P2Y$_1$ receptor responsiveness after

desensitization followed by apyrase-catalyzed removal of MRS2365. Additionally, we develop a radioligand binding assay for [^{32}P]MRS2500 binding to the P2Y$_1$ receptor of washed human platelets and apply this binding assay to measure the agonist-promoted loss of surface P2Y$_1$ receptors in human platelets.

Phosphorylation and internalization of the P2Y$_1$ receptor have been reported in platelets and 1321N1 human astrocytoma cells (Mundell *et al.*, 2006a). However, whether or not phosphorylation is required for internalization has not been determined and the molecular determinants of P2Y$_1$ receptor internalization have yet to be identified. In Chapter 4, we establish an intact cell binding assay for [^{32}P]MRS2500 binding to endogenous and recombinant P2Y$_1$ receptors in MDCK(II) epithelial cells to assess agonist-dependent internalization of the P2Y$_1$ receptor. Using this system, we express various mutant constructs of the P2Y$_1$ receptor lacking putative phosphorylation sites in the C-terminus to determine if phosphorylation in this region is required for agonist-induced internalization.

The findings presented in this work indicate that we have generated a novel, useful tool for studying P2Y$_1$ receptors in a variety of model systems. Furthermore, we use this ligand to address agonist-promoted regulation of P2Y$_1$ receptors in two very different cell types. In Chapter 5, we present a model of cell-type specific agonist-promoted regulation of P2Y$_1$ receptors and describe potential future directions that will more clearly define the mechanisms involved in each cell type.

Name	Tissue Distribution[#]	G-protein Coupling	Agonists[*]	Antagonists[*]
P2Y$_1$	Platelets, brain, placenta, prostate, heart, skeletal muscle	Gα_q, Gα_{11}	MRS2365 > 2MeSADP > ADP > 2MeSATP; ATP and ADPβS are partial agonists	MRS2500 > MRS2279 > MRS2179 > A3P5P > suramin > PPADS
P2Y$_2$	Lung, skeletal muscle, heart, brain, liver, stomach, pancreas, vascular smooth muscle, endothelial cells	Gα_q, Gα_{11}; rat can also couple to G$\alpha_{i/o}$	UTP > ATP > Ap$_4$A > ATPγS > 5BrUTP	Suramin
P2Y$_4$	Intestine, pituitary gland, brain, monocytes and lymphocytes	Gα_q, Gα_{11}	Human:UTP, UTPγS, 5BrUTP, UDP is a partial agonist; Rat: UTP = ATP	Human: PPADS, ATP; Rat: reactive blue 2
P2Y$_6$	Bone, spleen, placenta, kidney, lung, intestine, bone heart, brain, adipose tissue	Gα_q, Gα_{11}	UDP, UDPβS >> 5BrUTP > UTP > ADP >> 2MeSATP	Reactive blue 2 > PPADS > suramin
P2Y$_{11}$	Ubiquitous with highest levels in brain, spleen, lymphocytes and intestines and lowest levels in liver, cartilage and bone	Gα_q, Gα_{11}; Gα_s	PI hydrolysis: BzATP > ATPγS > dATP > ATP > ADPβS; cAMP formation: ATPγS > BzATP > dATP > ATP > ADPβS > 2MeSATP	PI hydrolysis: suramin > reactive blue 2; cAMP formation: suramin
P2Y$_{12}$	Platelets, spinal cord, brain	Gα_{i2}, >> Gα_{i1}, Gα_{i3}	2MeSADP = 2MeSATP > ADP; ATP is a partial agonist	Thienopyridines (see Section 1.5.3), Reactive blue 2, suramin, ARC66096, ARC69931MX
P2Y$_{13}$	Spleen, brain, liver, pancreas, bone marrow, heart, peripheral leukocytes	Gα_i, Gα_{16}	2MeSADP = ADPβS = 2MeSATP > ADP > Ap$_3$A > ATP	AR-C69931MX > PPADS > suramin
P2Y$_{14}$	Placenta, stomach, intestine, brain, spleen, lung, heart, bone marrow, immune cells	Gα_i, Gα_o, Gα_{16}	UDP-glucose > UDP-galactose > UDP-glucuronic acid > UDP-N-acetylglucosamine	

[#]Tissue distribution refers to mRNA expression pattern.
[*]Agonists and antagonists are listed in order of decreasing potency.
Table adapted from Abbracchio *et al*., 2006.

Table 1.1 Summary of P2Y receptor pharmacology and tissue distribution.

Agonist	K_i (μM)	Reference		Reference
2MeSADP	0.10 ± 0 0.015 (5)	Waldo, *et al*., 2004		
ADP	0.92 ± 0.23 (5)	Waldo, *et al*., 2004		
ATPγS	1.33 ± 0.42 (5)	Waldo, *et al*., 2004		
2MeSATP	1.87 ± 0.48 (5)	Waldo, *et al*., 2004		
ADPβS	2.42 ± 0.44 (5)	Waldo, *et al*., 2004		
ATP	17.7 ± 2.39 (5)	Waldo, *et al*., 2004		
Antagonist	K_i (nM)		K_B (nM)	
MRS2500	0.79	Kim, *et al*., 2003	1.6	Kim, *et al*., 2003
MRS2279	11 ± 3.0 (3)	Waldo, *et al*., 2004	8.0	Boyer, *et al*., 2002
MRS2179	15 ± 2.5 (3)	Waldo, *et al*., 2004	100	Boyer, *et al*., 1998
A3P5P	754 ± 100 (3)	Waldo, *et al*., 2004	900	Boyer, *et al*., 1996
PPADS	6200 ± 2600 (3-5)	Waldo, *et al*., 2002	1200	Boyer, *et al*., 1994
Suramin	4900 ± 1600 (3-5)	Waldo, *et al*., 2002		

Values reported are the average of 3 or more experiments ± S.E.M.

Table 1.2. Affinity constants for agonists and antagonists of the human P2Y$_1$ receptor.
Values were determined in competition binding assays with [^{3}H]MRS2279. Values of *n* for
each determination are noted in parentheses.
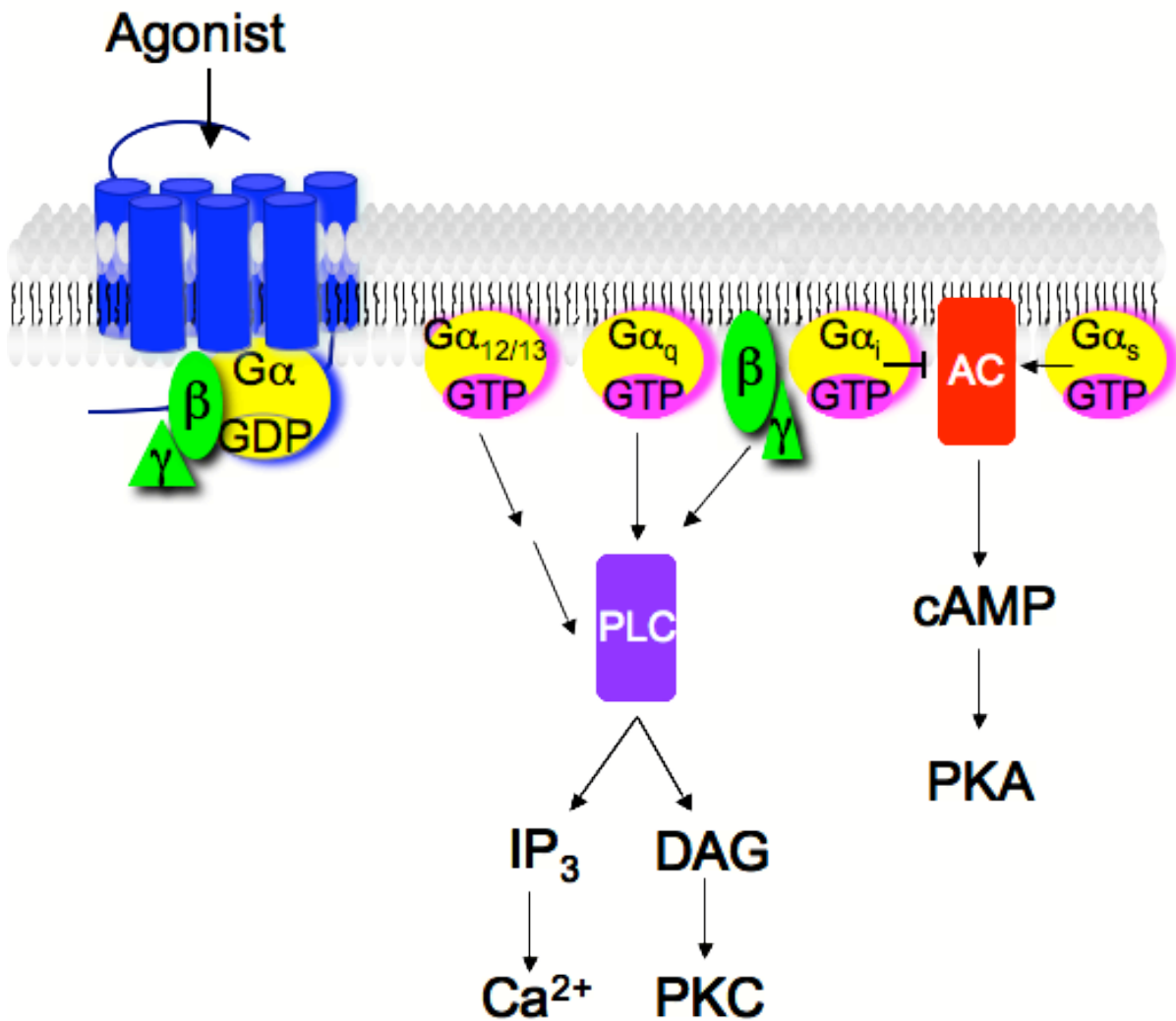
Figure 1.1. GPCR signal propagation. Agonist binding to GPCRs initiates the exchange of GDP for GTP on the Gα subunit and subsequent release of the G-protein heterotrimer from the receptor. GTP-bound Gα subunits of four different classes – G_s, G_i, G_q, and $G_{12/13}$ – and Gβγ subunits signal to various effector proteins (select effectors are depicted) to regulate levels of second messengers and thereby modify downstream signaling.
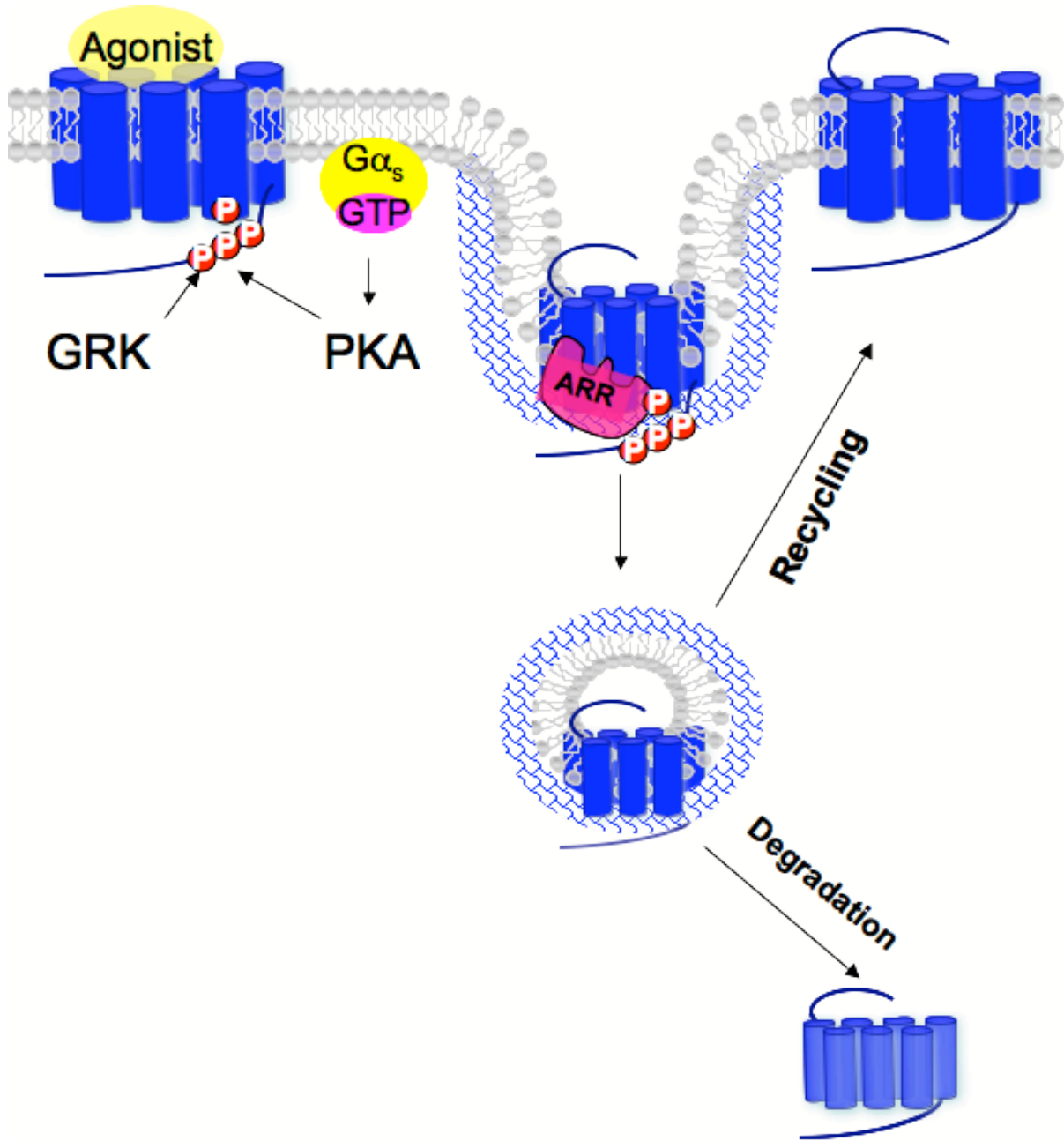
Figure 1.2. Agonist-promoted regulation of GPCR signaling. The canonical model of GPCR desensitization and internalization is based on studies of the β2 adrenergic receptor. Upon agonist binding, G-protein signaling results in the activation of second messenger kinases which can phosphorylate the receptor and prevent further-G protein coupling. GRKs can also phosphorylate the activated receptor. The phosphorylated receptor is a substrate for arrestin (ARR) which facilitates internalization into clathrin-coated pits. The internalized receptor may be targeted for degradation or recycled to the plasma membrane for subsequent rounds of stimulation.
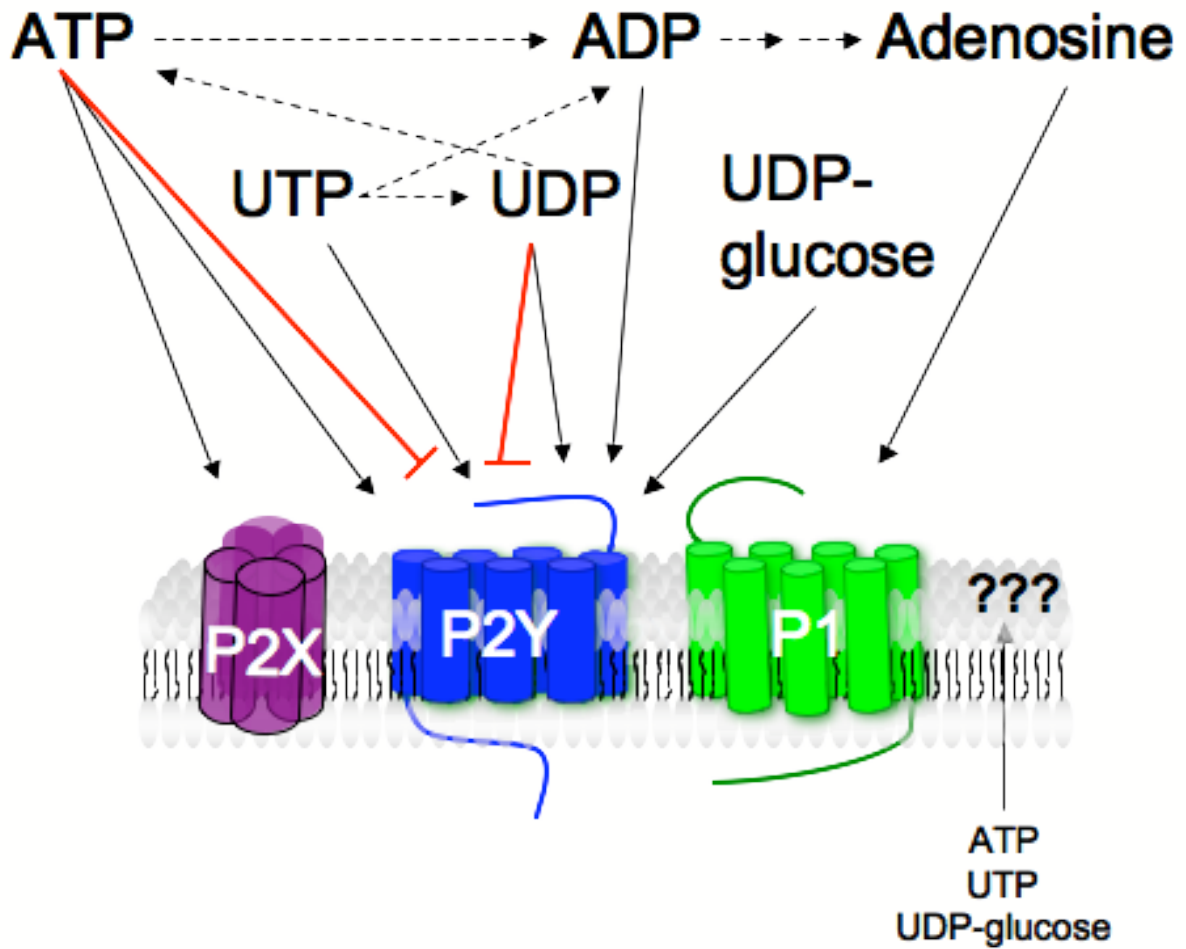
Figure 1.3. Purinergic signaling. Nucleotides are released from intracellular compartments into the extracellular milieu by as yet largely unknown mechanisms where they are capable of activating P1 adenosine receptors, P2X cation channels, or P2Y receptors. Nucleotides may also undergo nucleotide metabolism and interconversion (dashed lines) by a diverse group of ectoenzymes which alters ligand availability for the various nucleotide-activated receptors.
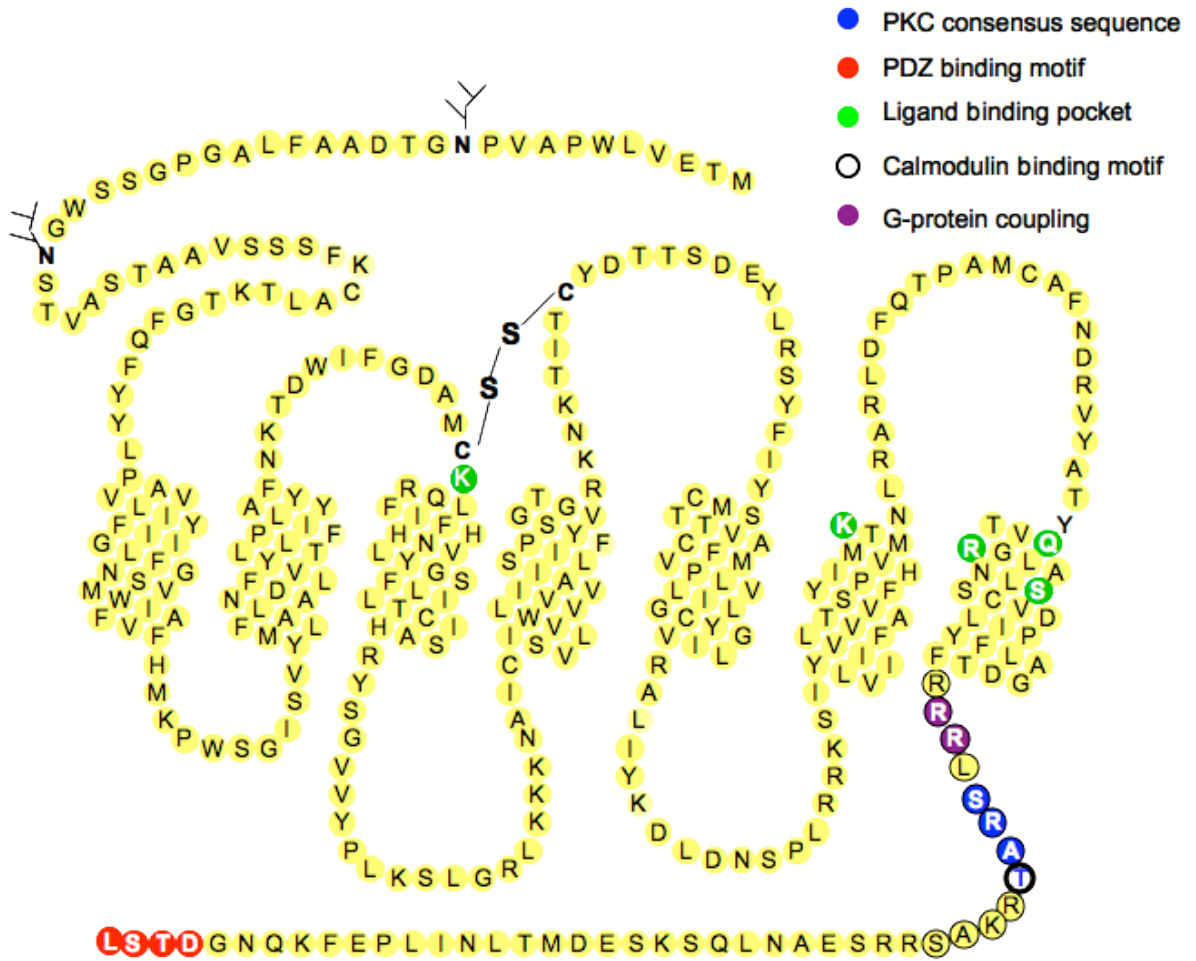
Figure 1.4. Bubble diagram of the amino acid sequence of the P2Y$_1$ receptor.
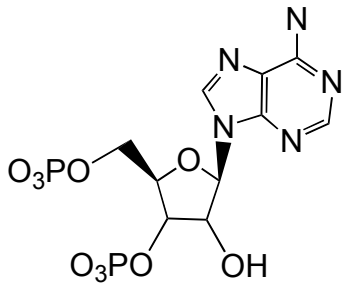
Adenosine-3',5'-bisphosphate (A3P5P)
K_B: 900 nM
Boyer *et al.*, 1996
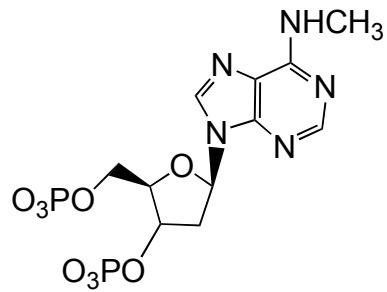
N^6-methyl-2'-deoxyadenosine-3',5'-bisphosphate (MRS2179)
K_B: 102 nM
Boyer, *et al.*, 1998
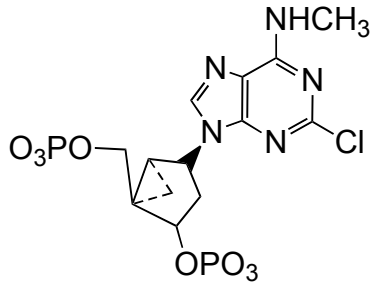
2-chloro-N^6-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate (MRS2279)
K_B: 8 nM
Boyer, *et al.*, 2002
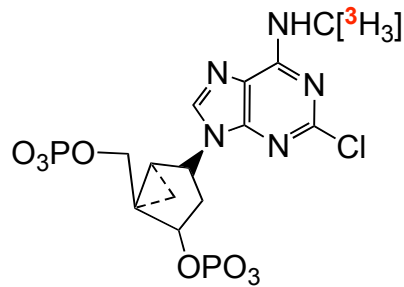
[^{3}H]2-chloro-N^6-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate ([^{3}H]MRS2279)
K_D: 7.05 nM
Waldo, *et al.*, 2002
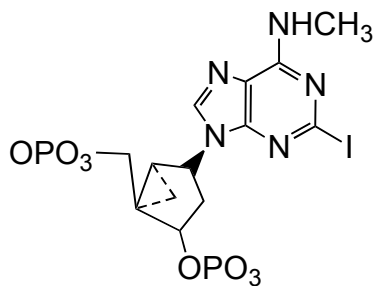
2-iodo-N^6-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate (MRS2500)
K_B: 1.6 nM
Kim, *et al.*, 2003

Figure 1.5. Selective, high-affinity antagonists of the P2Y$_1$ receptor.

CHAPTER 2

[^{32}P]2-IODO-N^6-METHYL-(N)-METHANOCARBA-2′-DEOXYADENOSINE-3′,5′-BISPHOSPHATE ([^{32}P]MRS2500), A NOVEL RADIOLIGAND FOR QUANTIFICATION OF P2Y$_1$ RECEPTORS

2.1 Introduction

Extracellular nucleotides signal through two classes of membrane-bound receptors to mediate a multiplicity of intracellular responses. The P2X receptors are ligand-gated ion channels and are primarily activated by ATP. The P2Y receptors are seven-transmembrane spanning G-protein coupled receptors and are activated by adenine and uridine nucleotides.   The P2Y receptor family consists of eight members which can be subclassified based on selectivity of G protein coupling and sequence homology. P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, and P2Y$_{11}$ receptors couple to the Gα_q class of guanine nucleotide-binding proteins, which signal downstream to trigger inositol lipid hydrolysis and subsequent mobilization of intracellular calcium. The P2Y$_{11}$ receptor also couples to the Gαs family of G proteins to stimulate adenylyl cyclase. The P2Y$_{12}$, P2Y$_{13}$, and P2Y$_{14}$ receptors exhibit high sequence homology and couple to the Gα_i family of G proteins resulting in inhibition of adenylyl cyclase activity (Burnstock, 2004; Burnstock, 1996).

The P2Y$_1$ receptor is preferentially activated by ADP, while ATP is a weak partial agonist, and UTP and UDP are inactive (Leon *et al.*, 1996; Palmer *et al.*, 1998; Schachter *et al.*, 1996).   This receptor plays an essential role in ADP-promoted platelet aggregation by triggering shape change and an initial, reversible phase of aggregation (Jantzen *et al.*, 1999). P2Y$_1$ receptor mRNA has been detected in numerous tissues (Janssens *et al.*, 1996; Leon *et*

al., 1996); however, direct study of this receptor and its related physiology historically has been difficult due to the lack of a reliable radioligand binding assay.

We have developed a series of competitive antagonists that selectively inhibit P2Y$_1$ receptor-promoted signaling. Adenosine derivatives with phosphate groups at the 5´ and 2´ or 3´ position of the ribose ring were initially identified as selective, competitive antagonists (Boyer *et al.*, 1996). Structure-activity studies for adenosine bisphosphate derivatives substituted at various positions of the adenine and ribose rings along with molecular modeling and site-directed mutagenesis have led to the development of non-nucleotide antagonists that are highly selective for the P2Y$_1$ receptor, exhibit low nanomolar potency for inhibiting downstream receptor signaling, and display limited susceptibility to metabolism by surface-localized nucleotide hydrolyzing enzymes (Jiang *et al.*, 1997; Boyer *et al.*, 1998; Camaioni *et al.*, 1998; Moro *et al.*, 1998; Nandanan *et al.*, 1999; Kim *et al.*, 2000; Nandanan *et al.*, 2000; Kim *et al.*, 2001; Boyer *et al.*, 2002; Kim *et al.*, 2002). One of these molecules, 2-chloro -N^6-methyl-(*N*)-methanocarba -2´-deoxyadenosine-3´,5´-bisphosphate ([^{3}H]MRS2279), was developed into an antagonist radioligand for the P2Y$_1$ receptor by a multi-step radiosynthetic scheme (Waldo *et al.*, 2002). While the development of a radioligand binding assay using this molecule provides a reliable tool for quantification of recombinant P2Y$_1$ receptors and screening of new P2Y$_1$ receptor ligands, its low specific activity (89 Ci mmol^{-1}) and intermediate affinity for the P2Y$_1$ receptor (K_D: 8 nM) limit its general application for broadly quantifying P2Y$_1$ receptors in native mammalian tissues.

Recently, 2-iodo -N^6-methyl-(*N*)-methanocarba-2´deoxyadenosine-3´5´-bisphosphate (MRS2500) was synthesized as a competitive P2Y$_1$ receptor antagonist that inhibited [^{3}H]MRS2279 binding to the P2Y$_1$ receptor with a Ki value (0.79 nM), ten times greater than

MRS2279 (Kim *et al.*, 2003). We have chosen this molecule as a template to develop a higher-affinity, high-specific-radioactivity antagonist radioligand for the $P2Y_1$ receptor. MRS2500 was synthesized in radioactive form by the facile, single-step kinase-catalyzed phosphorylation of a precursor molecule to yield $[^{32}P]MRS2500$ with a theoretical specific activity of 9120 Ci $mmol^{-1}$. In this study, we describe the synthesis of this novel radioligand, the development of a high-specific-activity radioligand binding assay for the $P2Y_1$ receptor, and the quantification of $P2Y_1$ receptors in various tissues of the adult rat.

2.2 Materials and Methods

Animals

Adult male Harlan Sprague-Dawley rats weighing 300-400 g were group housed and maintained on a 12:12 h light:dark cycle with access to food and water ad libitum. Animals were sacrificed by decapitation by a trained laboratory animal technician. All procedures were carried out in accordance with the guidelines of the University of North Carolina Institutional Animal Care and Use Committee.

Precursor for synthesis of MRS2500

The general synthetic approach for 2-iodo-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′-monophosphate, MRS2608, a precursor of MRS2500 was described (Kim *et al.*, 2003). The detailed synthesis will be published separately.

Enzymatic Synthesis of $[^{32}P]MRS2500$ from MRS2608

MRS2608 (50 nmol, 5 μl of a 10 mM solution in Tris pH 7.5) was combined with 1.5 μl of 10x reaction buffer (500 mM Tris HCl, 100 mM $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine, and 1 mM EDTA, pH 7.5), 1 mCi of $[\gamma^{32}P]ATP$ (7 ul, 0.16 nmol, 150 mCi ml^{-1})

and 2 μl (20 U) of 3′-phosphatase-free polynucleotide kinase. The sample was mixed by pipetting and the kinase-catalyzed reaction was incubated at 37° C for 1 h. The entire reaction volume was then injected onto a Luna 5μ C18(2) column (4.6 x 250 mm) at a flow rate of 1 ml min^{-1} in a mobile phase of 5% acetonitrile/95% 0.1 M triethylammonium acetate (5% A/95% B). The column was washed for 30 min in 5% A/95% B to remove free [γ^{32}P]ATP, and [^{32}P]MRS2500 was eluted using a linear gradient of 5%A/95%B to 60%A/40%B over 50 min. [^{32}P]MRS2500 eluted at 48 min, i.e. 18 min after the start of the gradient (approximately 75%A/25%B). The precursor molecule, MRS2608, which was detected by UV (275 nM) eluted at 50 min. One ml fractions were collected during purification, and radioactivity in each fraction was quantified by liquid scintillation counting of a 5 μl aliquot of each fraction. [^{32}P]MRS2500 has been purified by this procedure approximately 10 times with a typical yield of approximately 20%. [^{32}P]MRS2500 was stored at -20 C until use.

P2Y$_1$ receptor expression in Sf9 insect cells

Sf9 insect cell membranes expressing recombinant P2Y receptors were prepared as described in detail previously (Waldo *et al.*, 2002). Briefly, recombinant baculoviruses encoding epitope-tagged constructs of the human P2Y$_1$, P2Y$_2$, or P2Y$_{12}$ receptors, or an avian P2Y receptor (Boyer *et al.*, 1997) were constructed using established protocols. Suspension cultures of Sf9 cells were infected with recombinant baculoviruses, and plasma membranes were prepared from uninfected (wild type) or infected cells after cell lysis and high speed centrifugation. The membranes were frozen in aliquots at ─80°C.

Preparation of membranes from rat tissues

Adult male Harlan Sprague Dawley rats were sacrificed and organs were harvested and placed in 5 ml homogenization buffer (20 mM Hepes, pH 7.5, 145 mM NaCl, 5 mM

MgCl$_2$) per gram wet weight tissue. Whole organs or combined brain regions from groups of 2-6 rats were homogenized with a Polytron tissue disrupter for 45-60 sec. Homogenized samples were centrifuged at 35,000g for 10 min. The resulting pellets were resuspended in 3 ml homogenization buffer per gram wet weight tissue and centrifugation was repeated 2 times. Final resuspensions were in homogenization buffer plus 5% glycerol and the samples were stored at -80° C. Protein concentrations were determined using the BCA protein assay.

Radioligand binding assay

Membranes were typically incubated with 0.1-0.25 nM [^{32}P]MRS2500 in assay buffer (20 mM Hepes, 145 mM NaCl, 5 mM MgCl$_2$, pH 7.5) in a 25 μl reaction volume in 12x75 mm conical polypropylene tubes. Saturation binding isotherms were performed at concentrations of [^{32}P]MRS2500 ranging from 0.01 nM to 6 nM in a total volume of 20 μl. Incubations were from 15 to 45 min in an ice-water bath and were terminated by the addition of 3.5 ml of ice cold assay buffer followed by vacuum filtration over Whatman GF/A glass microfiber filters. The filters were washed with 7 ml ice cold assay buffer and radioactivity on each filter was quantified by liquid scintillation counting. Specific binding was defined as total [^{32}P]MRS2500 bound minus binding occurring in the presence of 10 or 100 μM MRS2179.

Materials

3′-phosphatase free polynucleotide kinase was from Roche Diagnostics Corp., Indianapolis, IN. MRS2179 was from Tocris-Cookson, Inc., Ellisville, MO. [γ^{32}P]ATP was from PerkinElmer, Inc., Boston, MA. All other drugs were from Sigma-Aldrich Corp., St. Louis, MO. The Luna 5μ C18(2) HPLC column was from Phenomenex, Inc., Torrence, CA.

Data analysis

All experiments were carried out in duplicate or triplicate assays and were carried out at least three times or on samples from three individual animals. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Data are presented as the mean ± S.E.M. from combined multiple experiments or in some cases as a data set from a typical experiment.

2.3 Results

Structure-activity relationships for a series of synthetic adenine nucleotide analogues have led to the development of a class of non-nucleotide adenosine bisphosphate derivatives that selectively inhibit the P2Y$_1$ receptor (Boyer *et al.*, 1998; Moro *et al.*, 1998; Nandanan *et al.*, 1999; Kim *et al.*, 2002; Kim *et al.*, 2003). The replacement of the ribose ring of adenosine 3′,5′-bisphosphate with a Northern-constrained cyclopentane structure and other modifications of the adenine base, including an N^6-methyl addition, have yielded molecules that are highly selective for P2Y$_1$ over other P2Y, P2X, and adenosine receptors. These non-nucleotide molecules are also presumed to circumvent the problem of nonspecific binding to the large number of other nucleotide-binding proteins present in mammalian cells. Recently, one of these molecules, 2-iodo-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2500), was found to interact with the P2Y$_1$ receptor with subnanomolar affinity. This molecule was selected as the template for development of a high-specific-activity, ^{32}P-labeled radioligand to quantify endogenous P2Y$_1$ receptors in mammalian tissues.

Synthesis of [^{32}P]MRS2500

MRS2500 inhibited binding of the $P2Y_1$ receptor radioligand [³H]MRS2279 with a K_i value of 0.79 nM and inhibited 2MeSADP-promoted inositol phosphate accumulation with a calculated K_B value of 1.74 nM (Kim *et al.*, 2003). A precursor to MRS2500 was generated with the goal of synthesizing a high-specific-activity radioligand. The precursor molecule, MRS2608, contains a phosphate group at the 3′-position and a hydroxyl group at the 5′-position, which potentially allows phosphorylation by polynucleotide kinase using [γ³²P]ATP as the 5′-phosphate donor.

Reaction conditions for polynucleotide kinase-catalyzed radiophosphorylation were optimized using unlabeled ATP and adenosine-3′-monophosphate (A3′MP) as the phosphate acceptor. The extent of phosphorylation was quantified using ion exchange chromatography. Since polynucleotide kinase is known to exhibit small amounts of 3′-phosphatase activity, reactions were carried out with a mutant form of the enzyme containing a C-terminal deletion that results in ablation of its 3′-phosphatase activity (Wang *et al.*, 2002). Lack of 3′-phosphatase activity was confirmed using A3′MP as substrate (data not shown). A3′MP was stable in the presence of 3′-phosphatase free polynucleotide kinase in the absence of ATP at 37ºC for up to 24 h; incubation of A3′MP with unmodified polynucleotide kinase under identical conditions resulted in the appearance of a small amount of adenosine (data not shown).

Reaction conditions that resulted in optimal phosphorylation of A3′MP were applied to ³²P-phosphorylate MRS2608 to generate [³²P]MRS2500 (Fig. 2.1). Approximately twenty percent of the added [³²P] radioactivity was routinely recovered in a single peak that eluted from the reversed-phase column with a retention time of 48 min. The retention time of the radioactive product corresponded to the retention time of purified, unlabeled MRS2500 (Kim

et al., 2003) under the same mobile phase conditions. Contamination of purified [^{32}P]MRS2500 with the precursor molecule MRS2608 was less than 1% in multiple purification procedures.

Selectivity of [^{32}P]MR2500 for the P2Y$_1$ receptor

To determine selectivity of the novel radioligand for the P2Y$_1$ receptor, [^{32}P]MRS2500 binding was evaluated in membranes from wild type Sf9 (Sf9-wt) insect cells or Sf9 insect cells expressing human P2Y$_1$ (Sf9-P2Y$_1$), P2Y$_2$ (Sf9-P2Y$_2$), or P2Y$_{12}$ (Sf9-P2Y$_{12}$) receptors or the avian P2Y receptor (Sf9-P2Y$_a$) (Boyer *et al.*, 1997). As shown in Fig. 2.2, [^{32}P]MRS2500 binding in Sf9-P2Y$_1$ membranes was 15-fold higher than binding observed in Sf9-wt membranes and was inhibited by 90% in the presence of the P2Y1-selective antagonist MRS2179 (10 μM). In contrast, [^{32}P]MRS2500 binding in Sf9-P2Y$_2$, Sf9-P2Y$_{12}$ and Sf9-P2Y$_a$ membranes was essentially identical to that observed Sf9-wt membranes and was not affected by MRS2179. These results indicate that [^{32}P]MRS2500 binds specifically to P2Y$_1$ receptors but not to other P2Y receptors in Sf9 membranes.

High affinity binding of [^{32}P]MRS2500 to the P2Y$_1$ receptor

Optimal conditions for radioligand binding were determined in preliminary experiments. Specific binding occurring at 4º C was at least as great as that observed at room temperature (data not shown), and therefore all subsequent binding analyses were carried out at 4º C. Time course experiments revealed rapid association of [^{32}P]MRS2500 such that steady state binding occurred within two min at 4º C. Moreover, prebound radioligand dissociated rapidly upon addition of a saturating concentration of MRS2179 (10 μM), with half of bound radioligand dissociating within approximately 90 sec.

Saturation binding analysis was performed to determine the affinity of $[^{32}P]$MRS2500 for the recombinant human $P2Y_1$ receptor expressed in Sf9 membranes (Fig. 2.3). Saturation binding isotherms exhibited one-site binding kinetics with a K_D of 1.1 ± 0.35 nM (n=3) and an average B_{max} of 4.8 ± 2.2 pmol receptor mg^{-1} protein in three experiments from a single membrane preparation.

Pharmacological selectivity of $[^{32}P]$MRS2500 binding

The capacity of several agonists and antagonists of the $P2Y_1$ receptor and other P2Y receptors to compete with $[^{32}P]$MRS2500 for binding was investigated in Sf9-$P2Y_1$ membranes. Because of the high specific activity of $[^{32}P]$MRS2500, competition curves could be generated with minimal amounts of protein (250-500 ng), limiting the alteration of added nucleotides by membrane-bound nucleotide-metabolizing enzymes. Agonists known to bind to the $P2Y_1$ receptor inhibited binding of $[^{32}P]$MRS2500 in a concentration-dependent manner (Fig. 2.4a). The rank order of potency observed was 2MeSADP > 2MeSATP > ADP > ATPγS > ADPβS > ATP. This order was in agreement with the predicted potencies for the $P2Y_1$ receptor based on previous observations of agonist-promoted $P2Y_1$ receptor second messenger signaling in cells continuously superfused with drug-containing medium (Palmer *et al.*, 1998). Moreover, K_i values (Table 2.1) were in excellent agreement with values determined in competition assays with $[^3H]$MRS2279 and the human $P2Y_1$ receptor purified to homogeneity (Waldo *et al.*, 2004). The $P2Y_1$ receptor is known to bind adenine nucleotides specifically and is not activated by UTP or UDP; accordingly, uridine nucleotides did not compete with $[^{32}P]$MRS2500 for binding to the $P2Y_1$ receptor.

P2Y_1 receptor antagonists were also investigated for their capacity to compete with $[^{32}P]$MRS2500 for binding to the $P2Y_1$ receptor. MRS2179, MRS2279 and MRS2500

inhibited [^{32}P]MRS2500 binding with Ki values in good agreement with K_B values determined for these same antagonists for inhibition of P2Y$_1$ receptor-promoted second messenger signaling (Fig. 4b, Table 2.1).

[^{32}P]MRS2500 binding in rat brain

One of the potential advantages of a high-specific-activity radioligand is high sensitivity for detection of receptors in native tissues. To determine the utility of [^{32}P]MRS2500 for detection of P2Y$_1$ receptors in native tissues, membranes were prepared from brains of adult male Sprague Dawley rats. As shown in Fig. 2.5a, saturation binding analysis revealed binding of [^{32}P]MRS2500 to a homogenous population of binding sites in rat brain with high affinity (K_D: 0.33 ± 0.02 nM). An average B_{max} value of 48.9 ± 8.7 fmol receptor mg^{-1} protein was determined (n=3). To confirm the identity of this high affinity binding site as the rat P2Y$_1$ receptor, pharmacological selectivity of P2Y$_1$ receptor antagonists was examined. The P2Y$_1$ selective antagonists, MRS2179, MRS2279 and MRS2500 competed for binding of [^{32}P]MRS2500 in rat brain membranes with K_i values of 1.97 ± 0.74, 27.4 ± 8.4, and 267 ± 72, respectively (n=3). These values were in agreement with values obtained at the recombinant human P2Y$_1$ receptor. Taken together, these data demonstrate that [^{32}P]MRS2500 is useful for quantification of P2Y$_1$ receptors in adult rat brains. Preliminary studies revealed a large amount of breakdown of nucleotides by brain homogenates; therefore we have not pursued agonist competition binding further in these studies of native P2Y$_1$ receptors.

Tissue distribution of the rat P2Y$_1$ receptor

Having confirmed the utility of [^{32}P]MRS2500 for labeling P2Y$_1$ receptors in rat brain, we determined relative density of P2Y$_1$ receptors in a variety of rat tissues (Fig. 2.6).

Among tissues examined with a submaximal concentration of $[^{32}P]$MRS2500 (4 nM), lung, liver, and brain exhibited the highest relative amounts of specific binding, with 55 ± 10, 31 ± 3, and 31 ± 5 fmol $[^{32}P]$MRS2500 bound mg^{-1} protein, respectively. Heart, abdominal muscle, spleen and stomach exhibited moderate receptor levels. Testes and kidney bound the least amount of radioligand, 6.5 ± 2.4 and 2.7 ± 1.7 fmol $[^{32}P]$MRS2500 bound mg^{-1} protein, respectively, and in some cases, specific binding in these tissues was undetectable.

P2Y$_1$ receptor distribution in rat brain

P2Y$_1$ receptor mRNA is abundantly expressed in brain, and this receptor has been implicated in a number of neuronal physiologies including regulation of neurotransmission, anxiolysis, and protection of astrocytes from oxidative stress-induced damage (Kittner *et al.*, 2003; Luthardt *et al.*, 2003; Shinozaki *et al.*, 2005). Saturation binding analyses were performed in five major brain regions – cerebellum, cortex, midbrain, hypothalamus, and hippocampus. Among the brain regions examined, cerebellum exhibited the highest number of binding sites with a B_{max} value of 112 ± 17 fmol $[^{32}P]$MRS2500 bound per mg protein (Table 2.2). Midbrain, hypothalamus, and hippocampus displayed intermediate densities of binding sites, and cortex displayed the lowest number of binding sites with a B_{max} value of 21.7 ± 2.4 fmol $[^{32}P]$MRS2500 bound per mg protein. Thus, P2Y$_1$ receptor expression varies by approximately six-fold among the major brain regions examined.

2.4 Discussion

Study of the P2Y$_1$ receptor has been significantly advanced by the development of selective pharmacological tools that directly target this signaling protein. In this report, we describe the synthesis and confirm the utility of $[^{32}P]$MRS2500 as a novel high-affinity, high-

specific-radioactivity antagonist radioligand for the $P2Y_1$ receptor. $[^{32}P]MRS2500$ binds selectively to the human $P2Y_1$ receptor with a K_D of 1.2 nM. We have used this high-affinity radioligand to quantify $P2Y_1$ receptors in a variety of rat tissues, and among the tissues examined, relative receptor levels were highest in lung, liver, and brain. We also examined receptor levels in several major brain regions and found a six-fold range of expression, with the highest and lowest densities of receptors found in the cerebellum and cortex, respectively. To our knowledge, this is the first unambiguous demonstration of a broadly useful high-specific-activity radioligand for a P2Y receptor natively expressed in mammalian tissues. Given the availability of the precursor, MRS2608, the preparative method is sufficiently simple to allow its convenient synthesis.

Development of selective $P2Y_1$ receptor antagonists began with the identification of adenosine bisphosphate molecules as competitive antagonists. The presence of a 5′ phosphate group and an accompanying 2′ or 3′ phosphate group on the ribose moiety allowed recognition of these molecules by the $P2Y_1$ receptor without receptor activation (Boyer *et al.*, 1996). Removal of the 2′-hydroxyl group of the ribose entity eliminated interactions of adenosine 3′,5′ bisphosphate analogues with adenosine receptors, and addition of a methyl group at the N^6 position conferred an increase in $P2Y_1$ receptor binding affinity (Boyer *et al.*, 1998). The discovery that interaction with the $P2Y_1$ receptor was retained in bisphosphate analogues in which the ribose was replaced by acylic or heterocyclic moieties (Kim *et al.*, 2000; Kim *et al.*, 2001) was extended to the use of carbocyclic ribose-substituted heterocyclic bisphosphate analogues constrained in either the Northern or Southern conformation by fusion of cyclopropane to a pseudosugar cyclopentane ring (Marquez *et al.*, 1996). These bisphosphate methanocarba analogues retained affinity for the

P2Y$_1$ receptor, and *N*-methanocarba derivatives of P2Y$_1$ receptor agonists and antagonists were more than 100-fold more potent than their corresponding *S*-isomers (Nandanan *et al.*, 2000; Kim *et al.*, 2002). Molecular modeling studies of the P2Y$_1$ receptor based on the structure of rhodopsin confirmed that the Northern conformation was energetically favored by ligands docked in the putative P2Y$_1$ receptor ligand recognition site (Nandanan *et al.*, 2000).

One goal of the development of non-nucleotide P2Y$_1$ receptor antagonists was to reduce interaction of these molecules with other nucleotide binding proteins, which hypothetically should be of value in our secondary goal of developing a useful radioligand for the P2Y$_1$ receptor. Indeed, our studies of methanocarba analogues led to the synthesis of [^{3}H]2-chloro-*N*^{6}-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate ([^{3}H]MRS2279), and the binding of [^{3}H]MRS2279 to membranes prepared from Sf9 insect cells expressing recombinant human P2Y$_1$ receptors fit the pharmacological properties of the P2Y$_1$ receptor (Waldo *et al.*, 2002). The [^{3}H]MRS2279 radioligand binding assay has allowed efficient screening of novel ligands for the P2Y$_1$ receptor (Waldo *et al.*, 2002; Kim *et al.*, 2002) and has been applied to quantify the P2Y$_1$ receptor during purification to homogeneity (Waldo & Harden, 2004). Although [^{3}H]MRS2279 proved useful for quantification of P2Y$_1$ receptors in human platelets (Waldo *et al.*, 2002), its relatively low specific activity (89 Ci mmol^{-1}) has limited its use in other tissues in which the receptor is endogenously expressed. Thus, development of [^{32}P]MRS2500, which exhibits 10-fold higher affinity and 100-fold higher specific radioactivity than [^{3}H]MRS2279, represents an important step in ligand development for unambiguous study of P2Y$_1$ receptor binding sites in mammalian tissues.

Previous work has investigated the tissue distribution of the rodent $P2Y_1$ receptor using in situ hybridization techniques (Janssens *et al.*, 1996; Leon *et al.*, 1996; Moran-Jimenez *et al.*, 2000; Tokuyama *et al.*, 1995). These studies suggest a broad expression pattern for the $P2Y_1$ receptor among peripheral tissues and in rodent brain. Although *in situ* hybridization studies provide important insight into the relative distribution of this signaling protein, the relationship of mRNA to expressed functional receptors is unknown and is not likely to be constant. Antibodies that specifically recognize P2Y receptors would allow direct immunocytochemical quantification of receptor protein but these tools also do not necessarily identify functional receptor binding sites. Moreover, although antibodies against the $P2Y_1$ receptor have been reported (Fong *et al.*, 2002; Franke *et al.*, 2005; Scheibler *et al.*, 2004; Yoshioka *et al.*, 2002), no unequivocal demonstration of their selectivity has been published, and their application to study $P2Y_1$ receptor distribution is therefore suspect.

The results described here illustrate that $[^{32}P]MRS2500$ is a useful radioligand for quantification of functional $P2Y_1$ receptor binding sites across a wide range of mammalian tissues, and the remarkably high ratio of specific to nonspecific binding of this high-affinity, high-specific-activity radioligand allows reliable detection of binding sites to at least 1 fmol mg^{-1} protein. Application of $[^{32}P]MRS2500$ revealed a broad expression pattern for the functional receptor protein among peripheral tissues and rodent brain. Interestingly, this pattern is similar to that previously reported for messenger RNA (Tokuyama *et al.*, 1995; Janssens *et al.*, 1996; Leon *et al.*, 1996; Moran-Jimenez *et al.*, 2000).

Tissue distribution data from our studies and other studies suggests potentially important physiological consequences of $P2Y_1$ receptor signaling. The role of the $P2Y_1$ receptor in ADP-promoted platelet aggregation is now well-established (Gachet, 2001).

However, its function remains largely undefined in the majority of tissues. Several studies have investigated the importance of $P2Y_1$ receptor signaling in the central nervous system. ATP released from nerve terminals acts as an excitatory neurotransmitter through ionotropic P2X receptors (Cunha *et al.*, 2000). Roles for adenine nucleotides in other neural processes have been proposed, and potentially important consequences of signaling involving the $P2Y_1$ receptor have been suggested. For example, activation of the $P2Y_1$ receptor inhibits glutamate release, and $P2Y_1$ receptor mediated inhibition of NMDA receptor-promoted signaling occurs in prefrontal and parietal cortex (Luthardt *et al.*, 2003; Rodrigues *et al.*, 2005). Activation of the $P2Y_1$ receptor also has been associated with anxiolysis, astrocyte protection, and oligodendrocyte proliferation and migration in rats (Agresti *et al.*, 2005; Kittner *et al.*, 2003; Shinozaki *et al.*, 2005).

Our work illustrates that $[^{32}P]MRS2500$ can be utilized to quantify $P2Y_1$ receptors in very small tissue samples, and the relatively high affinity and high specific radioactivity of this radioligand also make it a good candidate for detection of these receptors using autoradiographic techniques. Previous studies have claimed autoradiographic detection of the rat $P2Y_1$ receptor using $[\alpha^{33}P]dATP$ or $[^{35}S]dATP\alpha S$ as radioligands (Fong *et al.*, 2002; Simon *et al.*, 1995), but we previously have shown that the enormous amount of binding (10-50 pmol mg^{-1} protein) observed with these radioligands is nonspecific (Schachter *et al.*, 1997). A ^{33}P-labeled radioligand, $[^{33}P]MRS2179$, was used previously to quantify $P2Y_1$ receptors in human platelets (Baurand *et al.*, 2001). We suspect that $[^{33}P]MRS2179$ may not be a generally applicable radioligand since its affinity for the $P2Y_1$ receptor is 100-fold lower affinity than the affinity of MRS2500. We have demonstrated here the high selectivity of

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$[^{32}P]$MRS2500 for the P2Y$_1$ receptor, and we predict that this selectivity will allow for a more accurate analysis of brain P2Y$_1$ receptor binding sites.

The work described here demonstrates development of a new molecular tool for quantification of the P2Y$_1$ receptor with high sensitivity and illustrates that active P2Y$_1$ receptor binding sites are broadly distributed across rat tissues and brain. A reliable means for quantification of the P2Y$_1$ receptor should lead to better understanding of the complex signaling and physiology associated with this important signaling protein.

2.5 Acknowledgements

2.6 Footnotes

Agonist	n	K_i (μM)	
2MeSADP	5	0.05 ± 0.01	
2MeSATP	5	0.49 ± 0.10	
ADP	4	0.56 ± 0.09	
ATPγS	3	1.07 ± 0.11	
ADPβS	3	2.30 ± 0.60	
ATP	3	14.0 ± 6.0	
UTP	3	>1000	
UDP	3	>1000	
Antagonist	n	K_i (nM)	K_B (nM)
MRS2500	3	2.35 ± 0.48	1.74 (Kim *et al.*, 2003)
MRS2279	5	46.5 ± 7.9	8.91 (Boyer *et al.*, 2002)
MRS2179	3	117 ± 9	102 (Boyer *et al.*, 1998)

Values reported are the average of 3 or more experiments ± S.E.M.

Table 2.1. K_i values for P2Y$_1$ receptor agonists and antagonists in P2Y$_1$ receptor-expressing Sf9 membranes.

Region	K_D (nM)	B_{max}, fmol mg^{-1} protein
whole brain	0.33 ± 0.02	48.9 ± 8.7
cerebellum	0.55 ± 0.07	112 ± 17
cortex	0.47 ± 0.06	21.7 ± 2.4
hippocampus	0.44 ± 0.09	32.2 ± 7.8
hypothalamus	0.43 ± 0.09	55.8 ± 11.0
midbrain	0.38 ± 0.01	74.8 ± 9.4

Values reported are the average of 3 experiments ± S.E.M.

Table 2.2. K_D and B_{max} values for [^{32}P]MRS2500 binding in rat brain regions.
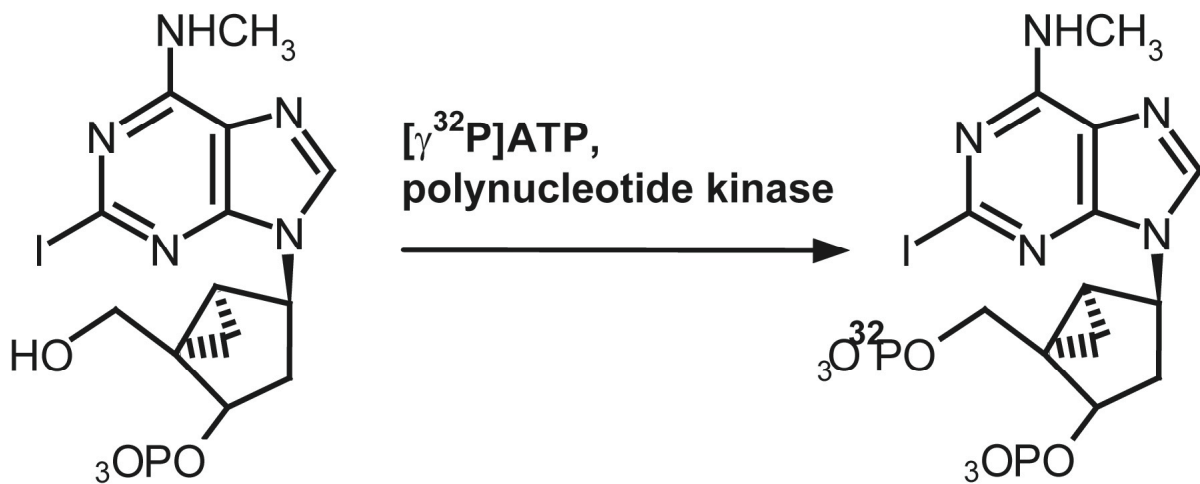
Figure 2.1. Synthesis of [³²P]MRS2500. MRS2608 (5 µl of a 10 mM solution) was combined with 1.5 µl of 10x reaction buffer, 1 mCi of [γ³²P]ATP (7 ul, 0.16 nmol, 150 mCi ml⁻¹) and 2 µl (20 U) of 3′-phosphatase-free polynucleotide kinase. The sample was mixed by pipetting and incubated at 37° C for 1 h. The entire reaction volume was then injected onto a Luna 5µ C18(2) column for purification under mobile phase conditions as described in *Methods*
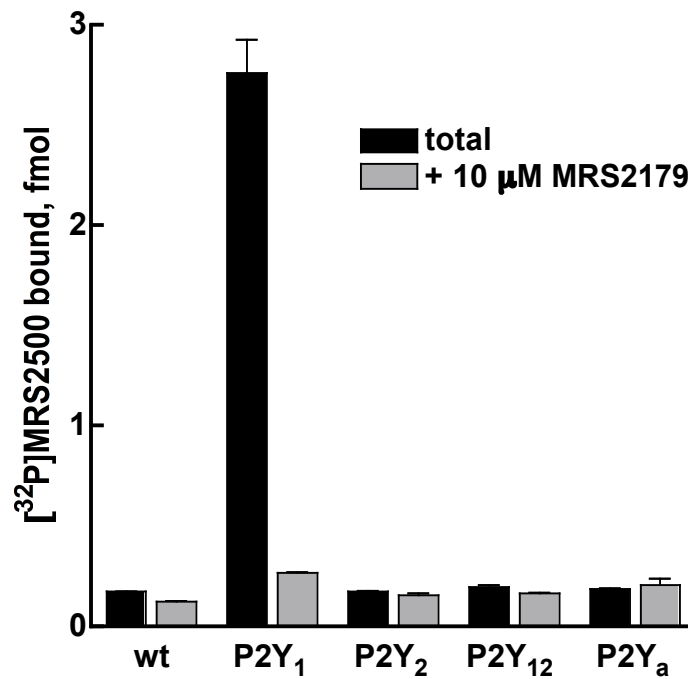
Figure 2.2. [^{32}P]MRS2500 binding in Sf9 membranes expressing P2Y receptors. Wild type Sf9 membranes or membranes expressing the human P2Y$_1$, P2Y$_2$, or P2Y$_{12}$ receptors or the avian P2Y receptor, P2Y$_a$, (10 μg each) were incubated with 220 pM [^{32}P]MRS2500 in the presence or absence of 10 μM MRS2179 to determine nonspecific binding. Values are reported as total fmol [^{32}P]MRS2500 bound ± S.E.M. from a representative experiment (n=3).
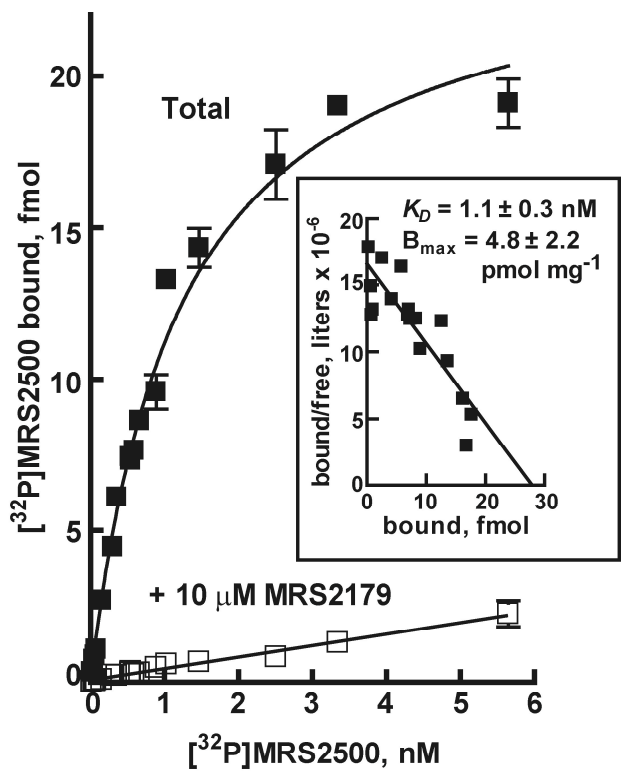
Figure 2.3. Saturation binding isotherm for [^{32}P]MRS2500 binding to the human P2Y$_1$ receptor. Sf9-P2Y$_1$ membranes (10 μg per assay) were incubated for 45 minutes with the indicated concentrations of [^{32}P]MRS2500 without or with the P2Y$_1$R selective antagonist MRS2179 (10 μM). Values are reported as total fmol [^{32}P]MRS2500 bound ± S.E.M. from a representative experiment (n=3). *Inset*, Scatchard transformation of the data.
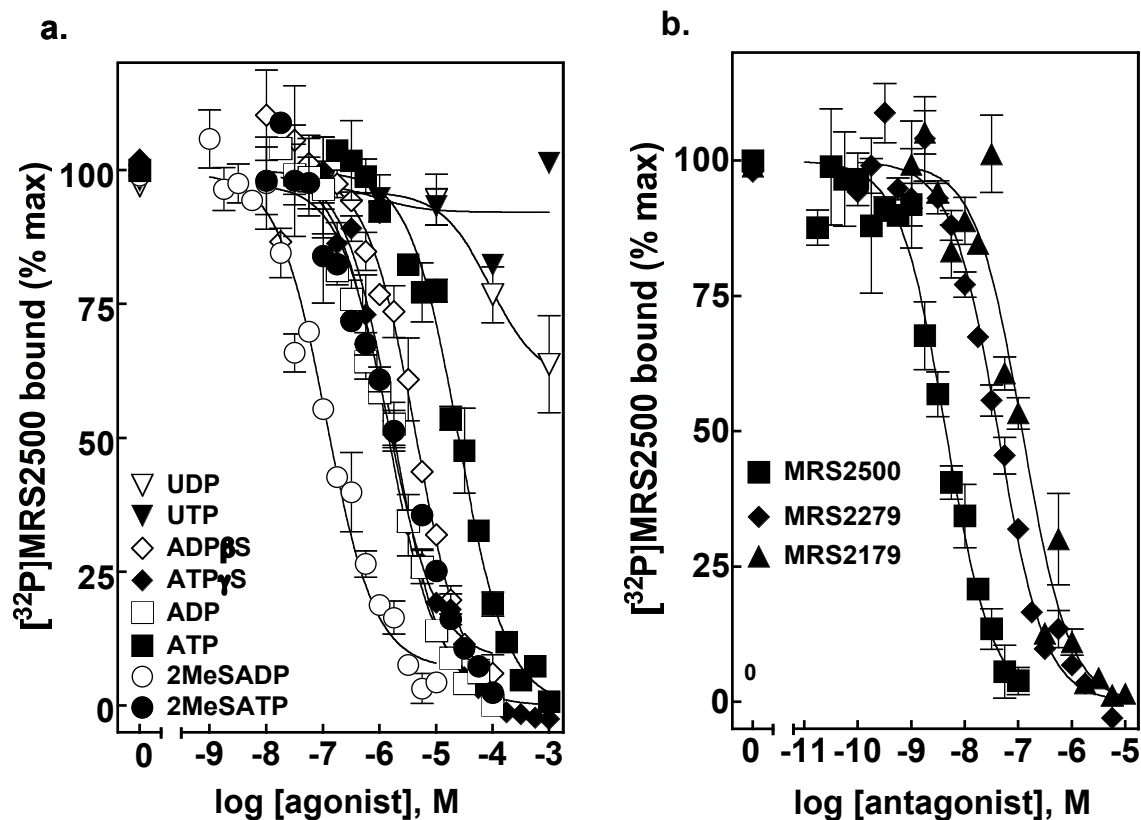
Figure 2.4. Competition of P2Y$_1$ receptor agonists and antagonists with [^{32}P]MRS2500 for binding to P2Y$_1$ receptor-expressing Sf9 membranes. *a,* Sf9-P2Y$_1$ membranes (250 ng per assay) were incubated with 100 pM [^{32}P]MRS2500 and the indicated concentrations of P2Y$_1$ receptor agonists. *b,* Sf9-P2Y$_1$ membranes (500 ng per assay) were incubated with 200 pM [^{32}P]MRS2500 and the indicated concentrations of the P2Y$_1$ receptor selective antagonists. Values are reported as % binding observed in the absence of competing ligand. Data shown are averages of triplicate samples ± S.E.M. from a representative experiment.
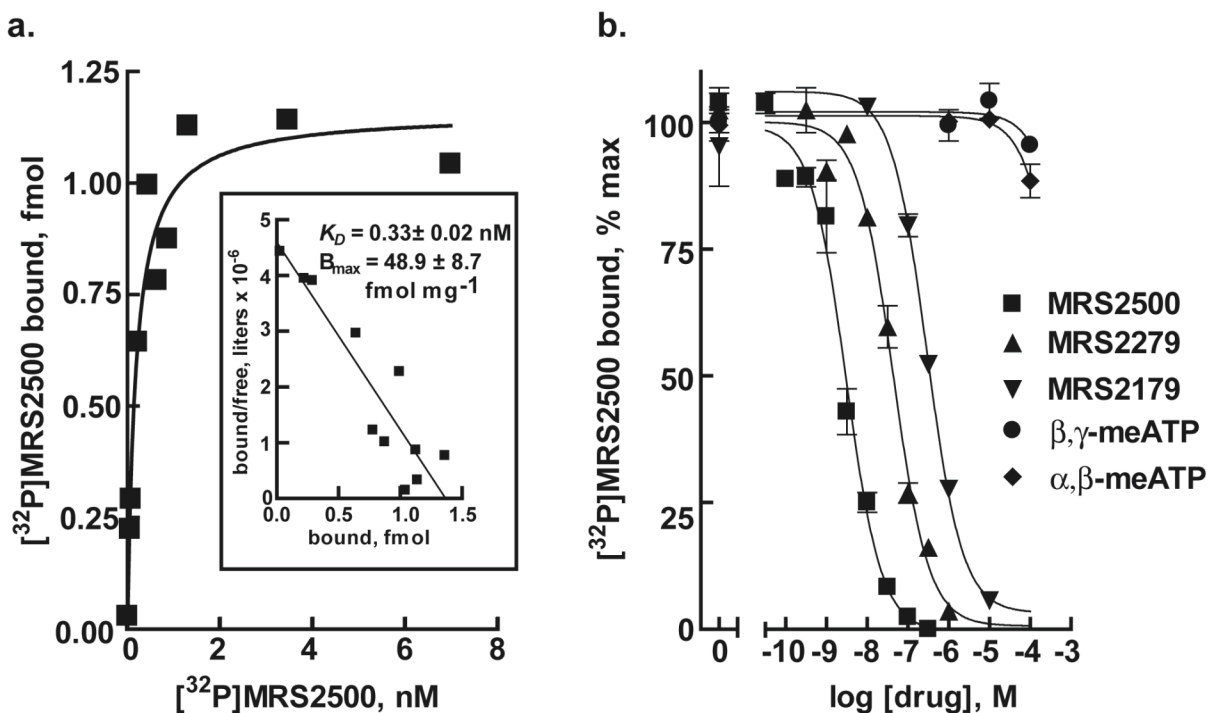
Figure 2.5. [^{32}P]MRS2500 binding in adult male rat brain. *a,* Membranes prepared from adult male rat brain (30 μg per assay) were incubated for 45 min with the indicated concentrations of [^{32}P]MRS2500 without or with the P2Y$_1$R selective antagonist MRS2179 (100 μM). *Inset,* Scatchard transformation of the data. *b,* Membranes from adult male rat brains (50 μg per assay) were incubated with 200 pM [^{32}P]MRS2500 and the indicated concentrations of the indicated P2Y$_1$ receptor antagonists. Values are reported as % binding observed in the absence of competing ligand. Data shown are averages of triplicate samples (a) or averages of triplicate samples ± S.E.M. (b) from a representative experiment (n=3).
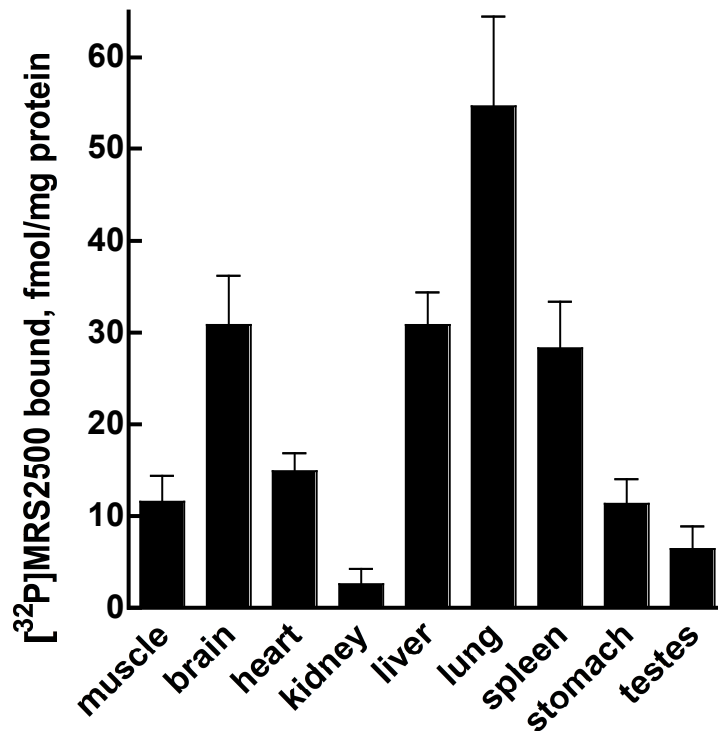
Figure 2.6. [^{32}]MRS2500 binding in adult rat tissues. Membranes prepared from various tissues of adult male Sprague Dawley rats were incubated with 4 nM [^{32}P]MRS2500 in the presence or absence of MRS2179. Specific binding was normalized to protein amounts. Values are reported as fmol [^{32}P]MRS2500 bound per mg protein. Data shown are averages of triplicate samples ± S.E.M. from a representative experiment (n=3).

CHAPTER 3

HUMAN PLATELET P2Y$_1$ RECEPTOR: QUANTIFICATION DURING DESENSITIZATION INDUCED BY A RECEPTOR SELECTIVE AGONIST

3.1 Introduction

Platelet aggregation is a complex physiological process that underlies many major physiological and pathological events including thrombosis, stroke, wound healing, hemostasis, and atherosclerosis. Consequently, understanding of the molecular mechanisms underlying platelet aggregation is of significant interest for the development of therapeutic interventions. Vascular injury results in platelet activation and, among other events, the subsequent release of platelet dense granule contents (Offermanns, 2006). Adenosine diphosphate (ADP), stored in platelet dense granules, activates the P2Y$_1$ and P2Y$_{12}$ G-protein coupled receptors, which act synergistically to promote platelet aggregation. Activation of the Gα_q-coupled (G$_q$) P2Y$_1$ receptor results in platelet shape change and the initiation of a transient wave of aggregation (Hechler *et al.*, 1998). Activation of the Gα_i-coupled (G$_i$) P2Y$_{12}$ receptor results in activation of the receptor for fibrinogen and sustained aggregation (Yang *et al.*, 2002). Activation of both of these receptors is necessary for complete platelet aggregation, and each is an important potential target for treatment of disorders involving dysregulated platelet function.

The P2Y$_1$ and P2Y$_{12}$ receptors are members of the P2Y family of nucleotide-activated G-protein coupled receptors. Currently, there are eight recognized P2Y receptors, and these are activated by adenine and uridine nucleotide di- and triphosphates and nucleotide sugars

(Burnstock, 2004). Clear description of the signaling properties of P2Y receptors has historically been difficult due to extracellular nucleotide metabolism and a lack of selective ligands. In the case of the $P2Y_1$ and $P2Y_{12}$ receptors, the distinction between individual receptors mediating G_q and G_i signaling pathways in platelets was made possible by the cloning of a G_q-coupled ADP receptor on platelets and identification of a $P2Y_1$-receptor selective antagonist, adenosine 3′, 5′ bisphosphate (Boyer *et al.*, 1996; Janssens *et al.*, 1996; Jantzen *et al.*, 1999; Leon *et al.*, 1996; Schachter *et al.*, 1996). Subsequently, the $P2Y_{12}$ receptor was cloned and identified to be the target of thienopyridine compounds, such as clopidogrel, successful at inhibiting platelet aggregation in vivo (Hollopeter *et al.*, 2001).

Molecular modeling, site-directed mutagenesis, and structure activity relationships for adenosine bisphosphate analogues have led to the development of several selective $P2Y_1$ receptor antagonists (Boyer *et al.*, 2002; Boyer *et al.*, 1998; Camaioni *et al.*, 1998; Hoffmann *et al.*, 1999; Jacobson *et al.*, 1999; Jacobson *et al.*, 2001; Jiang *et al.*, 1997; Kim *et al.*, 2001; Kim *et al.*, 2003; Kim *et al.*, 2002; Kim *et al.*, 2000; Moro *et al.*, 1998; Nandanan *et al.*, 1999; Nandanan *et al.*, 2000; Ravi *et al.*, 2002). Of these, two have been synthesized as radiolabeled ligands: [^{3}H]MRS2279, with a specific radioactivity of 89 Ci/mmol (Waldo *et al.*, 2002) and [^{32}P]MRS2500, with a specific radioactivity of 9000 Ci/mmol (Houston *et al.*, 2006; Waldo *et al.*, 2002). Recently, we demonstrated the utility of [^{32}P]MRS2500 for labeling endogenous $P2Y_1$ receptors in a variety of mammalian tissues. Its high specific radioactivity, high affinity and high selectivity for the $P2Y_1$ receptor make it a useful tool for studying $P2Y_1$ receptors in systems expressing multiple nucleotide receptors, such as platelets.

Recently, we reported the identification of a high affinity, $P2Y_1$ receptor-selective agonist, (*N*)-methanocarba-2-methylthioadenosine diphosphate (MRS2365) (Bourdon *et al.*, 2006; Chhatriwala *et al.*, 2004). Activated platelets are known to become refractory to further stimulation with ADP. Selective activation of $P2Y_1$ receptors in washed human platelets by MRS2365 revealed that loss of platelet responsiveness to ADP occurs as a result of rapid desensitization of the $P2Y_1$ receptor. Studies by Baurand *et al* have also demonstrated that a rapid loss of surface $P2Y_1$ receptor immunoreactivity also occurs after treatment with a nonselective agonist (Baurand *et al.*, 2005). While the refractoriness of platelets to ADP can be reversed by removal of extracellular ADP with apyrase, the time course for this recovery is still undefined. Additionally, whether desensitization and resensitization of the ADP response of human platelets is temporally associated with changes in surface $P2Y_1$ receptor expression is as yet undetermined.

In the current study, we explored the phenomenon of agonist-induced desensitization and sequestration of the $P2Y_1$ receptor using the combination of a $P2Y_1$ receptor-selective agonist and a high affinity, high-specific radioactivity $P2Y_1$ receptor radioligand. We illustrate that rapid desensitization occurs with the G_q-coupled $5\text{-}HT_{2A}$ receptor in platelets and that receptor desensitization is not paralleled by a significant loss of surface $P2Y_1$ receptor binding sites, suggesting that platelets have devised unique mechanisms for desensitizing components of the platelet aggregation pathway while preserving platelet responsiveness overall.

3.2 Materials and Methods

Materials

ADP, 2MeSADP, prostacyclin, fibrinogen, apyrase, bovine serum albumin and other reagents were purchased from Sigma Chemical Company, St. Louis, MO, USA. MRS2179 was from Tocris-Cookson, Inc., Ellisville, MO, USA. Heparin (1000 U ml^{-1}, Baxter Healthcare, Deerfield, IL, USA) was obtained from the University of North Carolina Hospital.

Synthesis of MRS2365 and [^{32}P]MRS2500

Synthesis and purification of the P2Y$_1$-selective ligands (*N*)-methanocarba-2MeSADP (MRS2365) (Ravi *et al.*, 2002) and [^{32}P]2-iodo-N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate ([^{32}P]MRS2500) were as described previously (Chapter 2).

Preparation and assay of washed human platelets

Suspensions of washed human platelets were prepared using a modification of the method described by Cazenave, et al (Cazenave *et al.*, 2004). Briefly, 150 ml of blood was drawn from healthy volunteers into 60 ml syringes containing one-sixth final blood volume of 65 mM citric acid, 85 mM sodium citrate, 110 mM dextrose and aliquoted into sterile 50 ml conical tubes. After a 30 minute incubation at 37ºC, tubes were centrifuged at 275 x *g* for 16 minutes. Supernatants from the first centrifugation (platelet-rich plasma) were pooled and centrifuged for 13 minutes at 2200 x *g*. The resulting pellet containing platelets was resuspended in modified Tyrode's buffer (137 mM NaCl, 2.7 mM CaCl$_2$, 1 mM MgCl$_2$, 3 mM NaH$_2$PO$_4$, 5 mM glucose, 10 mM HEPES, and 0.36 % bovine serum albumin, pH 7.35)

containing an additional 10 U ml^{-1} heparin and 5 μM prostacyclin (PGI$_2$). Following a 10 min incubation at 37ºC, prostacyclin was replenished to 5 μM. Platelets were centrifuged for 8 min at 1900 x *g* and washed as before except without heparin. After the second wash, platelets were resuspended in modified Tyrode's solution containing 0.05 U ml^{-1} apyrase to a final density of 5 x 10^8 platelets ml^{-1}. Platelets were maintained at 37ºC for 90 minutes prior to experiments.

Platelet aggregation was quantified using a two-channel Chrono-Log aggregometer (Model 560-VS; Chrono-Log Corporation, Havertown, PA, USA) in optical mode. For each measurement, 425-450 μl washed platelets were stirred at 1000 rpm at 37ºC and light transmission was recorded relative to platelet resuspension buffer for 6-8 minutes. For all measurements, 25 μl of 20 mg ml^{-1} fibrinogen was added to the cuvette prior to recording and test compounds were added at least 1 minute after commencement of recording.

Radioligand binding assay

Washed platelets were typically incubated for 20 minutes in an ice water bath with 0.1 -0.25 nM [^{32}P]MRS2500 in radioligand binding assay buffer (20 mM Hepes, 145 mM NaCl, 5 mM MgCl$_2$, pH 7.4) in a 25 μl reaction volume in 12x75 mm conical polypropylene test tubes. Saturation binding isotherms were performed at concentrations of [^{32}P]MRS2500 from 0.01 nM to 6 nM in a 25 μl reaction volume. Specific binding was defined as total [^{32}P]MRS2500 bound minus binding occurring in the presence of 100 μM MRS2179. Reactions were terminated by the addition of 3.5 ml of ice cold assay buffer followed by vacuum filtration over Whatman GF/A glass microfiber filters. The filters were washed twice with 3.5 ml ice cold assay buffer and radioactivity on each filter was quantified by liquid scintillation counting.

Measurement of surface binding sites with [^{32}P]MRS2500

For the detection of changes in surface P2Y$_1$ receptor binding sites on washed platelets, 200 µl aliquots of a washed human platelet suspension (5 x 10^8 ml^{-1}) were incubated at 37ºC for ten minutes, followed by the addition of 100 µM ADP or 10 µM MRS2365 for various times. Reactions were stopped by the addition of 1 ml of ice cold modified Tyrode's solution and immediate centrifugation for ten minutes at 3000 x *g* at 4ºC. Samples were maintained at 4ºC for all subsequent procedures. Centrifuged platelets were washed once with Tyrode's solution and again with radioligand binding buffer and resuspended after the final wash in 250 µl of radioligand binding buffer. Total and nonspecific binding of [^{32}P]MRS2500 was determined in triplicate for each sample as described above.

Data Analysis

All binding assays were conducted in triplicate and were carried out at least three times or on samples from three individual donors. Aggregometer traces are presented as representative results from a typical experiment. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Binding data are presented as the mean ± S.E.M. from combined multiple experiments or in some cases as a data set from a typical experiment.

3.3 Results

Desensitization of the P2Y$_1$ receptor in human platelets is rapid and reversible

Platelet aggregation requires the synergistic activation of the P2Y$_1$ and P2Y$_{12}$ receptors by ADP released from platelet dense granules. After stimulation with ADP,

platelets become refractory to further ADP stimulation. We recently reported the rapid desensitization of the $P2Y_1$ receptor using the selective agonist (*N*)-methanocarba-2-MeSADP (MRS2365) (Bourdon *et al.*, 2006). We have extended our studies of agonist-induced desensitization of the $P2Y_1$ receptor of human platelets with the goals of 1) determining if the remarkably rapid desensitization of the $P2Y_1$ receptor in platelets is unique to the $P2Y_1$ receptor by examining the desensitization of another platelet G_q-coupled receptor, the $5-HT_{2A}$ receptor; 2) defining the time course of resensitization of the platelet response to ADP following removal of a $P2Y_1$ selective agonist; 3) establishing a radioligand binding assay in washed human platelets using the high specific radioactivity radioligand $[^{32}P]MRS2500$; and 4) applying this radioligand binding assay to examine agonist-induced changes in surface $P2Y_1$ receptor expression.

Quantification of desensitization and resensitization of $P2Y_1$ receptors in washed human platelets was carried out by measuring light transmission in cuvettes containing stirred washed platelets to which various agonists were added. Experiments examining the rate of recovery of platelet responsiveness to ADP required removal of the $P2Y_1$ receptor selective agonist, MRS2365 with apyrase, followed by induction of the aggregation response using the non-hydrolyzable ADP analogue ADPβS. Consequently, all experiments measuring platelet aggregation were carried out using ADPβS as the agonist for activation of the $P2Y_1$ and $P2Y_{12}$ receptors. To assess the efficacy of ADPβS to promote platelet aggregation, we compared aggregation induced by ADPβS to that of the endogenous agonist, ADP, in washed human platelets. ADPβS was previously shown to induce submaximal platelet aggregation in washed human platelets and was identified as a partial agonist of the purified recombinant $P2Y_1$ and $P2Y_{12}$ receptors (Bodor *et al.*, 2003; Park *et al.*, 1999; Waldo

et al., 2004). In platelet aggregation dose-response curves generated to ADP and ADPβS, partial agonist activity of ADPβS was observed with a maximal concentration of ADPβS producing 52% of the aggregation observed in the presence of a similar dose of ADP (Fig. 3.1 a and b). ADPβS was approximately three-fold less potent than ADP for inducing aggregation.

Agonist-induced desensitization of the $P2Y_1$ receptor of human platelets was shown to occur with remarkably rapid kinetics, with full desensitization observed in less than two minutes (Bourdon *et al.*, 2006). To determine if agonist-induced desensitization is unique to the $P2Y_1$ receptor, we compared the rate of desensitization of the $P2Y_1$ receptor to the rate of desensitization of another G_q-coupled receptor expressed in platelets, the $5\text{-}HT_{2A}$ receptor. As shown in Fig. 3.2, pretreatment of platelets with the selective $P2Y_1$ receptor agonist MRS2365 for two minutes resulted in almost complete loss of responsiveness to a further challenge with the ADP analogue ADPβS. This desensitization was $P2Y_1$ receptor-specific since the platelet aggregation response to ADPβS still occurred in the presence of 5-HT, which activates the G_q signaling pathway necessary for aggregation independent of the $P2Y_1$ receptor. Desensitization of the $5\text{-}HT_{2A}$ receptor was assessed by pretreatment of platelets with 5-HT followed by induction of aggregation by the combination of 5-HT and the G_i-coupled $\alpha2_A$ receptor agonist epinephrine. While the combination of 5-HT and epinephrine induced platelet aggregation independent of the addition of adenine nucleotides, pretreatment of platelets for two minutes with 5-HT resulted in a loss of the ability of 5-HT and epinephrine to produce platelet aggregation. Partial desensitization of the $5\text{-}HT_{2A}$ receptor was observed within 15 seconds and full loss of responsiveness occurred within one minute (data not shown) similar to the kinetics observed with the $P2Y_1$ receptor. Activation of the

P2Y$_1$ receptor with MRS2365 produced robust platelet aggregation in combination with epinephrine after desensitization of the G$_q$-coupled 5-HT$_{2A}$ receptor (Fig 3.3). These data indicate that both the G$_q$-coupled P2Y$_1$ and 5-HT$_{2A}$ receptors are rapidly desensitized after activation by their cognate agonists.

To determine if the rapidly occurring desensitization of the P2Y$_1$ receptor is reversed with similarly rapid kinetics, we examined the recovery of responsiveness after removal of the agonist with apyrase. In preliminary experiments, we determined that 1 μM MRS2365 is completely hydrolyzed by 2 U ml^{-1} apyrase within 2 minutes (data not shown). ADPβS-induced aggregation was abolished after two minutes of pretreatment with MRS2365. Recovery of ADPβS-induced platelet aggregation was then measured at time points following the addition of apyrase. As illustrated in Figure 3.4, removal of agonist with apyrase resulted in recovery of approximately half of the control ADPβS-induced platelet aggregation that was maximal in 10 minutes. The t½ for recovery under these experimental conditions was approximately 5 min (Fig. 3.4). These data indicate that after rapid desensitization of the P2Y$_1$ receptor with MRS2365, removal of the selective agonist results in a slow, partial recovery of ADPβS-induced platelet aggregation. Whether this partial recovery reflects partial resensitization of the P2Y$_1$ receptor is unclear.

[^{32}P]MRS2500 selectively labels P2Y$_1$ receptors in human platelets

We recently reported the synthesis of [^{32}P]MRS2500 and described its utility for labeling P2Y$_1$ receptors in native rat tissues (Houston *et al.*, 2006). To confirm the suitability of [^{32}P]MRS2500 for measuring changes in surface P2Y$_1$ receptors in washed platelets, we first established the affinity and pharmacological selectivity for [^{32}P]MRS2500 binding to the P2Y$_1$ receptor of washed platelets through a series of saturation binding and competition

binding analyses. In saturation binding experiments, [^{32}P]MRS2500 bound to the P2Y$_1$ receptor of human platelets with a K_D of 0.33 ± 0.08 nM and a B$_{max}$ of 45 ± 2.2 binding sites per platelet (Fig 3.5).

The pharmacological selectivity of a series of selective P2Y$_1$ receptor agonists and antagonists was established in competition binding assays with [^{32}P]MRS2500 in washed platelets (Fig 3.6, Table 3.1). MRS2179, MRS2279 and MRS2500 inhibited [^{32}P]MRS2500 binding with K_i values of 47.1, 19.0 and 4.2 nM, respectively. These values are in agreement with the apparent affinities of these compounds for inhibiting second messenger signaling and platelet aggregation (Baurand *et al.*, 2001; Boyer *et al.*, 2002; Boyer *et al.*, 1998; Hechler *et al.*, 2006).

K_i values were also determined for the P2Y$_1$ agonists 2MeSADP, ADP, ADPβS and MRS2365 in human platelets. ADP and ADPβS inhibited [^{32}P]MRS2500 binding in platelets with K_i values of 1.6 and 2.8 μM, respectively, while 2MeSADP and MRS2365 exhibited 5-fold and 50-fold greater affinities than ADP, respectively (Fig. 3.6b, Table 3.1). The K_i value for ADP was in excellent agreement with that observed in competition binding assays with [^{3}H]MRS2279 at the purified human P2Y$_1$ receptor. Collectively, these data indicate that [^{32}P]MRS2500 selectively labels P2Y$_1$ receptors in human platelets.

P2Y$_1$ receptor binding sites are minimally diminished during agonist treatment in human platelets.

Agonist-induced internalization has been reported to accompany agonist-induced desensitization. Previous reports suggest that, upon agonist treatment, P2Y$_1$ receptors are removed from the surface of platelets in a clathrin- and PKC-dependent manner (Baurand *et al.*, 2005) . Using the nonselective agonist, ADP, we measured agonist-promoted changes in

the number of surface binding sites for the selective radioligand, [^{32}P]MRS2500. Within one minute of agonist treatment, a 20% decrease in the number of surface [^{32}P]MRS2500 binding sites was observed that was maintained for at least five minutes (Fig. 3.5). For technical reasons, resolution of the time course of loss of surface P2Y$_1$ receptors was not possible. Treatment with the selective agonist, MRS2365, yielded similar results. Removal of either ADP or MRS2365 with apyrase resulted in recovery of surface binding sites to near control values within fifteen minutes. Thus, the change in surface P2Y$_1$ receptor expression correlated with the reappearance of functional response (Fig. 3.5b). These results suggest that internalization is not a major mechanism of signal termination for the P2Y$_1$ receptor of human platelets.

3.4 Discussion

The results presented here demonstrate the use of novel, highly selective pharmacological tools to study agonist-promoted regulation of P2Y$_1$ receptor function and surface expression in washed human platelets. We have demonstrated that rapid desensitization of the P2Y$_1$ receptor in platelets also occurs with another G$_q$-coupled receptor in platelets, the 5-HT$_{2A}$ receptor, and that removal of a P2Y$_1$-selective agonist with apyrase results in only a partial recovery of the observed aggregation response to ADPβS. Additionally, we illustrated that cell surface binding sites for the P2Y$_1$-selective radioligand, [^{32}P]MRS2500 are minimally diminished during treatment of human platelets with a P2Y$_1$ receptor agonist. Taken together, these data provide new insight into the regulatory responses employed by platelets to control platelet function in a dynamic signaling environment.

Previously, we reported the uniquely rapid desensitization of the $P2Y_1$ receptor of human platelets after incubation with the $P2Y_1$-selective, high-affinity agonist, MRS2365 (Bourdon *et al.*, 2006). This compound does not interact with the $P2Y_{12}$ receptor and induces shape change that is selectively inhibited by antagonists of the $P2Y_1$ receptor, suggesting that the $P2X_1$ receptor is not involved. Desensitization of the $P2Y_1$ receptor was remarkably fast, occurring with a $t_{1/2}$ of 18 seconds. The loss of platelet ADP-induced platelet aggregation was due to homologous $P2Y_1$ receptor desensitization since activation of another G_q-coupled receptor, the 5-HT_{2A} receptor, restored ADP-induced platelet aggregation. These data are consistent with the conclusion that desensitization of the $P2Y_1$ receptor-promoted response occurred at the level of the receptor rather than at the level of the G-protein or other downstream signaling machinery.

Having determined that agonist-induced desensitization of the $P2Y_1$ receptor is homologous and that platelets retain the capacity to aggregate through the G_q pathway, we examined the time course of desensitization of the G_q-coupled 5-HT_{2A} receptor to determine if the rapid kinetics of desensitization were indeed unique to the $P2Y_1$ receptor. We showed that platelet aggregation promoted by the 5-HT_{2A} receptor was completely abolished after a 2 minute pretreatment with 5-HT. As with the homologous desensitization of the $P2Y_1$ receptor, this loss of responsiveness was receptor-specific since induction of G_q-signaling through the $P2Y_1$ receptor was sufficient to produce robust platelet aggregation after pretreatment of platelets with 5-HT.

Our data suggest two important notions about platelet signaling. First, desensitization of receptor-promoted G_q responses is remarkably rapid and is not unique to a single platelet G_q-coupled receptor. Studies on agonist-induced desensitization of G_q–coupled receptors

have been limited, and the rapid and complete desensitization observed after less than two minutes of agonist treatment suggests that platelets have devised a mechanism to rapidly terminate responsiveness to G_q agonists after initial exposure. Whether this response occurs with all platelet G_q-coupled receptors obviously requires further study. Secondly, desensitization of the platelet G_q-linked receptor does not appear to be heterologous and does not permanently affect pathways downstream of the G_q response. Second-messenger kinases are implicated in rapid, negative feedback of agonist-promoted signals through G-protein coupled receptors and protein kinase C has been implicated in the desensitization of both the 5-HT_{2A} and $P2Y_1$ receptors. However, if activation of PKC is involved in negative feedback, its action occurs in a receptor-specific manner. How this receptor specificity is achieved is unclear.

The time course of recovery of $P2Y_1$ receptor responsiveness was also investigated in these studies, taking advantage of the hydrolyzable, $P2Y_1$-selective agonist MRS2365 and the non-hydrolyzable, non-selective agonist of platelet aggregation, ADPβS. The aggregation response to ADPβS was only partially recovered following desensitization of the $P2Y_1$ receptor. Partial recovery of ADPβS-induced platelet aggregation after $P2Y_1$-receptor desensitization could reflect another unique mechanism devised by platelets to prevent repetitive stimulation in the presence of ADP. Results using the selective radioligand [^{32}P]MRS2500 indicate that the number of cell surface $P2Y_1$ receptors is not greatly reduced after agonist treatment, leaving the possibility that receptors retained on the cell surface exist in a prolonged desensitized state. For many GPCRs, internalization serves as a means to restore the ability of the receptor to respond to agonist, by dephosphorylation of the receptor and recycling to the cell surface, or to permanently terminate receptor signaling by receptor

degradation. The platelet is a small, anucleated cell with a singular function – to form aggregates in response to vascular injury – and a short lifespan of only 10 days. A system in which the majority of $P2Y_1$ receptors are not internalized but remain desensitized on the cell surface possibly reflects the design for the platelet to respond once and only once to vascular injury and reduces the amount of trafficking machinery necessary for this small cell to carry out its function for its short lifespan. The observed rapid loss of a small number of surface $P2Y_1$ receptors during agonist treatment and the partial recovery of the ADPβS-induced aggregation response may reflect constitutive cycling of unactivated, unphosphorylated $P2Y_1$ receptors.

Binding data presented here clearly indicate that [^{32}P]MRS2500 is a selective radioligand for the $P2Y_1$ receptor of human platelets. This work provides the first determination of agonist affinities in human platelets and was made possible by the high specific radioactivity of [^{32}P]MRS2500. Nucleotides are not significantly degraded under the conditions of our binding assays which were carried out at 4º C with small amounts of protein from the low number of platelets used for each sample. The accuracy of these values for agonist binding is underscored by the fact that K_i values determined for agonists in these studies mirror those obtained with the purified, recombinant human $P2Y_1$ receptor (Waldo *et al.*, 2004). One future direction of this work will be to determine whether GTP-sensitive agonist binding is observed in competition binding assays with [^{32}P]MRS2500 using membranes from washed platelets. GTP-sensitive agonist binding has been historically difficult to demonstrate for G_q-coupled receptors, but the high affinity of [^{32}P]MRS2500 and MRS2365 along with preliminary data from our lab suggest that a shift to a low-affinity binding state for MRS2365 can be observed in the presence of GTP in platelet membranes.

One possible advantage of the demonstration of GTP-sensitive agonist binding will be to use this methodology to examine the state of receptor-G-protein coupling in membranes from agonist-treated platelets. Such studies should reveal whether the receptor is uncoupled from G protein in the agonist-desensitized state and may help resolve the question of whether the relatively small changes in surface $[^{32}P]$MRS2500 binding sites observed during conditions of almost complete loss of $P2Y_1$ receptor signaling reflect the maintenance of desensitized receptors at the cell surface.

In these studies, we demonstrate the first use of a selective radioligand $[^{32}P]$MRS2500 to quantify surface binding sites before and after agonist treatment in human platelets. This technique has an advantage over antibody labeling since platelets were not fixed during preparation and receptors likely retained their normal binding characteristics; additionally, the use of a selective radioligand obviates ambiguous labeling of $P2Y_{12}$ receptors which are regulated distinctly from $P2Y_1$ receptors in response to agonist. While previous studies suggest a rapid and significant loss of $P2Y_1$ receptors from the plasma membrane upon agonist treatment, our results suggest that receptor internalization is indeed rapid, but does not occur to a significant extent (Baurand *et al.*, 2005). Discrepancies between these studies and previous reports most likely result from differences in technical approach and each leave room for further investigation. Interestingly, treatment with the selective agonist MRS2365 and the non-selective agonist ADP resulted in similar results for receptor internalization, suggesting that trafficking of $P2Y_1$ receptors is independent of activation and trafficking of $P2Y_{12}$ receptors.

Taken together, these data demonstrate the usefulness of selective pharmacological tools for studying $P2Y_1$ receptor signaling and trafficking in human platelets and lend to our

understanding of the regulatory mechanisms employed by platelets to adjust to a dynamic signaling environment. Based on our results, we propose a model in which ADP activates the $P2Y_1$ and $P2Y_{12}$ receptors to promote platelet aggregation leading to rapid phosphorylation and desensitization but not internalization of the $P2Y_1$ receptor, and that the majority of $P2Y_1$ receptors remain in a permanently desensitized state on the cell surface for the short lifespan of the platelet. These and future studies will yield important information about platelet $P2Y_1$ receptor signaling and potentially aid in the development of novel anti-platelet therapeutics.

3.5 Acknowledgements

3.6 Footnotes

Agonist	n	K_i (μM)
MRS2365	3	0.037 ± 0.01
2MeSADP	3	0.41 ± 0.11
ADP	4	1.8 ± 0.53
ADPβS	4	2.6 ± 0.76
Antagonist	n	K_i (nM)
MRS2500	3	4.2 ± 1.7
MRS2279	5	19 ± 6.7
MRS2179	3	47.1 ± 11

Values reported are the average of 3 or more experiments $\pm$ S.E.M.

Table 3.1. K_i values for P2Y$_1$ receptor agonists and antagonists in washed human platelets.
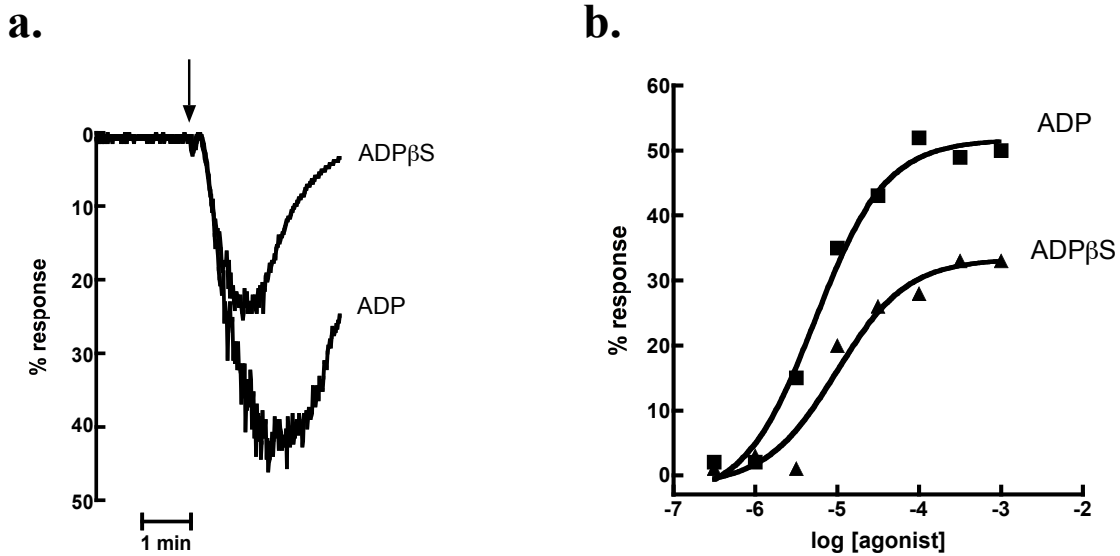
Figure 3.1. Partial agonist activity of ADPβS in platelet aggregation. *a*) Suspensions of washed human platelets were treated with 100 μM ADP or ADPβS and platelet aggregation was recorded. *b*) Maximal aggregation was measured in response to the indicated concentrations of ADP (■) or ADPβS (▲). Maximal aggregation and EC50 values were determined using nonlinear regression. Data are from a single experiment and are representative of at least three experiments.
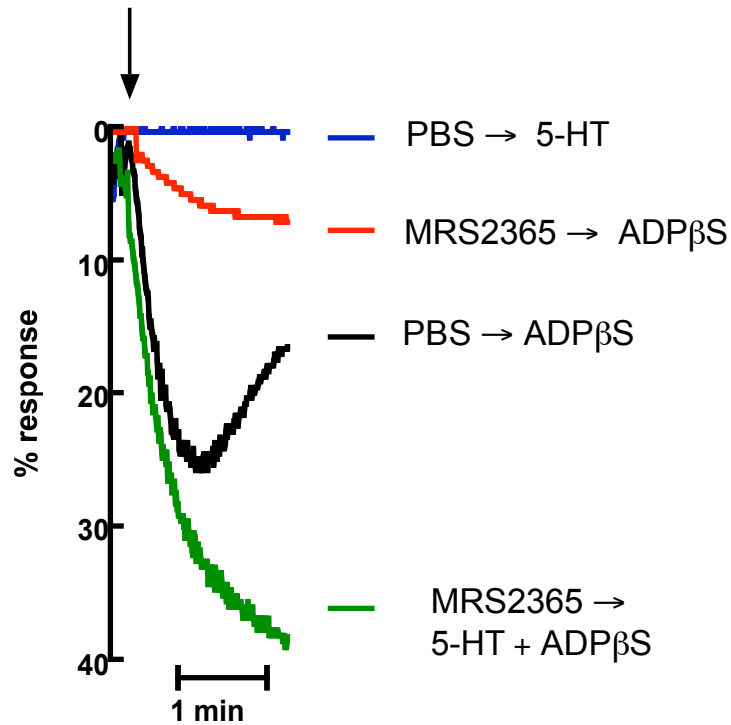
Figure 3.2. Rapid desensitization of the P2Y$_1$ receptor in washed human platelets.
Suspensions of washed human platelets were pretreated with PBS (blue and black traces) or 1 μM MRS2365 (red and green traces) for two minutes. The black arrow indicates the time of addition of reagents following the arrow in each label: 10 μM 5-HT (blue trace), 100 μM ADPβS (black and red traces) or 10 μM 5-HT + 100 μM ADPβS (green trace). Aggregation traces presented are from a single experiment representative of at least three experiments.
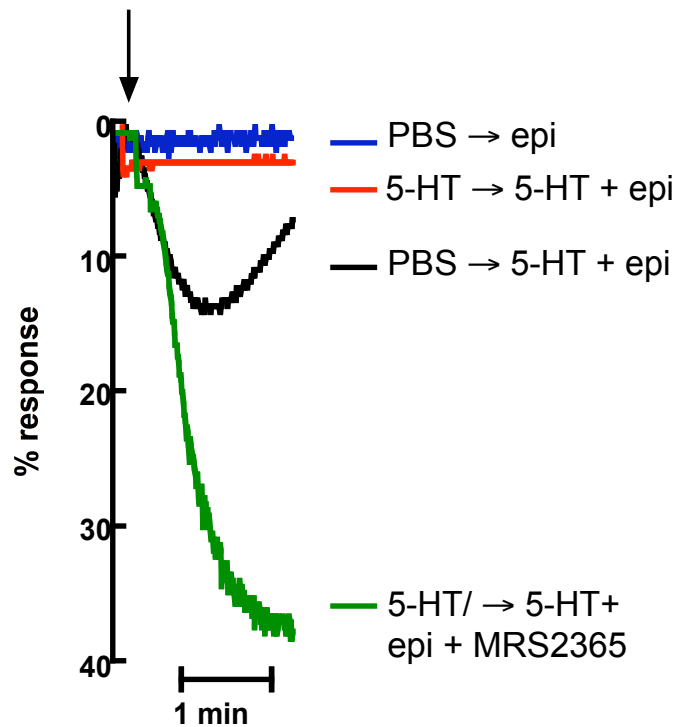
Figure 3.3. Rapid desensitization of the 5-HT$_{2A}$ receptor in washed human platelets. Suspensions of washed human platelets were pretreated with PBS (blue and black traces) or 10 μM 5-HT (red and green traces) for two minutes. The black arrow indicates the time of addition of reagents following the arrow in each label: 1 μM epinephrine (blue trace), 10 μM 5-HT + 1 μM epinephrine (black and red traces) or 10 μM 5-HT + 1 μM epinephrine + 1 μM MRS2365 (green trace). Aggregation traces presented are from a single experiment representative of at least three experiments.
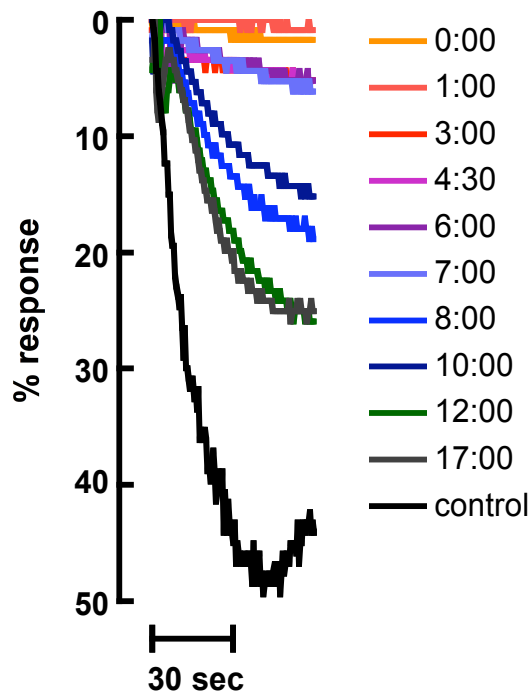
Figure 3.4. Recovery of functional response after removal of a P2Y$_1$ receptor-selective agonist in human platelets. Suspensions of washed human platelets were treated with MRS2365 for two minutes followed by the addition of 2 U/ml apyrase. 100 µM ADPβS was added at the indicated times after apyrase. *Control*, Aggregation in response to 100 µM ADPβS was measured after treatment with apyrase for the longest indicated time point. Data are from a single experiment and are representative of three separate experiments.
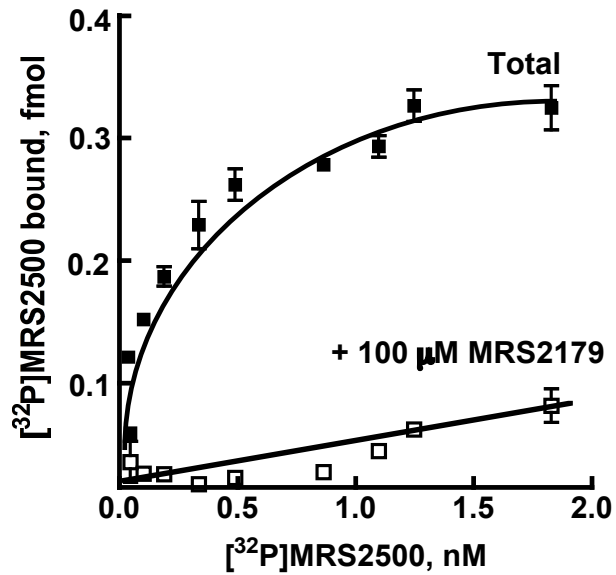
Figure 3.5. Saturation binding isotherm for [^{32}P]MRS2500 binding to P2Y$_1$ receptors in washed human platelets. Washed human platelets (5x10^6/assay) were incubated with the indicated concentrations of [^{32}P]MRS2500 without (■)or with (□) the P2Y$_1$R selective antagonist MRS2179 (100 μM). Values are reported as fmol [^{32}P]MRS2500 bound observed in the absence of competing ligand. Data shown are averages of triplicate samples ± S.E.M. from a representative experiment.
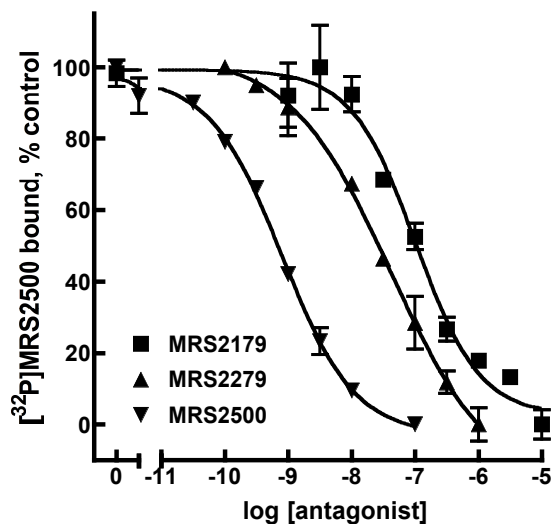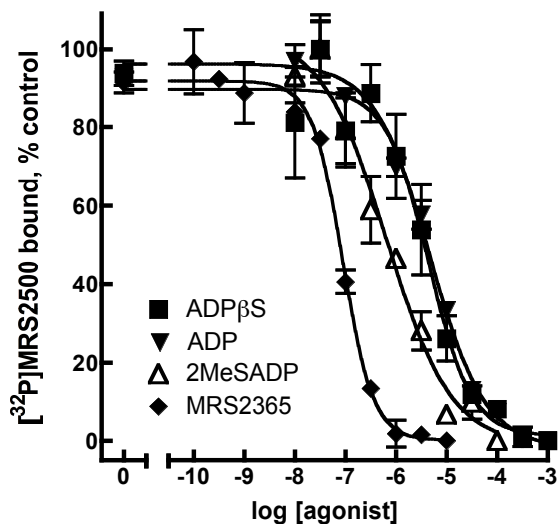
Figure 3.6. Pharmacological selectivity of [^{32}P]MRS2500 binding to the P2Y1 receptor of washed human platelets. *a*, Washed human platelets (5x10^6 washed platelets per assay) were incubated with 200 pM [^{32}P]MRS2500 and increasing concentrations of the P2Y$_1$ receptor antagonists MRS2179 (■), MRS2279 (▲) and MRS2500 (▼). *b*, Washed human platelets (5x10^6 washed platelets per assay) were incubated with 200 pM [^{32}P]MRS2500 and increasing concentrations of the P2Y$_1$ receptor agonists ADPβS (■), ADP (▼), 2MeSADP (△) and MRS2365 (◆). Values are reported as % binding observed in the absence of competing ligand. Data shown are averages of triplicate samples ± S.E.M. from a representative experiment.

a.

b.
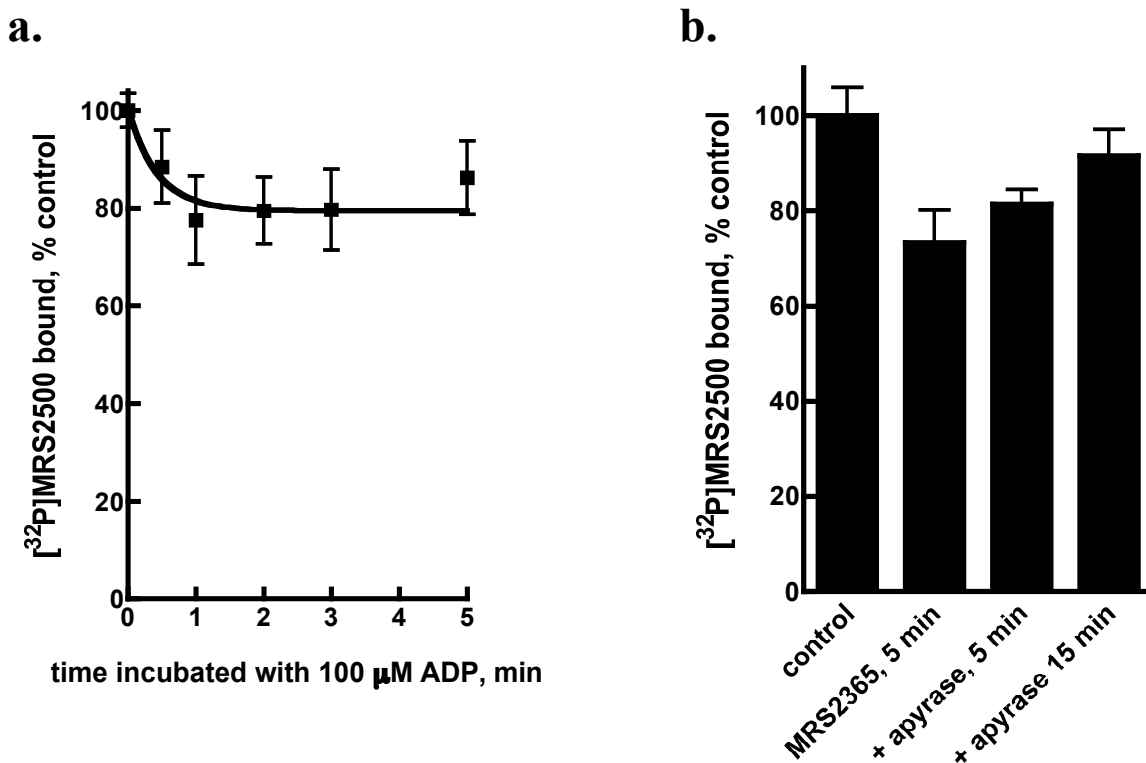
Figure 3.7. Changes in surface [^{32}P]MRS2500 binding in washed human platelets after treatment with P2Y$_1$ receptor agonists. *a*, Suspensions of washed human platelets were incubated with 100 μM ADP for the indicated times. Cells were chilled to 4°C and washed with repeated centrifugation, and surface binding sites were quantified with [^{32}P]MRS2500. Data shown are averages of triplicate samples ± S.E.M. from a representative experiment. *b*, Washed human platelets treated with 10 μM MRS2365 at 37°C for the indicated times after which agonist was removed with 0.2 U/ml apyrase and cells were placed at 37°C for an additional 5 or 15 minutes. Cell surface receptors were quantified as described in *a*. All values are normalized to the total amount of [^{32}P]MRS2500 binding in the absence of agonist treatment. Data shown are average values ± S.E.M. pooled from three experiments.

CHAPTER 4

SER-352 AND SER-354 IN THE CARBOXYL TERMINUS OF THE HUMAN P2Y$_1$ RECEPTOR ARE REQUIRED FOR AGONIST-DEPENDENT INTERNALIZATION

4.1 Introduction

A clear understanding of the mechanisms governing agonist-promoted regulation of G-protein coupled receptors is critical to the complete evaluation of the actions of current and potential therapeutics. Cells have evolved a number of ways to terminate the agonist-promoted signaling of G-protein coupled receptors, including a system of desensitization and internalization, preventing further coupling to and activation of G-proteins. The current model for GPCR desensitization and internalization, which involves phosphorylation by G-protein-coupled receptor kinases, arrestin binding, and internalization into clathrin-coated pits, is based on studies of the β2-adrenergic receptor, although many other GPCRs have been shown to desensitize and internalize by this and alternative mechanisms (Bhattacharyya *et al.*, 2002; Paing *et al.*, 2002; Pierce *et al.*, 2002; Waugh *et al.*, 1999).

The P2Y family of G-protein coupled receptors are activated by extracellular adenine and uridine nucleotide di- and triphosphates and nucleotide sugars. The eight recognized mammalian P2Y receptors can be subclassified based on sequence homology and G-protein coupling. The P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, and P2Y$_{11}$ receptors couple to the G$_q$ class of Gα subunits to activate phospholipase C beta. P2Y$_{11}$ is also capable of stimulating adenylyl cyclase activity through coupling to G$_s$. The P2Y$_{12}$, P2Y$_{13}$ and P2Y$_{14}$ receptors couple to the G$_i$ family of Gα subunits to inhibit adenylyl cyclase activity (Abbracchio *et al.*, 2006a;

Costanzi *et al.*, 2004; Qi *et al.*, 2001). Historically, investigation of the signaling and physiology of P2Y receptors has been impeded by a lack of selective, metabolically stable pharmacological and other biochemical agents. As a result, information regarding the agonist-promoted regulation of these receptors is limited.

The $P2Y_1$ receptor is activated by ADP and, in platelets, responds simultaneously with the ADP-activated $P2Y_{12}$ receptor to initiate platelet aggregation (Hechler *et al.*, 1998; Hollopeter *et al.*, 2001; Jantzen *et al.*, 1999; Jin *et al.*, 1998). Like other P2Y receptors, few studies exist that address the mechanisms of desensitization and internalization of the $P2Y_1$ receptor. We showed recently that the $P2Y_1$ receptor in platelets desensitizes rapidly in response to the selective agonist (*N*)-methanocarba-2-methylthioadenosine-diphosphate (MRS2365) (Bourdon *et al.*, 2006). Recent reports by others also have indicated that the $P2Y_1$ receptor is phosphorylated and undergoes rapid, reversible internalization in human platelets and astrocytoma cells and that this activity requires the activation of conventional and novel protein kinase C isoforms but not G-protein coupled receptor kinases (Baurand *et al.*, 2000; Hardy *et al.*, 2005; Mundell *et al.*, 2006a). However, the domains and specific residues of the receptor responsible for facilitation of these activities have not been determined.

In Chapter 2, synthesis of the selective, high-affinity, high-specific radioactivity radioligand $[^{32}P]$MRS2500 is described, and its utility is demonstrated for studying endogenous $P2Y_1$ receptor binding sites in mammalian tissues. Here, we apply this novel molecule to quantify $P2Y_1$ receptors in studies designed to determine the molecular determinants of agonist-induced internalization of this signaling protein. the molecular determinants of $P2Y_1$ receptor internalization. In Madin-Darby canine kidney (MDCK)

cells, we have taken advantage of the high specific radioactivity of $[^{32}P]$MRS2500 to quantify the agonist-promoted loss of surface binding sites of endogenous and stably expressed receptors.   We have used a series of mutant $P2Y_1$ receptors with putative phosphorylation sites mutated to alanine or lacking regions of the carboxyl terminus to identify the regions necessary for agonist-promoted internalization. These studies indicate a clear role for serine residues in the carboxyl terminus of the receptor in agonist-promoted internalization.

4.2 Materials and Methods

Construction of Mutant $P2Y_1$ Receptor cDNAs

The human $P2Y_1$ receptor was cloned into the pLXSN retroviral expression vector with an amino-terminal HA epitope tag, YPYDVPDYA, following the initiating methionine residue as described previously (Wolff *et al.*, 2005). Previous studies have demonstrated that incorporation of an amino-terminal HA-tag does not interfere with P2Y receptor function (Sromek *et al.*, 1998). Truncation mutants and a C-tail serine/threonine to alanine mutant ($P2Y_1$-340/0P) were constructed by PCR amplification with *Pfu* polymerase (Stratagene, La Jolla, CA) followed by restriction digest and ligation into pLXSN vector.   Truncation mutants were constructed using PCR amplification with 5′ primers containing an *EcoRI* restriction site and 3′ primers containing a stop codon after Thr-339 (Δ339), Asn-349 (Δ349), Asn-359 (Δ359) or Gly-369 (Δ369) and a *XhoI* restriction site to enable cloning into similarly digested pLXSN. The $P2Y_1$-340/0P mutant was constructed using long overlapping primers up to 60 bases in length overlapping by approximately 18 bases containing the indicated mutations.   The sense primer contained a *XhoI* restriction site and the antisense primer

contained a *BamHI* restriction site. The primers were annealed and filled in with the Klenow fragment of DNA polymerase followed by digestion and ligation into a P2Y$_1$-Δ339 truncation mutant containing silent mutations to incorporate the necessary restriction sites. Individual point mutants S343A, S346A, S352A, S354A, T354A, and the S336/T339A (ARAA) and S352/354A mutants were constructed using the Stratagene QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA) with HA-P2Y$_1$ in pLXSN as the template and reverse complementary primers between 30 and 45 bases in length with alanine substitutions at the indicated sites.   Constructs were confirmed by DNA sequencing at the UNC DNA Sequencing facility and purified using the Qiagen maxiprep kit (Qiagen, Valencia, CA).

Cell Culture and Expression of Receptor Constructs

MDCK(II) epithelial cells were maintained in 50/50 DMEM/F12 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO). PA317 retroviral packaging cells were maintained in DMEM supplemented with 10% fetal bovine serum. Purified cDNAs of each construct were stably expressed in MDCK(II) cells by retroviral infection of target cells using the method of Comstock *et al.,* (Comstock *et al.*, 1997). Briefly, PA317 retroviral packaging cells were plated at a density of 1x10^6 cells per well in 25 cm^2 tissue culture dishes and transfected 24 h later. Using calcium phosphate transfection, each flask was transfected with 20 μg of purified cDNA construct for the HA-P2Y$_1$ receptor or mutant receptors or mock-transfected with buffer alone. Transfected cells were incubated overnight at 37º C, and the medium was replaced with tissue culture medium containing 5 mM sodium butyrate (Sigma, St. Louis, MO). Cells were incubated for an additional 48 h at 30º C. Medium containing virus was harvested and added to MDCK(II) cells at 70% confluence along with 2 μl of 4 mg ml^{-1} polybrene (Sigma,

St. Louis, MO). The infection reaction was incubated for 2.5 h at 37º C. Viral medium was replaced with culture medium and cells were incubated at 37º C for 48 hours. Following the initial 48 hours, cells were maintained in culture medium supplemented with 1 mg ml^{-1} G418 antibiotic (Invitrogen, Grand Island, NY) with medium changes every other day. After approximately seven days and death of mock-infected control cells, antibiotic concentration was reduced to 0.2 mg ml^{-1}, and cells were cultured as normal and used for experiments.

Synthesis of [^{32}P]MRS2500

The enzymatic synthesis and purification of [^{32}P]2-iodo-N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate ([^{32}P]MRS2500) is described in Chapter 2.

Intact Cell Radioligand Binding Assay.

For assay of endogenous P2Y$_1$ receptors in MDCK(II) cells, MDCK(II) cells were plated at 5 x 10^5 cells per well in 6-well plates (Corning/Costar) and assayed 24 hours later at approximately 80-90% confluence. On the day of assay, tissue culture medium was replaced with 1 ml assay buffer (DMEM/F12 medium supplemented with 20 mM Hepes, pH 7.4) and cell culture plates were placed in a 37º C water bath for 15 minutes for temperature and pH equilibration prior to the assay. The assay was started with the addition of 250 µl of 50 µM 2MeSADP to a final assay concentration of 10 µM and cells were incubated at 37º C for various times. The reaction was stopped by placing cells on an ice-water slurry and adding 8 ml ice cold wash buffer (1% BSA in PBS, pH 7.4) to each well to rapidly cool cells to approximately 4º C. Cells were washed two additional times with 8 ml wash buffer to thoroughly remove agonist from the medium. To quantify cell surface receptors, cells were incubated for 10 minutes on an ice-water slurry with 0.1-0.5 nM of [^{32}P]MRS2500 in 500 µl

assay buffer without or with 100 μM MRS2179 (Tocris-Cookson, Ellisville, MO) to determine nonspecific binding. Cells were then washed once with 8 ml per well wash buffer and lysed in a solution of 1% SDS in PBS. Radioactivity in each lysate was quantified by scintillation counting. For assay of MDCK(II) cells expressing recombinant $P2Y_1$ receptors, a similar procedure was used except cells stably expressing various $P2Y_1$ receptor constructs were plated at a density of 1.5 x 10^5 cells per well in 24-well plates and assayed in 250 μl assay volume for incubation with agonist, 100 μl volume incubation with radioligand on ice, and 2 ml volume of wash buffer.

In vivo labeling and immunoprecipitation of HA-P2Y₁ receptors

Phosphorylation of $P2Y_1$ receptors in MDCK(II) cells was detected as previously described (Brinson *et al.*, 2001).

Data analysis

All measurements were carried out in triplicate and each experiment was carried out at least three times. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Data are presented as the mean ± S.E.M. from a data set from a typical experiment.

4.3 Results

The $P2Y_1$ receptor was reported previously to undergo agonist-dependent phosphorylation and internalization in platelets and 1321N1 human astrocytoma cells (Mundell *et al.*, 2006a). However, the domains of the receptor required for agonist-promoted trafficking are unknown. Recently, we synthesized the high-affinity, high-specific radioactivity radioligand [^{32}P]MRS2500 and demonstrated its use as a selective tool for

quantifying $P2Y_1$ receptors in a variety of mammalian tissues. In the current study, we apply this radioligand to quantify loss of surface $P2Y_1$ receptor binding sites in MDCK(II) epithelial cells. MDCK(II) epithelial cells express endogenous $P2Y_1$ receptors at a density of approximately 8 fmol mg^{-1} protein (Wolff *et al.*, 2005). Here, we use non-polarized MDCK(II) cells as a model system because the native $P2Y_1$ receptor expression in these cells suggests that they employ the proper machinery for trafficking of $P2Y_1$ receptors.

We developed an intact cell binding assay to measure agonist-promoted loss of $[^{32}P]$MRS2500 binding sites on MDCK(II) cells. Cells were treated with agonist at 37° C permitting receptor activation and internalization. After agonist incubation, cells were transferred to an ice water bathto prevent receptor trafficking. Agonist was removed by washing and remaining receptors were quantified by surface radioligand binding As shown in Fig. 4.1a, treatment of wild type MDCK(II) cells with the P2Y receptor agonist 2MeSADP resulted in a 56% decrease in the number of surface $P2Y_1$ receptor binding sites labeled by the selective radioligand $[^{32}P]$MRS2500. The loss of surface receptors reached an apparent steady state with a t½ of 7.8 min and remained diminished for up to one hour. Because a major goal of this research was to examine changes in cell surface expression of overexpressed $P2Y_1$ receptor mutants in response to agonist, we compared the internalization of a stably expressed human HA-tagged $P2Y_1$ (HA-$P2Y_1$) receptor to that of the endogenous receptor in MDCK(II) cells. The method of retroviral infection used to introduce the HA-$P2Y_1$ receptor into MDCK(II) cells has been shown to generate approximately 20-fold overexpression of recombinant receptors relative to endogenous receptor expression levels (Wolff *et al.*, 2005). As shown in Fig. 4.1b, surface levels of recombinant $P2Y_1$ receptor decreased to similar extent and with similar kinetics as the endogenous receptor in response

to agonist treatment, with a t½ of 10.4 min and a 54% maximal loss of surface $[^{32}P]$MRS2500 binding sites. HA-P2Y$_1$ receptor-expressing cells treated with agonist on ice did not show a reduction in surface $[^{32}P]$MRS2500 binding sites, indicating that the loss of receptor binding sites was not due to competition of 2MeSADP for radioligand binding. These results suggest that native and overexpressed P2Y$_1$ receptors in MDCK(II) cells undergo agonist-dependent relocalization to an intracellular compartment with similar kinetics. Recombinantly expressed P2Y$_1$ receptors in MDCK(II) cells appear to undergo similar trafficking to the endogenous P2Y$_1$ receptor and are an appropriate system for evaluation of receptor mutants.

To further explore the characteristics of P2Y$_1$ receptor internalization in MDCK(II) cells, we determined if agonist-dependent loss of $[^{32}P]$MRS2500 binding sites in HA-P2Y$_1$ receptor-expressing MDCK(II) cells is concentration-dependent. The 2-MeSADP-promoted loss of surface $[^{32}P]$MRS2500 binding sites observed after 15 minutes of agonist treatment was agonist concentration-dependent. The potency for inducing diminution of surface receptors (EC50: 46 nM) corresponded to the established potency of 2MeSADP at the P2Y$_1$ receptor, suggesting that P2Y$_1$ receptors undergo internalization as a direct consequence of agonist activation (Fig. 4.2a).

Clathrin-mediated endocytosis has been suggested to occur for many GPCRs including P2Y$_1$ receptors in human platelets (Baurand *et al.*, 2005). To determine if internalization of the P2Y$_1$ receptor in MDCK(II) cells occurs through a clathrin-dependent mechanism, we investigated the effect of sucrose, which inhibits the formation of clathrin-coated pits, on the agonist-promoted loss of $[^{32}P]$MRS2500 binding sites in HA-P2Y$_1$ receptor-expressing MDCK(II) cells. In cells pretreated with 450 mM sucrose,

[^{32}P]MRS2500 surface binding sites were unchanged over 60 minutes of agonist treatment, compared to a 56% decrease in binding sites in untreated cells (Fig. 4.2b). Total radioligand binding was similar in sucrose-treated and untreated cells. These data indicate a role for clathrin-coated pit formation in the internalization of P2Y$_1$ receptors in MDCK(II) cells.

Agonist-dependent phosphorylation and internalization of the P2Y$_1$ receptor was reported to require the activation of conventional and novel PKC isoforms in human platelets and astrocytoma cells (Mundell *et al.*, 2006a), although the domains of the receptor necessary for these processes have not been identified. To identify critical residues and regions of the P2Y$_1$ receptor required for internalization, we first determined whether the P2Y$_1$ receptor is phosphorylated in an agonist-dependent manner in MDCK(II) cells in order to implicate or exclude a role for intracellular serines and/or threonines in agonist-dependent regulation. Cells were prelabeled with [^{32}P]P$_i$ followed by incubation with agonist for 5 minutes. Agonist-treated HA-P2Y$_1$-expressing MDCK(II) cells displayed a marked increase in the amount of observed P2Y$_1$ receptor ^{32}P-phosphorylation, indicating that the P2Y$_1$ receptor is phosphorylated in response to agonist-treatment (Fig. 4.3).

Two putative phosphorylation sites are located within a PKC consensus sequence, Ser-Arg-Ala-Thr (SRAT) in the P2Y$_1$ receptor C-terminus (Fig. 4.4). S336 and T339 of this sequence were mutated to alanine (P2Y$_1$-ARAA) to determine if phosphorylation of residues in this motif is required for agonist-promoted internalization. Expression levels and agonist-promoted inositol phosphate accumulation for the P2Y$_1$-ARAA mutants and the other mutants described in Figure 4.4 were similar to that of the wild-type receptor (data not shown). Agonist-dependent loss of [^{32}P]MRS2500 binding sites was observed in P2Y$_1$-ARAA-expressing MDCK(II) cells (Fig. 4.5). Indeed, mutation of S336 and T339 to alanine

appeared to increase the rate of agonist-promoted internalization of the P2Y$_1$ receptor by approximately 3-fold (Fig. 4.5). The maximal loss of [^{32}P]MRS2500 binding sites was similar for both receptors. Thus, residues S336 and T339 do not appear to be required for agonist-promoted internalization of the P2Y$_1$ receptor.

The C-terminus of the P2Y$_1$ receptor contains seven serine and threonine residues carboxyl to T339 (Fig. 4.4) and the possibility that one or more of these residues plays a role in agonist-promoted internalization was examined. To determine if regions of the C-terminus are required for agonist-promoted internalization, truncation mutants of the P2Y$_1$ receptor were generated and expressed in MDCK(II) cells. One of these mutants, P2Y$_1$-Δ369, primarily lacks the PDZ binding motif, Asp-Thr-Ser-Leu (DTSL), located at the extreme C-terminus of the receptor. The Na$^+$/H$^+$ exchanger regulatory protein NHERF was shown to bind to this region and act as a scaffold for the receptor and its downstream effector PLCβ$_1$ (Fam *et al.*, 2005). Additionally, PDZ ligands in GPCRs have been suggested to play a role in surface retention time and targeting to subsets of clathrin-coated pits (Puthenveedu *et al.*, 2006). Truncation of the PDZ binding domain of the P2Y$_1$ receptor had no effect on its ability to undergo agonist-promoted internalization (Fig. 4.6a). Further truncation of the C-terminus however, did reveal a role for the region between residues 349 and 359, since truncation at residue 349 resulted in a marked reduction in the loss of [^{32}P]MRS2500 binding sites observed after 30 minutes of agonist treatment (Fig. 4.6a). To determine the contribution of serines and threonines of P2Y$_1$ receptor C-terminus to agonist-promoted internalization, agonist-induced loss of [^{32}P]MRS2500 binding sites was compared for the wild type receptor, the P2Y$_1$-Δ349 truncation mutant, and a mutant lacking the last seven serine and threonine residues in the receptor C-terminus (P2Y$_1$-340/0P, see Fig. 4.4). As

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shown in Fig. 4.6b, the agonist-promoted loss of surface [^{32}P]MRS2500 binding sites in the P2Y$_1$-Δ349 mutant occurred with much slower kinetics and to a lesser extent than that of the wild-type receptor. The loss of rapid, agonist-promoted internalization observed in the Δ349 truncation mutant was mimicked almost identically by the P2Y$_1$-340/0P mutant, indicating a clear role for serines and threonines in the receptor C-terminus in agonist-promoted internalization.

Two serines and one threonine residue are located between residues 349 and 359 of the P2Y$_1$ receptor C-terminus, and each of these residues was mutated individually to alanine to identify putative phosphorylation sites involved in receptor internalization. Mutation of residues S352, S354, or T358 to alanine had minimal effect on the agonist-promoted loss of [^{32}P]MRS2500 binding sites (Fig. 4.7a). Since serine clusters in the C-terminus of several GPCRs, including the P2Y$_4$ receptor (Brinson *et al.*, 2001; Oakley *et al.*, 2001), have been shown to mediate internalization, we generated a double mutant, P2Y$_1$-S352/354A, and measured the rate and extent of agonist-induced loss of surface [^{32}P]MRS2500 binding sites. Similar to P2Y$_1$-Δ349 and P2Y$_1$-340/0P mutants, the S352/354A mutant showed a significant impairment in its ability to undergo rapid agonist-induced internalization compared to the wild-type receptor (Fig. 4.7b). These results, taken together, indicate that residues Ser352 and Ser354 in the C-terminus of the P2Y$_1$ receptor are required for agonist-promoted internalization and suggest a role for receptor phosphorylation in trafficking.

4.4 Discussion

A fundamental understanding of the mechanisms governing agonist-promoted regulation of the P2Y family of G-protein coupled receptors is critical to the further

evaluation of these receptors as biological intermediates and therapeutic targets. Though historically impeded by a lack of selective, high-affinity pharmacological reagents to discriminate among receptor subtypes in a broad range of physiological systems, recent developments have expanded the pharmacological toolkit for studying P2Y receptors, and made possible further investigation of P2Y receptor signaling and physiology. In the studies presented here, we describe the use of a selective, high-affinity radioligand, [^{32}P]MRS2500, to examine changes in surface P2Y$_1$ receptor expression in response to agonist treatment. We have illustrated that the endogenous P2Y$_1$ receptor in MDCK(II) cells undergoes rapid, agonist-induced internalization and that overexpressed receptors are similarly sequestered away from the cell surface in response to agonist treatment. This internalization appears to be correlated to agonist activation and dependent on the formation of clathrin-coated pits. Furthermore, we have demonstrated agonist-dependent phosphorylation of the P2Y$_1$ receptor and identified two serine residues in the C-terminus of the P2Y$_1$ receptor, Ser352 and Ser354, that are required for rapid, agonist-promoted internalization. These studies represent the first identification of the regions and residues of the P2Y$_1$ receptor necessary for agonist-promoted internalization and suggest an important role for phosphorylation of the receptor C-terminus in agonist-dependent regulation.

The development of the selective radioligand [^{32}P]MRS2500 is the result of a series of structure-activity relationships and molecular modeling studies for the P2Y$_1$ receptor that have yielded a number of high-affinity antagonists and radioligands, including MRS2500, a potent *in vivo* antiplatelet agent, and [^{3}H]MRS2279, the first non-nucleotide antagonist radioligand for a P2Y receptor, with specific radioactivity of 89 Ci mmol^{-1} (Hechler *et al.*, 2006; Waldo *et al.*, 2002). These reagents have proved indispensable for studying the activity

of P2Y$_1$ receptors in various physiological systems and mammalian cell lines. However, the development of the high specific radioactivity radioligand [^{32}P]MRS2500 provides the requisite sensitivity to quantify P2Y$_1$ receptors in an almost limitless diversity of tissues, as evidenced by data in Chapter 2 illustrating the detection of P2Y$_1$ receptors in rat tissues of receptor density less than 10 fmol mg^{-1} of protein. As a result, we have utilized this ligand to compare agonist-promoted internalization of endogenous receptors in MDCK(II) cells to overexpressed wild type and mutant receptors. The observation of agonist-induced internalization of the endogenous receptor indicates that this cell lines expresses the proper machinery for agonist-induced trafficking of P2Y$_1$ receptors, and the similarities in the kinetic profiles of internalization for the endogenous receptor versus the recombinant receptor suggest that this system is appropriate for the evaluation of the behavior of overexpressed mutant receptors.

A role for PKC in P2Y$_1$ receptor desensitization, phosphorylation and internalization in platelets and 1321N1 human astrocytoma cells has been reported. Thr339 in the C-terminus of the P2Y$_1$ receptor is located within a PKC consensus motif and was reported to be required for desensitization (Fam *et al.*, 2003; Hardy *et al.*, 2005; Mundell *et al.*, 2006a). Our data indicate that Thr339 is not required for P2Y$_1$ receptor internalization and preliminary data using inhibitors of various PKC isoforms suggests that PKC is not required for agonist-promoted internalization of P2Y$_1$ receptor in MDCK(II) cells, although simultaneous inhibition of multiple classes of PKCs has not been attempted (Aidong Qi, personal communication). The observation of agonist-promoted phosphorylation of the P2Y$_1$ receptor and the requirement of serine residues in the receptor C-terminus for agonist-promoted internalization strongly suggest that phosphorylation is involved in agonist-

promoted P2Y$_1$ receptor trafficking. However, the direct requirement of Ser352 and Ser354 in receptor phosphorylation must be demonstrated followed by identification of the kinase involved.

The increased rate of internalization of the P2Y$_1$-ARAA mutant should be noted, and the possibility exists that this sequence plays a role in cell surface retention of the receptor. The ARAA sequence is located in the middle of a purported calmodulin binding motif (Arthur *et al.*, 2006), and a P2Y$_1$-Δ334 truncation mutant, which lacks all but the two amino-terminal residues of the calmodulin binding motif internalized similarly to the ARAA mutant. These findings indicate that alternate mechanisms of receptor internalization that do not require serines in the receptor C-terminus are possible. Binding of calmodulin or another adaptor protein in the region of the receptor containing residues 336-339 may play a role in the association of the receptor with the plasma membrane, such that disrupting this interaction has a positive effect on P2Y$_1$ receptor internalization. Also, many GPCRs are phosphorylated at sites in the third intracellular loop, an activity that is required for agonist-promoted internalization (Lee *et al.*, 2000a; Tran *et al.*, 2004). The third intracellular loop of the P2Y$_1$ receptor is relatively short and contains two serines, separated by six amino acids. Mutation of each or both of those serines to alanine had no effect on the agonist-dependent loss of [^{32}P]MRS2500 binding sites.

The data presented in these studies delineate the molecular determinants of rapid, agonist-promoted internalization of the P2Y$_1$ receptor but also raise additional questions about the mechanisms involved. We propose that agonist-promoted internalization is mediated by S352 and S354 in the C-terminus of the receptor and is likely to require direct phosphorylation at these residues, potentially by a G-protein receptor kinase or second

messenger kinase other than PKC.   Cell surface $P2Y_1$ receptor expression is positively modulated by S336 and T339 in a membrane proximal region of the C-terminus, potentially by a protein-protein interaction that requires basal phosphorylation at these residues; however, each of these possibilities requires further investigation. A direct requirement for phosphorylation at residues 352 and 354 in agonist-promoted internalization of the $P2Y_1$ receptor and identification of the kinase involved are immediate future directions of this work.

4.5 Acknowledgements

4.6 Footnotes

a.

MDCK

b.

MDCK-P2Y$_1$
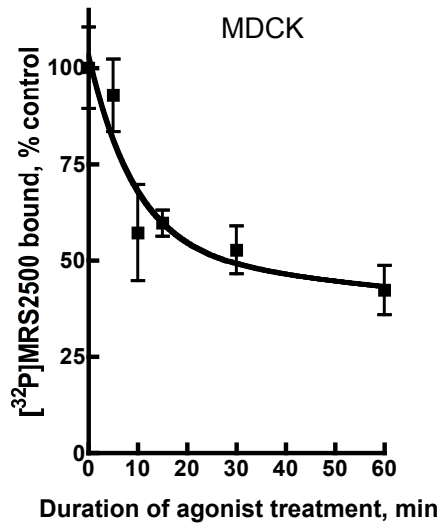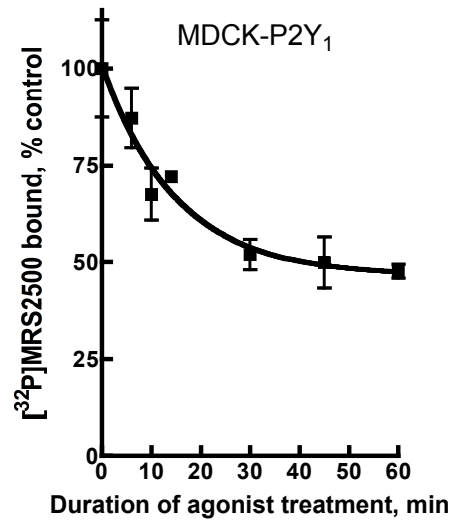
Figure 4.1. Agonist-dependent loss of surface [^{32}P]MRS2500 binding sites in wild type and HA-P2Y$_1$-expressing MDCK cells. MDCK cells expressing endogenous (*a*) or recombinant (*b*) P2Y$_1$ receptors were treated with 10 μM 2MeSADP for the indicated times. Cells were chilled to 4° C and washed thoroughly, and surface binding sites were quantified with [^{32}P]MRS2500 as described in *Methods*. All values are normalized to the total amount of [^{32}P]MRS2500 binding in the absence of agonist treatment. Data shown are averages of triplicate samples ± S.E.M. from a representative experiment (*n*=3).
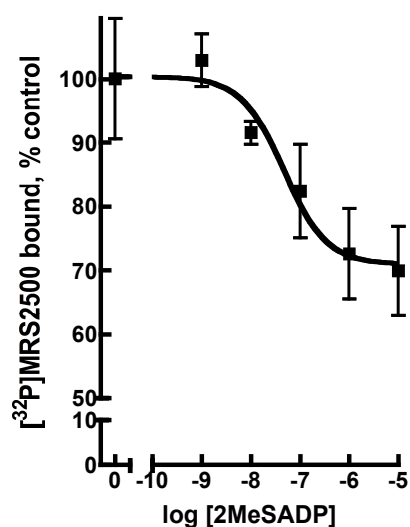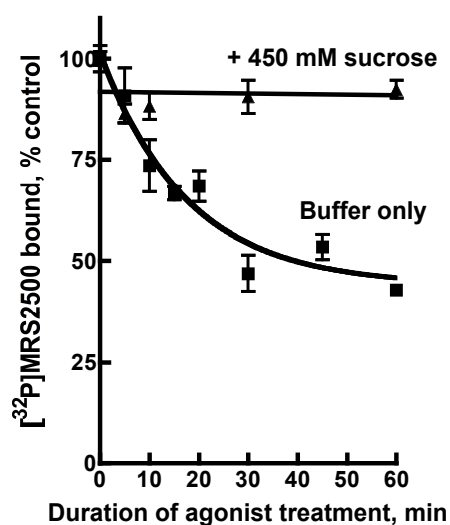
Figure 4.2. Internalization of HA-P2Y$_1$ receptors in MDCK cells. (*a*) MDCK cells stably expressing recombinant P2Y$_1$ receptors were incubated with increasing amounts of 2MeSADP for 15 minutes at 37° C and [^{32}P]MRS2500 binding sites were measured as described in *Methods*. (*b*) HA-P2Y$_1$ MDCK cells were incubated with 10 μM 2MeSADP for the indicated times in the absence (■) or presence (▲) of 450 mM sucrose. [^{32}P]MRS2500 binding sites were quantified as described in *Methods*. All values are normalized to the total amount of [^{32}P]MRS2500 binding in the absence of agonist treatment. Data shown are averages of triplicate samples ± S.E.M. from a representative experiment (*n*=3).
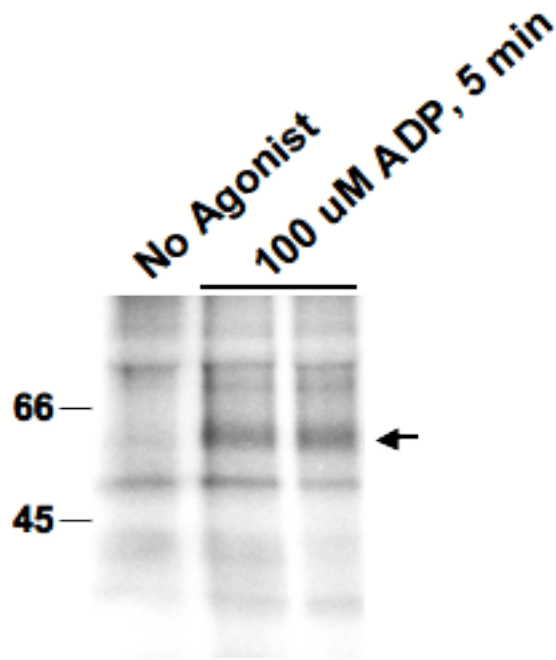
Figure 4.3. Agonist-dependent phosphorylation of the HA-P2Y$_1$ receptor in MDCK(II) cells. MDCK(II) cells stably expressing recombinant HA-tagged P2Y$_1$ receptors were prelabeled with 500 µCi [^{32}P]Pi followed by treatment with 100 µM ADP for 5 min. P2Y$_1$-receptors were immunoprecipitated, resolved by SDS-PAGE, and transferred to nitrocellulose. A PhosphorImager was used to detect radioactivity. Data shown is from a typical experiment (*n*=3).

P2Y$_1$ C-tail mutants:

		339	349	359	369

Wild type: 334RLSRATRKASRRSEANLQSKSEDMTLNILPEFKQNGDTSL-COOH

ARAA: 334RLARAARKASRRSEANLQSKSEDMTLNILPEFKQNGDTSL-COOH

340/0P: 334RLSRATRKAARRAEANLQAKAEDMALNILPEFKQNGDAAL-COOH

S352A: 334RLSRATRKASRRSEANLQAKSEDMTLNILPEFKQNGDTSL-COOH

S354A: 334RLSRATRKASRRSEANLQSKAEDMTLNILPEFKQNGDTSL-COOH

T358A: 334RLSRATRKASRRSEANLQSKSEDMALNILPEFKQNGDTSL-COOH

S352/354A: 334RLSRATRKASRRSEANLQAKAEDMTLNILPEFKQNGDTSL-COOH

Figure 4.4. P2Y$_1$ mutant constructs expressed in MDCK cells. Mutants of the P2Y$_1$ receptor were generated in pLXSN with an N-terminal HA tag and stably expressed in MDCK cells. Serines and threonines in the C-tail of the wild type receptor are highlighted in red. Serines and threonines mutated to alanine are highlighted in red for point mutants. Numbered residues indicate the last residue before insertion of a stop codon in truncation mutants.

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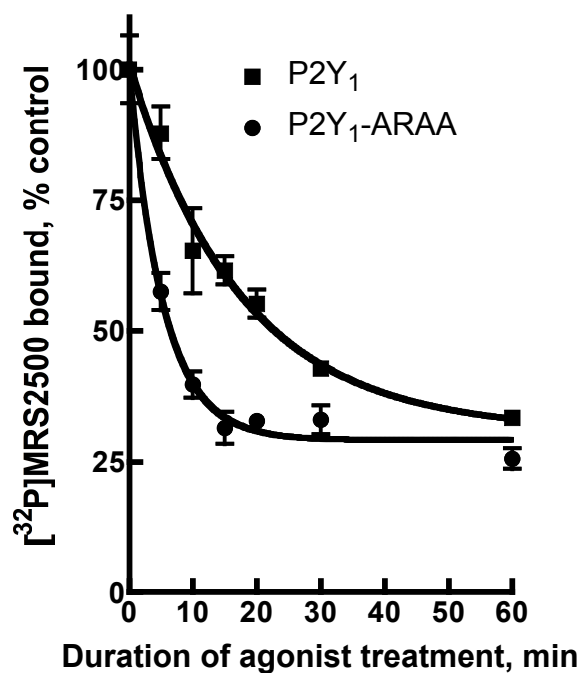
Figure 4.5. Ser-336 and Thr-339 of the P2Y$_1$ C-tail are not required for agonist-dependent internalization. MDCK cells stably expressing HA-P2Y$_1$ (■) or HA-P2Y$_1$-ARAA (●) receptors were treated with 10 µM 2MeSADP for the indicated times. Surface [^{32}P]MRS2500 binding sites were quantified as described in *Methods*. Values are reported as % binding ± S.E.M. observed in the absence of agonist treatment. Data are from a single representative experiment (*n*=3).

a.

b.
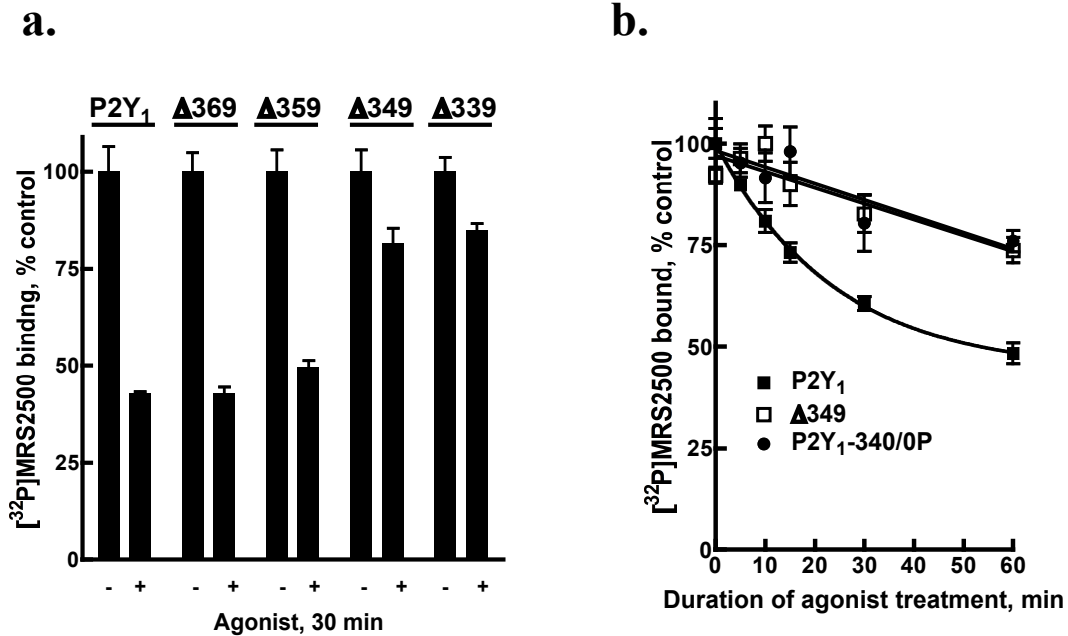
Figure 4.6. Serines and threonines in the C-terminus of the P2Y$_1$ receptor are required for agonist-dependent internalization. (*a*) MDCK cells stably expressing the indicated P2Y$_1$ receptor truncation mutants were treated with 10 μM 2MeSADP for 0 or 30 minutes and cell surface [^{32}P]MRS2500 binding sites were quantified as described in *Methods*. (*b*) MDCK cells stably expressing HA-P2Y$_1$ (■), HA-P2Y$_1$-Δ349 (□), or HA-P2Y$_1$-340/0P (●) receptors were treated with 10 μM 2MeSADP for the indicated times. Surface [^{32}P]MRS2500 binding sites were quantified as described in *Methods*. Values are reported as % binding ± S.E.M. observed in the absence of agonist treatment. Data are from a single representative experiment (*n*=3).

a.

b.
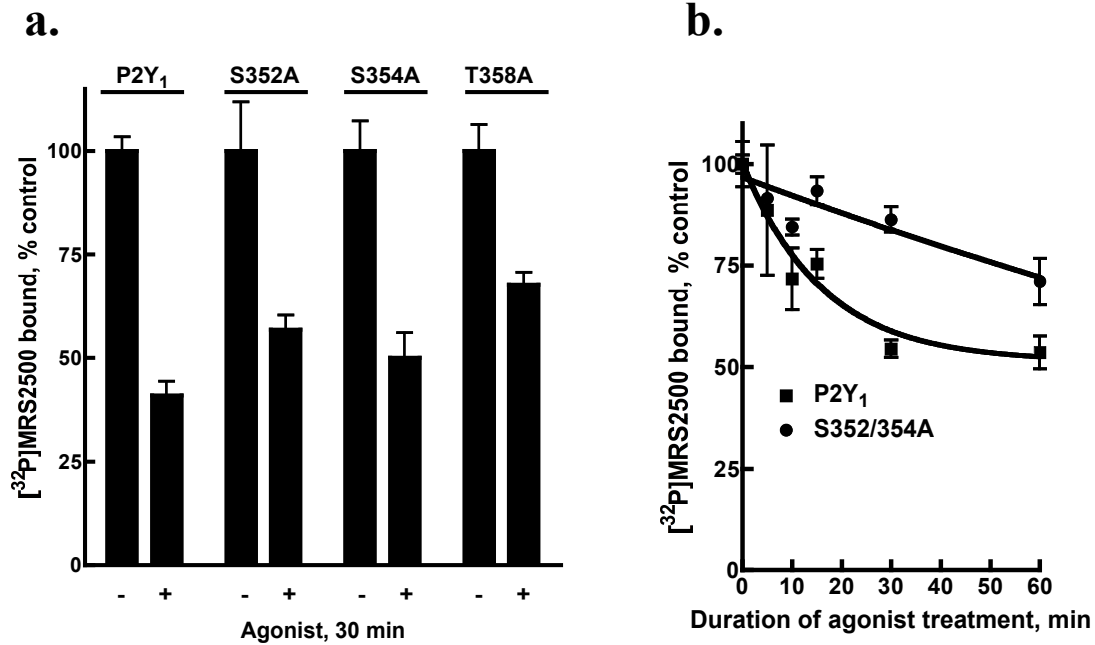
Figure 4.7. Ser-352 and Ser 354 in the C-terminus of the P2Y$_1$ receptor are required for agonist-dependent internalization. (*a*) MDCK cells stably expressing the indicated P2Y$_1$ receptor single point mutants were treated with 10 μM 2MeSADP for 0 or 30 minutes and cell surface [^{32}P]MRS2500 binding sites were quantified as described in *Methods*. (*b*) MDCK cells stably expressing HA-P2Y$_1$ (■) or HA-P2Y$_1$-S352/354A (●) receptors were treated with 10 μM 2MeSADP for the indicated times. Surface [^{32}P]MRS2500 binding sites were quantified as described in *Methods*. Values are reported as % binding ± S.E.M. observed in the absence of agonist treatment. Data are from a single representative experiment (*n*=3).

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Signaling through nucleotide-activated G-protein coupled receptors is involved in myriad physiological responses; however, without the aid of selective pharmacological tools, further study and successful manipulation of individual members of this important class of drug targets will be difficult. The work presented here describes the synthesis of a novel pharmacological tool, [^{32}P]MRS2500, which selectively labels P2Y$_1$ receptors in a variety of tissues with very high sensitivity. We have demonstrated that this radioligand can be synthesized and purified in a single-step, enzymatic reaction using a 3′-monophosphate precursor, MRS2608, commercially available [γ^{32}P]ATP, and polynucleotide kinase, and can be purified using reversed-phase HPLC for routine production with high yield. [^{32}P]MRS2500 bound selectively to the human P2Y$_1$ receptor overexpressed in Sf9 insect cell membranes with a K_D of 1.1 nM and the predicted pharmacological selectivity in competition binding assays with a series of P2Y$_1$ receptor agonists and antagonists. Additionally, [^{32}P]MRS2500 was used to determine the tissue distribution of functional P2Y$_1$ receptor binding sites in a panel of rat tissues and in regions of the adult rat brain, identifying regions of highest relative expression as lung, liver, and cerebellum.

A primary goal of the synthesis of [^{32}P]MRS2500 was its use as a tool to study the cellular redistribution of P2Y$_1$ receptors in response to agonist treatment as a part of a study to further define the mechanisms of agonist-promoted regulation of P2Y$_1$ receptors. We developed a binding assay with [^{32}P]MRS2500 for the P2Y$_1$ receptor in intact, washed

human platelets and used this assay to show that agonist treatment of platelets results in a loss of less than 20% of surface $P2Y_1$ receptors. Interestingly, $P2Y_1$ receptors are desensitized rapidly in human platelets and this rapid desensitization also occurs for another G_q-coupled GPCR, the $5\text{-}HT_{2A}$ receptor, pointing to unique regulation of G_q-signaling by human platelets. In contrast, in intact MDCK(II) cells, we showed rapid agonist-induced internalization of the $P2Y_1$ receptor, with a t ½ of approximately 10 minutes and a 50% decrease in surface $[^{32}P]MRS2500$ binding sites at steady state. This internalization required two serine residues, Ser352 and Ser354, in the carboxyl terminus of the $P2Y_1$ receptor.

5.1 Ligand Development and Applications

Further applications of $[^{32}P]MRS2500$ include receptor autoradiography to determine the cellular localization of the $P2Y_1$ receptor in brain. Expression of the $P2Y_1$ receptor on neurons and astrocytes has been demonstrated by mRNA expression and functional studies, and the $P2Y_1$ receptor is linked to a number of neurophysiological events including glutamate release, feeding behavior, and anxiety (Jourdain *et al.*, 2007; Kittner *et al.*, 2003; Kittner *et al.*, 2006). Autoradiographic analysis will define the cellular distribution of $P2Y_1$ receptors in brain and begin to answer questions about neurotransmitters and neurotransmitter receptors with which the $P2Y_1$ receptor is colocalized, possibly leading to conclusions about the mechanisms by which it exerts neurophysiological effects. $[^{32}P]MRS2500$ has the advantages over previous radioligands of high selectivity, solving problems of nonspecific labeling of other nucleotide receptors, and high affinity and high specific radioactivity, which will permit ease of use and high sensitivity for autoradiographic studies.

Since ADP-mediated platelet aggregation through the $P2Y_1$ and $P2Y_{12}$ receptors is a well-established physiological phenomenon, ideally, a $P2Y_1$ antagonist will broaden therapeutic choices for antiplatelet clinical use and will be advantageous in situations in which $P2Y_{12}$ blockade is insufficient or undesirable. While we have successfully developed $P2Y_1$ receptor antagonists for use in a variety of model systems, the effective blockade of $P2Y_1$ receptors in human subjects to inhibit platelet aggregation has not yet reached clinical practice. Due to concerns regarding increased bleeding observed with some $P2Y_{12}$ antagonists and in $P2Y_1$ knockout mice, and the broad tissue distribution of the $P2Y_1$ receptor, safety must be established and care taken to identify and address potential side effects. Additionally, a variety of cardiovascular conditions require antiplatelet therapy, including long-term treatment for coronary artery disease during and after percutaneous coronary intervention, and temporary platelet inhibition during surgery. Therefore, the suitability and efficacy of a reversible, competitive $P2Y_1$ antagonist for each of these outcomes must be established and compared to existing treatments and combination therapy.

The methodology applied to developing high affinity, selective compounds for the $P2Y_1$ receptor can be expanded for other P2Y receptors in order to obtain useful drugs. Structure-activity studies have been promising but as yet have not yielded high-affinity ligands for the majority of P2Y receptors. The $P2Y_{14}$ receptor is the only P2Y family member activated by uridine nucleotide sugars including UDP-glucose. As such, ligand development has relied on UDP-sugar derivatives and has yielded a high-affinity agonist, 2-thio-UDP-glucose (Ko *et al.*, 2007). The $P2Y_{12}$ receptor is also unique in its blockade by thienopyridine compounds and several available antagonists are currently in use or under clinical investigation. However, ligand development for the $P2Y_1$ subfamily, e.g. the $P2Y_2$,

$P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors and for the $P2Y_{13}$ receptor have been less successful, yielding few selective ligands of typically moderate potency and no successful radioligands to date. Molecular modeling studies for all of the P2Y receptors have identified commonalities in the putative ligand binding pockets for $P2Y_1$ subfamily members and $P2Y_{12}$ subfamily members with residues critical for coordinating features of the nucleotide ligands (Costanzi *et al.*, 2004). These molecular modeling studies are encouraging and form a platform to increase the affinity and potency of existing ligands and potentially circumvent the disadvantage of nucleotide metabolism, steps that will be essential to creating biologically useful agonists, antagonists, and radioligands.

5.2 Agonist-Dependent Regulation

Agonist-promoted regulation of G-protein coupled receptors is a complex process, and while the existing model is useful for the β2 adrenergic receptor and other G_s-coupled receptors, considerably more study is necessary to elucidate the mechanisms at work for phosphorylation, desensitization, and internalization of the $P2Y_1$ receptor. The $P2Y_1$ receptor mutants introduced in these studies will help determine whether direct phosphorylation is necessary for desensitization and/or internalization of the receptor and will demonstrate whether the same or distinct regions or residues of the receptor modulate each of these processes.

The findings presented here suggest interesting differences in agonist-promoted regulation of the $P2Y_1$ receptor in platelets and MDCK(II) epithelial cells, and phosphorylation may play an important role in each cell type. A model is illustrated in Figure 5.1 that describes potential pathways for $P2Y_1$ receptor agonist-dependent regulation

in each of these cell types. In platelets, our data suggests that desensitization of the $P2Y_1$ receptor occurs rapidly as a result of agonist activation and that the receptor may remain desensitized at the cell surface, unable to respond to further stimulation by agonist. Previous data implicating PKC in phosphorylation and desensitization of the $P2Y_1$ receptor in platelets suggests that PKC-mediated receptor phosphorylation is a rapid negative feedback mechanism designed to rapidly terminate $P2Y_1$ receptor signaling in platelets. While some recovery of $P2Y_1$ receptor activity was observed, this recovery may result from the insertion of naive receptors into the plasma membrane due to some constitutive receptor cycling.

If phosphorylation by PKC causes prolonged desensitization of surface-retained $P2Y_1$ receptors in platelets, our radioligand binding studies suggesting a lack of internalization coupled with a measurement of GTP-sensitive agonist competition binding will possibly reveal whether receptors remaining on the cell surface exist in a permanently desensitized state. One future direction of this work will be to assess whether GTP-sensitive binding for $P2Y_1$ receptor agonists in competition with $[^{32}P]MRS2500$ can be observed in platelet membranes. If so, a loss of GTP-sensitive agonist competition binding in membranes from platelets treated with the $P2Y_1$-receptor selective agonist MRS2365 will suggest that surface-localized receptors from membranes prepared from agonist-treated platelets exist in a permanently desensitized stated. Another future direction of this work will be to confirm that the loss of full recovery of $P2Y_1$ receptor responsiveness following desensitization and agonist removal is not the result of long term changes in downstream signaling pathways. To do this, we will assess whether the use of 5-HT, an agonist of another Gq-coupled receptor in platelets can promote full platelet aggregation when combined with ADP at longer time points after desensitization of the $P2Y_1$ receptor.

In contrast to platelets, P2Y$_1$ receptors in MDCK(II) cells internalize in an agonist-dependent manner and we have shown that this activity requires two serine residues in the C-terminus of the receptor. Additionally, experiments from our lab indicate that PKC is not required for agonist-induced internalization of the P2Y$_1$ receptor in MDCK(II) cells. This information, coupled with existing dogma regarding agonist-promoted trafficking of GPCRs, suggests that in epithelial cells, the P2Y$_1$ receptor may undergo a process similar to the canonical process of phosphorylation by a G-protein receptor kinase, association with adaptor proteins, and internalization into clathrin-coated pits from which it is recycled (Fig. 5.1). Many GPCRs bearing serine clusters in the third intracellular loop or C-terminus require arrestin association and GRKs for internalization, and a role for acidic amino acids flanking these serine clusters has been shown (Lee *et al.*, 2000a; Oakley *et al.*, 2001). Ser352 and S354 in the C-terminus of the P2Y$_1$ receptor are followed by the two acidic residues, Glu355 and Asp356 and studies from our lab in mouse fibroblast cells lacking β-arrestin expression suggest that arrestins are required for P2Y$_1$ receptor internalization. This model does not completely exclude a role for PKC in phosphorylation and internalization of the P2Y$_1$ receptor in epithelial cells; in the case of the **β**2 adrenergic receptor, PKA and GRKs phosphorylate distinct sites on the receptor and kinase specificity can be determined by receptor occupancy, such that PKA phosphorylation is observed at low agonist concentrations (Tran *et al.*, 2004). In order to confirm this model, experiments are required that show the necessity of GRK and arrestin expression for agonist-promoted internalization in MDCK(II) cells. Other adaptor proteins such as AP2 that regulate clathrin-dependent endocytosis are also possible candidates, since AP2 binds proteins through a dileucine-based motif and the P2Y$_1$ receptor contains an Ile361-Leu362 pair in its C-terminus.

Preliminary data from our lab also suggests that, while phosphorylation sites in the C-terminus of the $P2Y_1$ receptor are necessary for internalization, there may be alternate molecular determinants capable of allowing the receptor to internalize or, alternately interpreted, regions of the receptor that promote retention at the cell surface. Truncation of the $P2Y_1$ receptor at residue 334, paradoxically results in a receptor capable of agonist-promoted internalization, unlike the $\Delta349$ truncation mutant. The rate and extent of internalization are mimicked by the ARAA mutant presented in Chapter 4. These data strongly suggest a role for sequence between residues 334 and 339 in regulating cell surface expression of the $P2Y_1$ receptor. Amino acids in this region may play a role in constitutive internalization, phosphorylation, association with scaffolding proteins that help localize the receptor to the cell surface, or a combination of these possibilities, and each requires further investigation.

Nucleotide-activated G-protein coupled receptors are an important class of potential therapeutic targets. Consequently, thorough investigation of the signaling and physiology of these receptors, requiring high-affinity, selective pharmacological tools, is essential. The studies presented here describe the development of a novel tool for the study of the $P2Y_1$ receptor and add to our increasing body of knowledge regarding agonist-promoted regulation of nucleotide-receptor signaling.
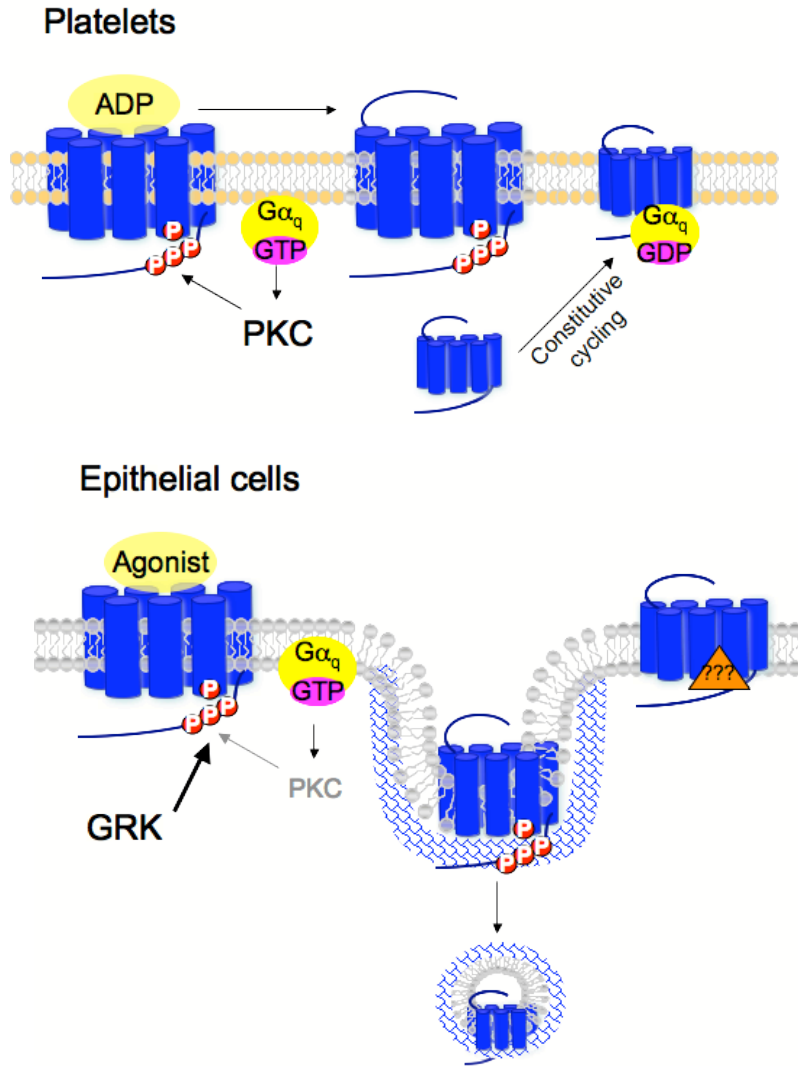
Figure 5.1.   Model of cell-type specific agonist-promoted regulation of the P2Y$_1$ receptor. In platelets, irreversible termination of P2Y$_1$ receptor signaling may occur by agonist-dependent phosphorylation of the receptor by PKC, after which desensitized receptors remain on the cell surface unable to respond to further agonist stimulation. A small number of naive receptors are inserted into the plasma membrane through constitutive receptor cycling. In epithelial cells, such as MDCK(II) cells, agonist activation of P2Y receptors results in the canonical pathway of C-terminal phosphorylation by GRK followed by internalization into clathrin-coated pits, facilitated by an as yet unidentified adaptor protein. Cell surface receptor expression may be controlled by a protein-protein interaction requiring S336 and T339 of the receptor C-terminus.

REFERENCES

ABBRACCHIO, M.P., BOEYNAEMS, J.M., BARNARD, E.A., BOYER, J.L., KENNEDY, C., MIRAS-PORTUGAL, M.T., KING, B.F., GACHET, C., JACOBSON, K.A., WEISMAN, G.A. & BURNSTOCK, G. (2003). Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci*, **24,** 52-5.

ABBRACCHIO, M.P., BRAMBILLA, R., CERUTI, S., KIM, H.O., von LUBITZ, D.K., JACOBSON, K.A. & CATTABENI, F. (1995). G protein-dependent activation of phospholipase C by adenosine A3 receptors in rat brain. *Mol Pharmacol*, **48,** 1038-45.

ABBRACCHIO, M.P., BURNSTOCK, G., BOEYNAEMS, J.M., BARNARD, E.A., BOYER, J.L., KENNEDY, C., KNIGHT, G.E., FUMAGALLI, M., GACHET, C., JACOBSON, K.A. & WEISMAN, G.A. (2006a). 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.

ABBRACCHIO, M.P. & VERDERIO, C. (2006b). Pathophysiological roles of P2 receptors in glial cells. *Novartis Found Symp*, **276,** 91-103; discussion 103-12, 275-81.

ABRAMS, C.S., ZHANG, J., DOWNES, C.P., TANG, X., ZHAO, W. & RITTENHOUSE, S.E. (1996). Phosphopleckstrin inhibits gbetagamma-activable platelet phosphatidylinositol-4,5-bisphosphate 3-kinase. *J Biol Chem*, **271,** 25192-7.

AGRESTI, C., MEOMARTINI, M.E., AMADIO, S., AMBROSINI, E., SERAFINI, B., FRANCHINI, L., VOLONTE, C., ALOISI, F. & VISENTIN, S. (2005). Metabotropic P2 receptor activation regulates oligodendrocyte progenitor migration and development. *Glia*, **50,** 132-44.

AOYAMA, S., KASE, H. & BORRELLI, E. (2000). Rescue of locomotor impairment in dopamine D2 receptor-deficient mice by an adenosine A2A receptor antagonist. *J Neurosci*, **20,** 5848-52.

ARSHAVSKY, V.Y., LAMB, T.D. & PUGH, E.N., JR. (2002). G proteins and phototransduction. *Annu Rev Physiol*, **64,** 153-87.

ARTHUR, J.F., SHEN, Y., MU, F.T., LEON, C., GACHET, C., BERNDT, M.C. & ANDREWS, R.K. (2006). Calmodulin interacts with the platelet ADP receptor P2Y1. *Biochem J*, **398,** 339-43.

ASAI, T., MIURA, S., SIBLEY, L.D., OKABAYASHI, H. & TAKEUCHI, T. (1995). Biochemical and molecular characterization of nucleoside triphosphate hydrolase isozymes from the parasitic protozoan Toxoplasma gondii. *J Biol Chem*, **270,** 11391-7.

ASCHERIO, A., ZHANG, S.M., HERNAN, M.A., KAWACHI, I., COLDITZ, G.A., SPEIZER, F.E. & WILLETT, W.C. (2001). Prospective study of caffeine consumption and risk of Parkinson's disease in men and women. *Ann Neurol*, **50,** 56-63.

AUCHAMPACH, J.A., JIN, X., MOORE, J., WAN, T.C., KRECKLER, L.M., GE, Z.D., NARAYANAN, J., WHALLEY, E., KIESMAN, W., TICHO, B., SMITS, G. & GROSS, G.J. (2004). Comparison of three different A1 adenosine receptor antagonists on infarct size and multiple cycle ischemic preconditioning in anesthetized dogs. *J Pharmacol Exp Ther*, **308,** 846-56.

BAILEY, M.A., TURNER, C.M., HUS-CITHAREL, A., MARCHETTI, J., IMBERT-TEBOUL, M., MILNER, P., BURNSTOCK, G. & UNWIN, R.J. (2004). P2Y receptors present in the native and isolated rat glomerulus. *Nephron Physiol*, **96,** p79-90.

BAURAND, A., ECKLY, A., BARI, N., LEON, C., HECHLER, B., CAZENAVE, J.P. & GACHET, C. (2000). Desensitization of the platelet aggregation response to ADP: differential down-regulation of the P2Y1 and P2cyc receptors. *Thromb Haemost*, **84,** 484-91.

BAURAND, A., ECKLY, A., HECHLER, B., KAUFFENSTEIN, G., GALZI, J.L., CAZENAVE, J.P., LEON, C. & GACHET, C. (2005). Differential regulation and relocalization of the platelet P2Y receptors after activation: a way to avoid loss of hemostatic properties? *Mol Pharmacol*, **67,** 721-33.

BAURAND, A., RABOISSON, P., FREUND, M., LEON, C., CAZENAVE, J.P., BOURGUIGNON, J.J. & GACHET, C. (2001). Inhibition of platelet function by administration of MRS2179, a P2Y1 receptor antagonist. *Eur J Pharmacol*, **412,** 213-21.

BAYES, M., RABASSEDA, X. & PROUS, J.R. (2003). Gateways to clinical trials. *Methods Find Exp Clin Pharmacol*, **25,** 831-55.

BELARDINELLI, L., SHRYOCK, J.C., SONG, Y., WANG, D. & SRINIVAS, M. (1995). Ionic basis of the electrophysiological actions of adenosine on cardiomyocytes. *Faseb J*, **9,** 359-65.

BELLI, S.I., SALI, A. & GODING, J.W. (1994). Divalent cations stabilize the conformation of plasma cell membrane glycoprotein PC-1 (alkaline phosphodiesterase I). *Biochem J*, **304 (Pt 1),** 75-80.

BELLI, S.I., VAN DRIEL, I.R. & GODING, J.W. (1993). Identification and characterization of a soluble form of the plasma cell membrane glycoprotein PC-1 (5'-nucleotide phosphodiesterase). *Eur J Biochem*, **217,** 421-8.

BERTHET, J., RALL, T.W. & SUTHERLAND, E.W. (1957). The relationship of epinephrine and glucagon to liver phosphorylase. IV. Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates. *J Biol Chem*, **224,** 463-75.

BERTONI, A., TADOKORO, S., ETO, K., PAMPORI, N., PARISE, L.V., WHITE, G.C. & SHATTIL, S.J. (2002). Relationships between Rap1b, affinity modulation of integrin alpha IIbbeta 3, and the actin cytoskeleton. *J Biol Chem*, **277,** 25715-21.

BEUKERS, M.W., CHANG, L.C., VON FRIJTAG DRABBE KUNZEL, J.K., MULDER-KRIEGER, T., SPANJERSBERG, R.F., BRUSSEE, J. & AP, I.J. (2004). New, non-adenosine, high-

potency agonists for the human adenosine A2B receptor with an improved selectivity profile compared to the reference agonist N-ethylcarboxamidoadenosine. *J Med Chem*, **47,** 3707-9.

BHATTACHARYYA, S., PURI, S., MILEDI, R. & PANICKER, M.M. (2002). Internalization and recycling of 5-HT2A receptors activated by serotonin and protein kinase C-mediated mechanisms. *Proc Natl Acad Sci U S A*, **99,** 14470-5.

BIANCHI, B.R., LYNCH, K.J., TOUMA, E., NIFORATOS, W., BURGARD, E.C., ALEXANDER, K.M., PARK, H.S., YU, H., METZGER, R., KOWALUK, E., JARVIS, M.F. & VAN BIESEN, T. (1999). Pharmacological characterization of recombinant human and rat P2X receptor subtypes. *Eur J Pharmacol*, **376,** 127-38.

BIGONNESSE, F., LEVESQUE, S.A., KUKULSKI, F., LECKA, J., ROBSON, S.C., FERNANDES, M.J. & SEVIGNY, J. (2004). Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-8. *Biochemistry*, **43,** 5511-9.

BODIN, P. & BURNSTOCK, G. (2001). Purinergic signalling: ATP release. *Neurochem Res*, **26,** 959-69.

BODOR, E.T., WALDO, G.L., HOOKS, S.B., CORBITT, J., BOYER, J.L. & HARDEN, T.K. (2003). Purification and functional reconstitution of the human P2Y12 receptor. *Mol Pharmacol*, **64,** 1210-6.

BOURDON, D.M., MAHANTY, S.K., JACOBSON, K.A., BOYER, J.L. & HARDEN, T.K. (2006). (N)-methanocarba-2MeSADP (MRS2365) is a subtype-specific agonist that induces rapid desensitization of the P2Y1 receptor of human platelets. *J Thromb Haemost*, **4,** 861-8.

BOYER, J.L., ADAMS, M., RAVI, R.G., JACOBSON, K.A. & HARDEN, T.K. (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,** 2004-10.

BOYER, J.L., MOHANRAM, A., CAMAIONI, E., JACOBSON, K.A. & HARDEN, T.K. (1998). Competitive and selective antagonism of P2Y1 receptors by N6-methyl 2'-deoxyadenosine 3',5'-bisphosphate. *Br J Pharmacol*, **124,** 1-3.

BOYER, J.L., ROMERO-AVILA, T., SCHACHTER, J.B. & HARDEN, T.K. (1996). Identification of competitive antagonists of the P2Y1 receptor. *Mol Pharmacol*, **50,** 1323-9.

BOYER, J.L., WALDO, G.L. & HARDEN, T.K. (1992). Beta gamma-subunit activation of G-protein-regulated phospholipase C. *J Biol Chem*, **267,** 25451-6.

BOYER, J.L., WALDO, G.L. & HARDEN, T.K. (1997). Molecular cloning and expression of an avian G protein-coupled P2Y receptor. *Mol Pharmacol*, **52,** 928-34.

BRACKETT, L.E. & DALY, J.W. (1994). Functional characterization of the A2b adenosine receptor in NIH 3T3 fibroblasts. *Biochem Pharmacol*, **47,** 801-14.

BRAMBILLA, R. & ABBRACCHIO, M.P. (2001). Modulation of cyclooxygenase-2 and brain reactive astrogliosis by purinergic P2 receptors. *Ann N Y Acad Sci*, **939,** 54-62.

BRAMBILLA, R., BURNSTOCK, G., BONAZZI, A., CERUTI, S., CATTABENI, F. & ABBRACCHIO, M.P. (1999). Cyclo-oxygenase-2 mediates P2Y receptor-induced reactive astrogliosis. *Br J Pharmacol*, **126,** 563-7.

BRAMBILLA, R., CERUTI, S., MALORNI, W., CATTABENI, F. & ABBRACCHIO, M.P. (2000). A novel gliotic P2 receptor mediating cyclooxygenase-2 induction in rat and human astrocytes. *J Auton Nerv Syst*, **81,** 3-9.

BRAMBILLA, R., NEARY, J.T., CATTABENI, F., COTTINI, L., D'IPPOLITO, G., SCHILLER, P.C. & ABBRACCHIO, M.P. (2002). Induction of COX-2 and reactive gliosis by P2Y receptors in rat cortical astrocytes is dependent on ERK1/2 but independent of calcium signalling. *J Neurochem*, **83,** 1285-96.

BRAUN, N., FENGLER, S., EBELING, C., SERVOS, J. & ZIMMERMANN, H. (2000). Sequencing, functional expression and characterization of rat NTPDase6, a nucleoside diphosphatase and novel member of the ecto-nucleoside triphosphate diphosphohydrolase family. *Biochem J*, **351 Pt 3,** 639-47.

BRINSON, A.E. & HARDEN, T.K. (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,** 11939-48.

BROWN, D.A. & LONDON, E. (1998). Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol*, **14,** 111-36.

BUCK, L.B. (2000). The molecular architecture of odor and pheromone sensing in mammals. *Cell*, **100,** 611-8.

BURNSTOCK, G. (2004). Introduction: P2 receptors. *Curr Top Med Chem*, **4,** 793-803.

BURNSTOCK, G. (1996). P2 purinoceptors: historical perspective and classification. *Ciba Found Symp*, **198,** 1-28; discussion 29-34.

BURNSTOCK, G. (1997). The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology*, **36,** 1127-39.

BURNSTOCK, G. (1972). Purinergic nerves. *Pharmacol Rev*, **24,** 509-81.

BURNSTOCK, G. (2006). Purinergic signalling. *Br J Pharmacol*, **147 Suppl 1,** S172-81.

BURNSTOCK, G., CAMPBELL, G., BENNETT, M. & HOLMAN, M.E. (1964). Innervation Of The Guinea-Pig Taenia Coli: Are There Intrinsic Inhibitory Nerves Which Are Distinct From Sympathetic Nerves? *Int J Neuropharmacol*, **3,** 163-6.

BUXTON, I.L., KAISER, R.A., OXHORN, B.C. & CHEEK, D.J. (2001). Evidence supporting the Nucleotide Axis Hypothesis: ATP release and metabolism by coronary endothelium. *Am J Physiol Heart Circ Physiol*, **281,** H1657-66.

CAMAIONI, E., BOYER, J.L., MOHANRAM, A., HARDEN, T.K. & JACOBSON, K.A. (1998). Deoxyadenosine bisphosphate derivatives as potent antagonists at P2Y1 receptors. *J Med Chem*, **41,** 183-90.

CATTANEO, M. (2005). The P2 receptors and congenital platelet function defects. *Semin Thromb Hemost*, **31,** 168-73.

CATTANEO, M. (2007). Platelet P2 receptors: old and new targets for antithrombotic drugs. *Expert Rev Cardiovasc Ther*, **5,** 45-55.

CATTANEO, M., LECCHI, A., LOMBARDI, R., GACHET, C. & ZIGHETTI, M.L. (2000). Platelets from a patient heterozygous for the defect of P2CYC receptors for ADP have a secretion defect despite normal thromboxane A2 production and normal granule stores: further evidence that some cases of platelet 'primary secretion defect' are heterozygous for a defect of P2CYC receptors. *Arterioscler Thromb Vasc Biol*, **20,** E101-6.

CATTANEO, M., LECCHI, A., RANDI, A.M., McGREGOR, J.L. & MANNUCCI, P.M. (1992). Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood*, **80,** 2787-96.

CATTANEO, M., ZIGHETTI, M.L., LOMBARDI, R., MARTINEZ, C., LECCHI, A., CONLEY, P.B., WARE, J. & RUGGERI, Z.M. (2003). Molecular bases of defective signal transduction in the platelet P2Y12 receptor of a patient with congenital bleeding. *Proc Natl Acad Sci U S A*, **100,** 1978-83.

CAZENAVE, J.P., OHLMANN, P., CASSEL, D., ECKLY, A., HECHLER, B. & GACHET, C. (2004). Preparation of washed platelet suspensions from human and rodent blood. *Methods Mol Biol*, **272,** 13-28.

CHAPMAN, E.R., AN, S., BARTON, N. & JAHN, R. (1994). SNAP-25, a t-SNARE which binds to both syntaxin and synaptobrevin via domains that may form coiled coils. *J Biol Chem*, **269,** 27427-32.

CHEN, C.C., AKOPIAN, A.N., SIVILOTTI, L., COLQUHOUN, D., BURNSTOCK, G. & WOOD, J.N. (1995). A P2X purinoceptor expressed by a subset of sensory neurons. *Nature*, **377,** 428-31.

CHHATRIWALA, M., RAVI, R.G., PATEL, R.I., BOYER, J.L., JACOBSON, K.A. & HARDEN, T.K. (2004). Induction of novel agonist selectivity for the ADP-activated P2Y1 receptor versus the ADP-activated P2Y12 and P2Y13 receptors by conformational constraint of an ADP analog. *J Pharmacol Exp Ther*, **311,** 1038-43.

CHORNA, N.E., SANTIAGO-PEREZ, L.I., ERB, L., SEYE, C.I., NEARY, J.T., SUN, G.Y., WEISMAN, G.A. & GONZALEZ, F.A. (2004). P2Y receptors activate neuroprotective mechanisms in astrocytic cells. *J Neurochem*, **91,** 119-32.

CLAIR, T., KRUTZSCH, H.C., LIOTTA, L.A. & STRACKE, M.L. (1997a). Nucleotide binding to autotaxin: crosslinking of bound substrate followed by lysC digestion identifies two labeled peptides. *Biochem Biophys Res Commun*, **236,** 449-54.

CLAIR, T., LEE, H.Y., LIOTTA, L.A. & STRACKE, M.L. (1997b). Autotaxin is an exoenzyme possessing 5'-nucleotide phosphodiesterase/ATP pyrophosphatase and ATPase activities. *J Biol Chem*, **272,** 996-1001.

CLAPHAM, D.E. & NEER, E.J. (1997). G protein beta gamma subunits. *Annu Rev Pharmacol Toxicol*, **37,** 167-203.

CLIFFORD, E.E., PARKER, K., HUMPHREYS, B.D., KERTESY, S.B. & DUBYAK, G.R. (1998). The P2X1 receptor, an adenosine triphosphate-gated cation channel, is expressed in human platelets but not in human blood leukocytes. *Blood*, **91,** 3172-81.

COCKAYNE, D.A., HAMILTON, S.G., ZHU, Q.M., DUNN, P.M., ZHONG, Y., NOVAKOVIC, S., MALMBERG, A.B., CAIN, G., BERSON, A., KASSOTAKIS, L., HEDLEY, L., LACHNIT, W.G., BURNSTOCK, G., MCMAHON, S.B. & FORD, A.P. (2000). Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature*, **407,** 1011-5.

COGE, F., GUENIN, S.P., RENOUARD-TRY, A., RIQUE, H., OUVRY, C., FABRY, N., BEAUVERGER, P., NICOLAS, J.P., GALIZZI, J.P., BOUTIN, J.A. & CANET, E. (1999). Truncated isoforms inhibit [3H]prazosin binding and cellular trafficking of native human alpha1A-adrenoceptors. *Biochem J*, **343 Pt 1,** 231-9.

COLEMAN, J.E. (1992). Structure and mechanism of alkaline phosphatase. *Annu Rev Biophys Biomol Struct*, **21,** 441-83.

COMMUNI, D., GOVAERTS, C., PARMENTIER, M. & BOEYNAEMS, J.M. (1997). Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J Biol Chem*, **272,** 31969-73.

COMSTOCK, K., WATSON, N. & OLSEN, J. (1997). *Recombinant gene expression protocols*. Totowa, N.J.: Humana Press.

COSTANZI, S., JOSHI, B.V., MADDILETI, S., MAMEDOVA, L., GONZALEZ-MOA, M.J., MARQUEZ, V.E., HARDEN, T.K. & JACOBSON, K.A. (2005). Human P2Y(6) receptor: molecular modeling leads to the rational design of a novel agonist based on a unique conformational preference. *J Med Chem*, **48,** 8108-11.

COSTANZI, S., MAMEDOVA, L., GAO, Z.G. & JACOBSON, K.A. (2004). Architecture of P2Y nucleotide receptors: structural comparison based on sequence analysis, mutagenesis, and homology modeling. *J Med Chem*, **47,** 5393-404.

COTRINA, M.L., LIN, J.H., ALVES-RODRIGUES, A., LIU, S., LI, J., AZMI-GHADIMI, H., KANG, J., NAUS, C.C. & NEDERGAARD, M. (1998). Connexins regulate calcium signaling by controlling ATP release. *Proc Natl Acad Sci U S A*, **95,** 15735-40.

CRESPO, P., XU, N., SIMONDS, W.F. & GUTKIND, J.S. (1994). Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature*, **369,** 418-20.

CUNHA, R.A., BRENDEL, P., ZIMMERMANN, H. & RIBEIRO, J.A. (2000). Immunologically distinct isoforms of ecto-5'-nucleotidase in nerve terminals of different areas of the rat hippocampus. *J Neurochem*, **74,** 334-8.

DALY, J.W., BUTTS-LAMB, P. & PADGETT, W. (1983). Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. *Cell Mol Neurobiol*, **3,** 69-80.

DAMM, E.M., PELKMANS, L., KARTENBECK, J., MEZZACASA, A., KURZCHALIA, T. & HELENIUS, A. (2005). Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. *J Cell Biol*, **168,** 477-88.

DELMAS, P., ABOGADIE, F.C., BUCKLEY, N.J. & BROWN, D.A. (2000). Calcium channel gating and modulation by transmitters depend on cellular compartmentalization. *Nat Neurosci*, **3,** 670-8.

DEVI, L.A. (2001). Heterodimerization of G-protein-coupled receptors: pharmacology, signaling and trafficking. *Trends Pharmacol Sci*, **22,** 532-7.

DIVIANI, D., SODERLING, J. & SCOTT, J.D. (2001). AKAP-Lbc anchors protein kinase A and nucleates Galpha 12-selective Rho-mediated stress fiber formation. *J Biol Chem*, **276,** 44247-57.

DODGE, K. & SCOTT, J.D. (2000). AKAP79 and the evolution of the AKAP model. *FEBS Lett*, **476,** 58-61.

DONALDSON, S.H., LAZAROWSKI, E.R., PICHER, M., KNOWLES, M.R., STUTTS, M.J. & BOUCHER, R.C. (2000). Basal nucleotide levels, release, and metabolism in normal and cystic fibrosis airways. *Mol Med*, **6,** 969-82.

DONG, Q., GINSBERG, H.N. & ERLANGER, B.F. (2001). Overexpression of the A1 adenosine receptor in adipose tissue protects mice from obesity-related insulin resistance. *Diabetes Obes Metab*, **3,** 360-6.

DOWD, F.J., LI, L.S. & ZENG, W. (1999). Inhibition of rat parotid ecto-ATPase activity. *Arch Oral Biol*, **44,** 1055-62.

DRAB, M., VERKADE, P., ELGER, M., KASPER, M., LOHN, M., LAUTERBACH, B., MENNE, J., LINDSCHAU, C., MENDE, F., LUFT, F.C., SCHEDL, A., HALLER, H. & KURZCHALIA,

T.V. (2001). Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science*, **293,** 2449-52.

DRIESSEN, B., BULTMANN, R., JURNA, I. & BALDAUF, J. (1998). Depression of C fiber-evoked activity by intrathecally administered reactive red 2 in rat thalamic neurons. *Brain Res*, **796,** 284-90.

DRIESSEN, B., REIMANN, W., SELVE, N., FRIDERICHS, E. & BULTMANN, R. (1994). Antinociceptive effect of intrathecally administered P2-purinoceptor antagonists in rats. *Brain Res*, **666,** 182-8.

DRURY, A.N. & SZENT-GYORGYI, A. (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J Physiol*, **68,** 213-37.

ELLENBOGEN, K.A., O'NEILL, G., PRYSTOWSKY, E.N., CAMM, J.A., MENG, L., LIEU, H.D., JERLING, M., SHREENIWAS, R., BELARDINELLI, L. & WOLFF, A.A. (2005). Trial to evaluate the management of paroxysmal supraventricular tachycardia during an electrophysiology study with tecadenoson. *Circulation*, **111,** 3202-8.

ENGLERT, M., QUITTERER, U. & KLOTZ, K.N. (2002). Effector coupling of stably transfected human A3 adenosine receptors in CHO cells. *Biochem Pharmacol*, **64,** 61-5.

ETO, K., MURPHY, R., KERRIGAN, S.W., BERTONI, A., STUHLMANN, H., NAKANO, T., LEAVITT, A.D. & SHATTIL, S.J. (2002). Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling. *Proc Natl Acad Sci U S A*, **99,** 12819-24.

EVANS, R.J., DERKACH, V. & SURPRENANT, A. (1992). ATP mediates fast synaptic transmission in mammalian neurons. *Nature*, **357,** 503-5.

EVANS, R.J., LEWIS, C., BUELL, G., VALERA, S., NORTH, R.A. & SURPRENANT, A. (1995). Pharmacological characterization of heterologously expressed ATP-gated cation channels (P2x purinoceptors). *Mol Pharmacol*, **48,** 178-83.

FABRE, J.E., NGUYEN, M., LATOUR, A., KEIFER, J.A., AUDOLY, L.P., COFFMAN, T.M. & KOLLER, B.H. (1999). Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat Med*, **5,** 1199-202.

FAM, S.R., GALLAGHER, C.J., KALIA, L.V. & SALTER, M.W. (2003). Differential frequency dependence of P2Y1- and P2Y2- mediated Ca 2+ signaling in astrocytes. *J Neurosci*, **23,** 4437-44.

FAM, S.R., PAQUET, M., CASTLEBERRY, A.M., OLLER, H., LEE, C.J., TRAYNELIS, S.F., SMITH, Y., YUN, C.C. & HALL, R.A. (2005). P2Y1 receptor signaling is controlled by interaction with the PDZ scaffold NHERF-2. *Proc Natl Acad Sci U S A*, **102,** 8042-7.

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FANG, X., HU, H.Z., GAO, N., LIU, S., WANG, G.D., WANG, X.Y., XIA, Y. & WOOD, J.D. (2006). Neurogenic secretion mediated by the purinergic P2Y1 receptor in guinea-pig small intestine. *Eur J Pharmacol*, **536,** 113-22.

FEOKTISTOV, I. & BIAGGIONI, I. (1995). Adenosine A2b receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. *J Clin Invest*, **96,** 1979-86.

FERGUSON, D.R., KENNEDY, I. & BURTON, T.J. (1997). ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes--a possible sensory mechanism? *J Physiol*, **505 (Pt 2),** 503-11.

FILTZ, T.M., LI, Q., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1994). Expression of a cloned P2Y purinergic receptor that couples to phospholipase C. *Mol Pharmacol*, **46,** 8-14.

FISCHER, B., BOYER, J.L., HOYLE, C.H., ZIGANSHIN, A.U., BRIZZOLARA, A.L., KNIGHT, G.E., ZIMMET, J., BURNSTOCK, G., HARDEN, T.K. & JACOBSON, K.A. (1993). Identification of potent, selective P2Y-purinoceptor agonists: structure-activity relationships for 2-thioether derivatives of adenosine 5'-triphosphate. *J Med Chem*, **36,** 3937-46.

FISHMAN, P., BAR-YEHUDA, S., MADI, L. & COHN, I. (2002). A3 adenosine receptor as a target for cancer therapy. *Anticancer Drugs*, **13,** 437-43.

FONG, A.Y., KRSTEW, E.V., BARDEN, J. & LAWRENCE, A.J. (2002). Immunoreactive localisation of P2Y1 receptors within the rat and human nodose ganglia and rat brainstem: comparison with [alpha 33P]deoxyadenosine 5'-triphosphate autoradiography. *Neuroscience*, **113,** 809-23.

FOSSETTA, J., JACKSON, J., DENO, G., FAN, X., DU, X.K., BOBER, L., SOUDE-BERMEJO, A., DE BOUTEILLER, O., CAUX, C., LUNN, C., LUNDELL, D. & PALMER, R.K. (2003). Pharmacological analysis of calcium responses mediated by the human A3 adenosine receptor in monocyte-derived dendritic cells and recombinant cells. *Mol Pharmacol*, **63,** 342-50.

FOSTER, C.J., PROSSER, D.M., AGANS, J.M., ZHAI, Y., SMITH, M.D., LACHOWICZ, J.E., ZHANG, F.L., GUSTAFSON, E., MONSMA, F.J., JR., WIEKOWSKI, M.T., ABBONDANZO, S.J., COOK, D.N., BAYNE, M.L., LIRA, S.A. & CHINTALA, M.S. (2001). Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest*, **107,** 1591-8.

FOZARD, J.R., ELLIS, K.M., VILLELA DANTAS, M.F., TIGANI, B. & MAZZONI, L. (2002). Effects of CGS 21680, a selective adenosine A2A receptor agonist, on allergic airways inflammation in the rat. *Eur J Pharmacol*, **438,** 183-8.

FRA, A.M., WILLIAMSON, E., SIMONS, K. & PARTON, R.G. (1994). Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J Biol Chem*, **269,** 30745-8.

FRANKE, H. & ILLES, P. (2005). Involvement of P2 receptors in the growth and survival of neurons in the CNS. *Pharmacol Ther*.

FRASER, I.D., CONG, M., KIM, J., ROLLINS, E.N., DAAKA, Y., LEFKOWITZ, R.J. & SCOTT, J.D. (2000). Assembly of an A kinase-anchoring protein-beta(2)-adrenergic receptor complex facilitates receptor phosphorylation and signaling. *Curr Biol*, **10,** 409-12.

FREDHOLM, B.B., AP, I.J., JACOBSON, K.A., KLOTZ, K.N. & LINDEN, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev*, **53,** 527-52.

FUKUHARA, S., MURGA, C., ZOHAR, M., IGISHI, T. & GUTKIND, J.S. (1999). A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. *J Biol Chem*, **274,** 5868-79.

GALLAGHER, C.J. & SALTER, M.W. (2003). Differential properties of astrocyte calcium waves mediated by P2Y1 and P2Y2 receptors. *J Neurosci*, **23,** 6728-39.

GALLAGHER, J.A. (2004). ATP P2 receptors and regulation of bone effector cells. *J Musculoskelet Neuronal Interact*, **4,** 125-7.

GALLEGO, D., HERNANDEZ, P., CLAVE, P. & JIMENEZ, M. (2006). P2Y1 receptors mediate inhibitory purinergic neuromuscular transmission in the human colon. *Am J Physiol Gastrointest Liver Physiol*, **291,** G584-94.

GALVEZ, T., DUTHEY, B., KNIAZEFF, J., BLAHOS, J., ROVELLI, G., BETTLER, B., PREZEAU, L. & PIN, J.P. (2001). Allosteric interactions between GB1 and GB2 subunits are required for optimal GABA(B) receptor function. *Embo J*, **20,** 2152-9.

GAO, Z.G., BLAUSTEIN, J.B., GROSS, A.S., MELMAN, N. & JACOBSON, K.A. (2003). N6-Substituted adenosine derivatives: selectivity, efficacy, and species differences at A3 adenosine receptors. *Biochem Pharmacol*, **65,** 1675-84.

GARTLAND, A., BUCKLEY, K.A., HIPSKIND, R.A., BOWLER, W.B. & GALLAGHER, J.A. (2003). P2 receptors in bone--modulation of osteoclast formation and activity via P2X7 activation. *Crit Rev Eukaryot Gene Expr*, **13,** 237-42.

GESSI, S., VARANI, K., MERIGHI, S., CATTABRIGA, E., PANCALDI, C., SZABADKAI, Y., RIZZUTO, R., KLOTZ, K.N., LEUNG, E., MAC LENNAN, S., BARALDI, P.G. & BOREA, P.A. (2005). Expression, pharmacological profile, and functional coupling of A2B receptors in a recombinant system and in peripheral blood cells using a novel selective antagonist radioligand, [3H]MRE 2029-F20. *Mol Pharmacol*, **67,** 2137-47.

GIFFIN, N.J., KOWACS, F., LIBRI, V., WILLIAMS, P., GOADSBY, P.J. & KAUBE, H. (2003). Effect of the adenosine A1 receptor agonist GR79236 on trigeminal nociception with blink reflex recordings in healthy human subjects. *Cephalalgia*, **23,** 287-92.

GODING, J.W., TERKELTAUB, R., MAURICE, M., DETERRE, P., SALI, A. & BELLI, S.I. (1998). Ecto-phosphodiesterase/pyrophosphatase of lymphocytes and non-lymphoid cells: structure and function of the PC-1 family. *Immunol Rev*, **161,** 11-26.

GOTTLIEB, S.S., BRATER, D.C., THOMAS, I., HAVRANEK, E., BOURGE, R., GOLDMAN, S., DYER, F., GOMEZ, M., BENNETT, D., TICHO, B., BECKMAN, E. & ABRAHAM, W.T. (2002). BG9719 (CVT-124), an A1 adenosine receptor antagonist, protects against the decline in renal function observed with diuretic therapy. *Circulation*, **105,** 1348-53.

GRIMMER, S., VAN DEURS, B. & SANDVIG, K. (2002). Membrane ruffling and macropinocytosis in A431 cells require cholesterol. *J Cell Sci*, **115,** 2953-62.

HAINES, W.R., TORRES, G.E., VOIGT, M.M. & EGAN, T.M. (1999). Properties of the novel ATP-gated ionotropic receptor composed of the P2X(1) and P2X(5) isoforms. *Mol Pharmacol*, **56,** 720-7.

HALL, R.A., OSTEDGAARD, L.S., PREMONT, R.T., BLITZER, J.T., RAHMAN, N., WELSH, M.J. & LEFKOWITZ, R.J. (1998a). A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na+/H+ exchanger regulatory factor family of PDZ proteins. *Proc Natl Acad Sci U S A*, **95,** 8496-501.

HALL, R.A., PREMONT, R.T., CHOW, C.W., BLITZER, J.T., PITCHER, J.A., CLAING, A., STOFFEL, R.H., BARAK, L.S., SHENOLIKAR, S., WEINMAN, E.J., GRINSTEIN, S. & LEFKOWITZ, R.J. (1998b). The beta2-adrenergic receptor interacts with the Na+/H+-exchanger regulatory factor to control Na+/H+ exchange. *Nature*, **392,** 626-30.

HANDA, M. & GUIDOTTI, G. (1996). Purification and cloning of a soluble ATP-diphosphohydrolase (apyrase) from potato tubers (Solanum tuberosum). *Biochem Biophys Res Commun*, **218,** 916-23.

HARDEN, T.K., LAZAROWSKI, E.R. & BOUCHER, R.C. (1997). Release, metabolism and interconversion of adenine and uridine nucleotides: implications for G protein-coupled P2 receptor agonist selectivity. *Trends Pharmacol Sci*, **18,** 43-6.

HARDY, A.R., CONLEY, P.B., LUO, J., BENOVIC, J.L., POOLE, A.W. & MUNDELL, S.J. (2005). P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood*, **105,** 3552-60.

HECHLER, B., LENAIN, N., MARCHESE, P., VIAL, C., HEIM, V., FREUND, M., CAZENAVE, J.P., CATTANEO, M., RUGGERI, Z.M., EVANS, R. & GACHET, C. (2003). A role of the fast ATP-gated P2X1 cation channel in thrombosis of small arteries in vivo. *J Exp Med*, **198,** 661-7.

HECHLER, B., LEON, C., VIAL, C., VIGNE, P., FRELIN, C., CAZENAVE, J.P. & GACHET, C. (1998). The P2Y1 receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. *Blood*, **92,** 152-9.

HECHLER, B., NONNE, C., ROH, E.J., CATTANEO, M., CAZENAVE, J.P., LANZA, F., JACOBSON, K.A. & GACHET, C. (2006). MRS2500 [2-iodo-N6-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate], a potent, selective, and stable antagonist of the platelet P2Y1 receptor with strong antithrombotic activity in mice. *J Pharmacol Exp Ther*, **316,** 556-63.

HELDIN, C.H. (1995). Dimerization of cell surface receptors in signal transduction. *Cell*, **80,** 213-23.

HENDEL, R.C., BATEMAN, T.M., CERQUEIRA, M.D., ISKANDRIAN, A.E., LEPPO, J.A., BLACKBURN, B. & MAHMARIAN, J.J. (2005). Initial clinical experience with regadenoson, a novel selective A2A agonist for pharmacologic stress single-photon emission computed tomography myocardial perfusion imaging. *J Am Coll Cardiol*, **46,** 2069-75.

HINRICHSEN, L., MEYERHOLZ, A., GROOS, S. & UNGEWICKELL, E.J. (2006). Bending a membrane: how clathrin affects budding. *Proc Natl Acad Sci U S A*, **103,** 8715-20.

HIRSCH, E., BOSCO, O., TROPEL, P., LAFFARGUE, M., CALVEZ, R., ALTRUDA, F., WYMANN, M. & MONTRUCCHIO, G. (2001). Resistance to thromboembolism in PI3Kgamma-deficient mice. *Faseb J*, **15,** 2019-21.

HOFFMANN, C., MORO, S., NICHOLAS, R.A., HARDEN, T.K. & JACOBSON, K.A. (1999). The role of amino acids in extracellular loops of the human P2Y1 receptor in surface expression and activation processes. *J Biol Chem*, **274,** 14639-47.

HOHENSTEIN, B., RENK, S., LANG, K., DANIEL, C., FREUND, M., LEON, C., AMANN, K.U., GACHET, C. & HUGO, C.P. (2007). P2Y1 Gene Deficiency Protects from Renal Disease Progression and Capillary Rarefaction during Passive Crescentic Glomerulonephritis. *J Am Soc Nephrol*, **18,** 494-505.

HOLLOPETER, G., JANTZEN, H.M., VINCENT, D., LI, G., ENGLAND, L., RAMAKRISHNAN, V., YANG, R.B., NURDEN, P., NURDEN, A., JULIUS, D. & CONLEY, P.B. (2001). Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature*, **409,** 202-7.

HOLTON, F.A. & HOLTON, P. (1953). The possibility that ATP is a transmitter at sensory nerve endings. *J Physiol*, **119,** 50P-51P.

HORIUCHI, J., POTTS, P.D., TAGAWA, T. & DAMPNEY, R.A. (1999). Effects of activation and blockade of P2x receptors in the ventrolateral medulla on arterial pressure and sympathetic activity. *J Auton Nerv Syst*, **76,** 118-26.

HOUSTON, D., OHNO, M., NICHOLAS, R.A., JACOBSON, K.A. & HARDEN, T.K. (2006). [32P]2-iodo-N6-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphat e ([32P]MRS2500), a novel radioligand for quantification of native P2Y1 receptors. *Br J Pharmacol*, **147,** 459-67.

HUSTED, S., EMANUELSSON, H., HEPTINSTALL, S., SANDSET, P.M., WICKENS, M. & PETERS, G. (2006). Pharmacodynamics, pharmacokinetics, and safety of the oral reversible P2Y12 antagonist AZD6140 with aspirin in patients with atherosclerosis: a double-blind comparison to clopidogrel with aspirin. *Eur Heart J*, **27,** 1038-47.

JACKSON, S.P., NESBITT, W.S. & KULKARNI, S. (2003). Signaling events underlying thrombus formation. *J Thromb Haemost*, **1,** 1602-12.

JACKSON, S.P., SCHOENWAELDER, S.M., GONCALVES, I., NESBITT, W.S., YAP, C.L., WRIGHT, C.E., KENCHE, V., ANDERSON, K.E., DOPHEIDE, S.M., YUAN, Y., STURGEON, S.A., PRABAHARAN, H., THOMPSON, P.E., SMITH, G.D., SHEPHERD, P.R., DANIELE, N., KULKARNI, S., ABBOTT, B., SAYLIK, D., JONES, C., LU, L., GIULIANO, S., HUGHAN, S.C., ANGUS, J.A., ROBERTSON, A.D. & SALEM, H.H. (2005). PI 3-kinase p110beta: a new target for antithrombotic therapy. *Nat Med*, **11,** 507-14.

JACOBSON, K.A., HOFFMANN, C., KIM, Y.C., CAMAIONI, E., NANDANAN, E., JANG, S.Y., GUO, D.P., JI, X.D., VON KUGELGEN, I., MORO, S., ZIGANSHIN, A.U., RYCHKOV, A., KING, B.F., BROWN, S.G., WILDMAN, S.S., BURNSTOCK, G., BOYER, J.L., MOHANRAM, A. & HARDEN, T.K. (1999). Molecular recognition in P2 receptors: ligand development aided by molecular modeling and mutagenesis. *Prog Brain Res*, **120,** 119-32.

JACOBSON, K.A., MORO, S., HOFFMANN, C., KIM, Y.C., KIM, H.S., RAVI, R.G., HARDEN, T.K. & BOYER, J.L. (2001). Structurally related nucleotides as selective agonists and antagonists at P2Y1 receptors. *Farmaco*, **56,** 71-5.

JANSSENS, R., COMMUNI, D., PIROTTON, S., SAMSON, M., PARMENTIER, M. & BOEYNAEMS, J.M. (1996). Cloning and tissue distribution of the human P2Y1 receptor. *Biochem Biophys Res Commun*, **221,** 588-93.

JANTZEN, H.M., GOUSSET, L., BHASKAR, V., VINCENT, D., TAI, A., REYNOLDS, E.E. & CONLEY, P.B. (1999). Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost*, **81,** 111-7.

JEONG, L.S., JIN, D.Z., KIM, H.O., SHIN, D.H., MOON, H.R., GUNAGA, P., CHUN, M.W., KIM, Y.C., MELMAN, N., GAO, Z.G. & JACOBSON, K.A. (2003). N6-substituted D-4'-thioadenosine-5'-methyluronamides: potent and selective agonists at the human A3 adenosine receptor. *J Med Chem*, **46,** 3775-7.

JI, X., KIM, Y.C., AHERN, D.G., LINDEN, J. & JACOBSON, K.A. (2001). [3H]MRS 1754, a selective antagonist radioligand for A(2B) adenosine receptors. *Biochem Pharmacol*, **61,** 657-63.

JIANG, Q., GUO, D., LEE, B.X., VAN RHEE, A.M., KIM, Y.C., NICHOLAS, R.A., SCHACHTER, J.B., HARDEN, T.K. & JACOBSON, K.A. (1997). A mutational analysis of residues essential for ligand recognition at the human P2Y1 receptor. *Mol Pharmacol*, **52,** 499-507.

JIN, J., DANIEL, J.L. & KUNAPULI, S.P. (1998). Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem*, **273,** 2030-4.

JONES, M.B., SIDEROVSKI, D.P. & HOOKS, S.B. (2004). The G{beta}{gamma} DIMER as a NOVEL SOURCE of SELECTIVITY in G-Protein Signaling: GGL-ing AT CONVENTION. *Mol Interv*, **4,** 200-214.

JORDAN, B.A. & DEVI, L.A. (1999). G-protein-coupled receptor heterodimerization modulates receptor function. *Nature*, **399,** 697-700.

JOURDAIN, P., BERGERSEN, L.H., BHAUKAURALLY, K., BEZZI, P., SANTELLO, M., DOMERCQ, M., MATUTE, C., TONELLO, F., GUNDERSEN, V. & VOLTERRA, A. (2007). Glutamate exocytosis from astrocytes controls synaptic strength. *Nat Neurosci*, **10,** 331-9.

KACZMAREK, E., KOZIAK, K., SEVIGNY, J., SIEGEL, J.B., ANRATHER, J., BEAUDOIN, A.R., BACH, F.H. & ROBSON, S.C. (1996). Identification and characterization of CD39/vascular ATP diphosphohydrolase. *J Biol Chem*, **271,** 33116-22.

KAMMERMEIER, P.J., RUIZ-VELASCO, V. & IKEDA, S.R. (2000). A voltage-independent calcium current inhibitory pathway activated by muscarinic agonists in rat sympathetic neurons requires both Galpha q/11 and Gbeta gamma. *J Neurosci*, **20,** 5623-9.

KARPA, K.D., LIN, R., KABBANI, N. & LEVENSON, R. (2000). The dopamine D3 receptor interacts with itself and the truncated D3 splice variant d3nf: D3-D3nf interaction causes mislocalization of D3 receptors. *Mol Pharmacol*, **58,** 677-83.

KAUFFENSTEIN, G., BERGMEIER, W., ECKLY, A., OHLMANN, P., LEON, C., CAZENAVE, J.P., NIESWANDT, B. & GACHET, C. (2001). The P2Y(12) receptor induces platelet aggregation through weak activation of the alpha(IIb)beta(3) integrin--a phosphoinositide 3-kinase-dependent mechanism. *FEBS Lett*, **505,** 281-90.

KAWAGOE, H., SOMA, O., GOJI, J., NISHIMURA, N., NARITA, M., INAZAWA, J., NAKAMURA, H. & SANO, K. (1995). Molecular cloning and chromosomal assignment of the human brain-type phosphodiesterase I/nucleotide pyrophosphatase gene (PDNP2). *Genomics*, **30,** 380-4.

KEGEL, B., BRAUN, N., HEINE, P., MALISZEWSKI, C.R. & ZIMMERMANN, H. (1997). An ecto-ATPase and an ecto-ATP diphosphohydrolase are expressed in rat brain. *Neuropharmacology*, **36,** 1189-200.

KENNEDY, C., QI, A.D., HEROLD, C.L., HARDEN, T.K. & NICHOLAS, R.A. (2000). ATP, an agonist at the rat P2Y(4) receptor, is an antagonist at the human P2Y(4) receptor. *Mol Pharmacol*, **57,** 926-31.

KHAKH, B.S., HUMPHREY, P.P. & SURPRENANT, A. (1995). Electrophysiological properties of P2X-purinoceptors in rat superior cervical, nodose and guinea-pig coeliac neurones. *J Physiol*, **484 (Pt 2),** 385-95.

KIM, H.S., BARAK, D., HARDEN, T.K., BOYER, J.L. & JACOBSON, K.A. (2001). Acyclic and cyclopropyl analogues of adenosine bisphosphate antagonists of the P2Y1 receptor: structure-activity relationships and receptor docking. *J Med Chem*, **44,** 3092-108.

KIM, H.S., OHNO, M., XU, B., KIM, H.O., CHOI, Y., JI, X.D., MADDILETI, S., MARQUEZ, V.E., HARDEN, T.K. & JACOBSON, K.A. (2003). 2-Substitution of adenine nucleotide analogues containing a bicyclo[3.1.0]hexane ring system locked in a northern conformation: enhanced potency as P2Y1 receptor antagonists. *J Med Chem*, **46,** 4974-87.

KIM, H.S., RAVI, R.G., MARQUEZ, V.E., MADDILETI, S., WIHLBORG, A.K., ERLINGE, D., MALMSJO, M., BOYER, J.L., HARDEN, T.K. & JACOBSON, K.A. (2002). Methanocarba modification of uracil and adenine nucleotides: high potency of Northern ring conformation at P2Y1, P2Y2, P2Y4, and P2Y11 but not P2Y6 receptors. *J Med Chem*, **45,** 208-18.

KIM, S., JIN, J. & KUNAPULI, S.P. (2004). Akt activation in platelets depends on Gi signaling pathways. *J Biol Chem*, **279,** 4186-95.

KIM, Y.C., GALLO-RODRIGUEZ, C., JANG, S.Y., NANDANAN, E., ADAMS, M., HARDEN, T.K., BOYER, J.L. & JACOBSON, K.A. (2000). Acyclic analogues of deoxyadenosine 3',5'-bisphosphates as P2Y(1) receptor antagonists. *J Med Chem*, **43,** 746-55.

KIRLEY, T.L. (1997). Complementary DNA cloning and sequencing of the chicken muscle ecto-ATPase. Homology with the lymphoid cell activation antigen CD39. *J Biol Chem*, **272,** 1076-81.

KITTNER, H., FRANKE, H., FISCHER, W., SCHULTHEIS, N., KRUGEL, U. & ILLES, P. (2003). Stimulation of P2Y1 receptors causes anxiolytic-like effects in the rat elevated plus-maze: implications for the involvement of P2Y1 receptor-mediated nitric oxide production. *Neuropsychopharmacology*, **28,** 435-44.

KITTNER, H., FRANKE, H., HARSCH, J.I., EL-ASHMAWY, I.M., SEIDEL, B., KRUGEL, U. & ILLES, P. (2006). Enhanced food intake after stimulation of hypothalamic P2Y1 receptors in rats: modulation of feeding behaviour by extracellular nucleotides. *Eur J Neurosci*, **24,** 2049-56.

KNOFEL, T. & STRATER, N. (1999). X-ray structure of the Escherichia coli periplasmic 5'-nucleotidase containing a dimetal catalytic site. *Nat Struct Biol*, **6,** 448-53.

KO, H., FRICKS, I., IVANOV, A.A., HARDEN, T.K. & JACOBSON, K.A. (2007). Structure Activity Relationship of Uridine 5′-Diphosphoglucose (UDP-Glucose). *J Med Chem*, **Accepted Manuscript**.

KOENIG, J.A. & EDWARDSON, J.M. (1996). Intracellular trafficking of the muscarinic acetylcholine receptor: importance of subtype and cell type. *Mol Pharmacol*, **49,** 351-9.

KRUPNICK, J.G. & BENOVIC, J.L. (1998). The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol*, **38,** 289-319.

KULL, B., SVENNINGSSON, P. & FREDHOLM, B.B. (2000). Adenosine A(2A) receptors are colocalized with and activate g(olf) in rat striatum. *Mol Pharmacol*, **58,** 771-7.

LAMMAS, D.A., STOBER, C., HARVEY, C.J., KENDRICK, N., PANCHALINGAM, S. & KUMARARATNE, D.S. (1997). ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. *Immunity*, **7,** 433-44.

LAZAROWSKI, E.R., BOUCHER, R.C. & HARDEN, T.K. (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,** 31061-8.

LAZAROWSKI, E.R., HOMOLYA, L., BOUCHER, R.C. & HARDEN, T.K. (1997a). Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *J Biol Chem*, **272,** 24348-54.

LAZAROWSKI, E.R., HOMOLYA, L., BOUCHER, R.C. & HARDEN, T.K. (1997b). Identification of an ecto-nucleoside diphosphokinase and its contribution to interconversion of P2 receptor agonists. *J Biol Chem*, **272,** 20402-7.

LAZAROWSKI, E.R., SHEA, D.A., BOUCHER, R.C. & HARDEN, T.K. (2003). Release of cellular UDP-glucose as a potential extracellular signaling molecule. *Mol Pharmacol*, **63,** 1190-7.

LE, K.T., BABINSKI, K. & SEGUELA, P. (1998). Central P2X4 and P2X6 channel subunits coassemble into a novel heteromeric ATP receptor. *J Neurosci*, **18,** 7152-9.

LEE, K.B., PTASIENSKI, J.A., BUNEMANN, M. & HOSEY, M.M. (2000a). Acidic amino acids flanking phosphorylation sites in the M2 muscarinic receptor regulate receptor phosphorylation, internalization, and interaction with arrestins. *J Biol Chem*, **275,** 35767-77.

LEE, S.B. & RHEE, S.G. (1995). Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. *Curr Opin Cell Biol*, **7,** 183-9.

LEE, S.P., O'DOWD, B.F., NG, G.Y., VARGHESE, G., AKIL, H., MANSOUR, A., NGUYEN, T. & GEORGE, S.R. (2000b). Inhibition of cell surface expression by mutant receptors demonstrates that D2 dopamine receptors exist as oligomers in the cell. *Mol Pharmacol*, **58,** 120-8.

LEE, S.P., O'DOWD, B.F., RAJARAM, R.D., NGUYEN, T. & GEORGE, S.R. (2003). D2 dopamine receptor homodimerization is mediated by multiple sites of interaction, including an intermolecular interaction involving transmembrane domain 4. *Biochemistry*, **42,** 11023-31.

LEON, C., HECHLER, B., FREUND, M., ECKLY, A., VIAL, C., OHLMANN, P., DIERICH, A., LEMEUR, M., CAZENAVE, J.P. & GACHET, C. (1999a). Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest*, **104,** 1731-7.

LEON, C., VIAL, C., CAZENAVE, J.P. & GACHET, C. (1996). Cloning and sequencing of a human cDNA encoding endothelial P2Y1 purinoceptor. *Gene*, **171,** 295-7.

LEON, C., VIAL, C., GACHET, C., OHLMANN, P., HECHLER, B., CAZENAVE, J.P., LECCHI, A. & CATTANEO, M. (1999b). The P2Y1 receptor is normal in a patient presenting a severe deficiency of ADP-induced platelet aggregation. *Thromb Haemost*, **81,** 775-81.

LEWIS, C., NEIDHART, S., HOLY, C., NORTH, R.A., BUELL, G. & SURPRENANT, A. (1995). Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. *Nature*, **377,** 432-5.

LI, J.Y., JAHN, R. & DAHLSTROM, A. (1996). Axonal transport and targeting of the t-SNAREs SNAP-25 and syntaxin 1 in the peripheral nervous system. *Eur J Cell Biol*, **70,** 12-22.

LIAN, L., WANG, Y., DRAZNIN, J., ESLIN, D., BENNETT, J.S., PONCZ, M., WU, D. & ABRAMS, C.S. (2005). The relative role of PLCbeta and PI3Kgamma in platelet activation. *Blood*, **106,** 110-7.

LIANG, Y., FOTIADIS, D., FILIPEK, S., SAPERSTEIN, D.A., PALCZEWSKI, K. & ENGEL, A. (2003). Organization of the G protein-coupled receptors rhodopsin and opsin in native membranes. *J Biol Chem*, **278,** 21655-62.

LINDEN, J. (2005). Adenosine in tissue protection and tissue regeneration. *Mol Pharmacol*, **67,** 1385-7.

LLORENTE, A., VAN DEURS, B., GARRED, O., EKER, P. & SANDVIG, K. (2000). Apical endocytosis of ricin in MDCK cells is regulated by the cyclooxygenase pathway. *J Cell Sci*, **113 (Pt 7),** 1213-21.

LOGOTHETIS, D.E., KURACHI, Y., GALPER, J., NEER, E.J. & CLAPHAM, D.E. (1987). The beta gamma subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. *Nature*, **325,** 321-6.

LONGENECKER, K.L., LEWIS, M.E., CHIKUMI, H., GUTKIND, J.S. & DEREWENDA, Z.S. (2001). Structure of the RGS-like domain from PDZ-RhoGEF: linking heterotrimeric g protein-coupled signaling to Rho GTPases. *Structure*, **9,** 559-69.

LU, J., PIERRON, A. & RAVID, K. (2003). An adenosine analogue, IB-MECA, down-regulates estrogen receptor alpha and suppresses human breast cancer cell proliferation. *Cancer Res*, **63,** 6413-23.

LU, Q., ATKISSON, M.S., JARVIS, S.E., FENG, Z.P., ZAMPONI, G.W. & DUNLAP, K. (2001). Syntaxin 1A supports voltage-dependent inhibition of alpha1B Ca2+ channels by Gbetagamma in chick sensory neurons. *J Neurosci*, **21,** 2949-57.

LUSTIG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc Natl Acad Sci U S A*, **90,** 5113-7.

LUTHARDT, J., BORVENDEG, S.J., SPERLAGH, B., POELCHEN, W., WIRKNER, K. & ILLES, P. (2003). P2Y(1) receptor activation inhibits NMDA receptor-channels in layer V pyramidal neurons of the rat prefrontal and parietal cortex. *Neurochem Int*, **42,** 161-72.

MADI, L., OCHAION, A., RATH-WOLFSON, L., BAR-YEHUDA, S., ERLANGER, A., OHANA, G., HARISH, A., MERIMSKI, O., BARER, F. & FISHMAN, P. (2004). The A3 adenosine receptor is highly expressed in tumor versus normal cells: potential target for tumor growth inhibition. *Clin Cancer Res*, **10,** 4472-9.

MAEMOTO, T., TADA, M., MIHARA, T., UEYAMA, N., MATSUOKA, H., HARADA, K., YAMAJI, T., SHIRAKAWA, K., KURODA, S., AKAHANE, A., IWASHITA, A., MATSUOKA, N. & MUTOH, S. (2004). Pharmacological characterization of FR194921, a new potent, selective, and orally active antagonist for central adenosine A1 receptors. *J Pharmacol Sci*, **96,** 42-52.

MAHAUT-SMITH, M.P., HUSSAIN, J.F. & MASON, M.J. (1999). Depolarization-evoked Ca2+ release in a non-excitable cell, the rat megakaryocyte. *J Physiol*, **515 (Pt 2),** 385-90.

MAIER, U., BABICH, A., MACREZ, N., LEOPOLDT, D., GIERSCHIK, P., ILLENBERGER, D. & NURNBERG, B. (2000). Gbeta 5gamma 2 is a highly selective activator of phospholipid-dependent enzymes. *J Biol Chem*, **275,** 13746-54.

MAMEDOVA, L.K., GAO, Z.G. & JACOBSON, K.A. (2006). Regulation of death and survival in astrocytes by ADP activating P2Y1 and P2Y12 receptors. *Biochem Pharmacol*, **72,** 1031-41.

MANES, S., ANA LACALLE, R., GOMEZ-MOUTON, C. & MARTINEZ, A.C. (2003). From rafts to crafts: membrane asymmetry in moving cells. *Trends Immunol*, **24,** 320-6.

MARGOLSKEE, R.F. (2002). Molecular mechanisms of bitter and sweet taste transduction. *J Biol Chem*, **277,** 1-4.

MARQUEZ, V.E., SIDDIQUI, M.A., EZZITOUNI, A., RUSS, P., WANG, J., WAGNER, R.W. & MATTEUCCI, M.D. (1996). Nucleosides with a twist. Can fixed forms of sugar ring pucker influence biological activity in nucleosides and oligonucleotides? *J Med Chem*, **39,** 3739-47.

MARTIN, P.L., WYSOCKI, R.J., JR., BARRETT, R.J., MAY, J.M. & LINDEN, J. (1996). Characterization of 8-(N-methylisopropyl)amino-N6-(5'-endohydroxy-endonorbornyl)-9-methyladenine (WRC-0571), a highly potent and selective, non-xanthine antagonist of A1 adenosine receptors. *J Pharmacol Exp Ther*, **276,** 490-9.

MASON, M.J. & MAHAUT-SMITH, M.P. (2001). Voltage-dependent Ca2+ release in rat megakaryocytes requires functional IP3 receptors. *J Physiol*, **533,** 175-83.

MATASI, J.J., CALDWELL, J.P., HAO, J., NEUSTADT, B., ARIK, L., FOSTER, C.J., LACHOWICZ, J. & TULSHIAN, D.B. (2005). The discovery and synthesis of novel adenosine receptor (A(2A)) antagonists. *Bioorg Med Chem Lett*, **15,** 1333-6.

MATEO, J., HARDEN, T.K. & BOYER, J.L. (1999). Functional expression of a cDNA encoding a human ecto-ATPase. *Br J Pharmacol*, **128,** 396-402.

McCUDDEN, C.R., HAINS, M.D., KIMPLE, R.J., SIDEROVSKI, D.P. & WILLARD, F.S. (2005). G-protein signaling: back to the future. *Cell Mol Life Sci*, **62,** 551-77.

McGARAUGHTY, S., COWART, M., JARVIS, M.F. & BERMAN, R.F. (2005). Anticonvulsant and antinociceptive actions of novel adenosine kinase inhibitors. *Curr Top Med Chem*, **5,** 43-58.

MOORE, C.A., MILANO, S.K. & BENOVIC, J.L. (2007). Regulation of Receptor Trafficking by GRKs and Arrestins. *Annu Rev Physiol*, **69,** 451-82.

MOORE, D., CHAMBERS, J., WALDVOGEL, H., FAULL, R. & EMSON, P. (2000). Regional and cellular distribution of the P2Y(1) purinergic receptor in the human brain: striking neuronal localisation. *J Comp Neurol*, **421,** 374-84.

MORAN-JIMENEZ, M.J. & MATUTE, C. (2000). Immunohistochemical localization of the P2Y(1) purinergic receptor in neurons and glial cells of the central nervous system. *Brain Res Mol Brain Res*, **78,** 50-8.

MORO, S., BACILIERI, M., FERRARI, C. & SPALLUTO, G. (2005). Autocorrelation of molecular electrostatic potential surface properties combined with partial least squares analysis as alternative attractive tool to generate ligand-based 3D-QSARs. *Curr Drug Discov Technol*, **2,** 13-21.

MORO, S., GAO, Z.G., JACOBSON, K.A. & SPALLUTO, G. (2006). Progress in the pursuit of therapeutic adenosine receptor antagonists. *Med Res Rev*, **26,** 131-59.

MORO, S., GUO, D., CAMAIONI, E., BOYER, J.L., HARDEN, T.K. & JACOBSON, K.A. (1998). Human P2Y1 receptor: molecular modeling and site-directed mutagenesis as tools to identify agonist and antagonist recognition sites. *J Med Chem*, **41,** 1456-66.

MULERO, J.J., YEUNG, G., NELKEN, S.T. & FORD, J.E. (1999). CD39-L4 is a secreted human apyrase, specific for the hydrolysis of nucleoside diphosphates. *J Biol Chem*, **274,** 20064-7.

MUNDELL, S.J. & BENOVIC, J.L. (2000). Selective regulation of endogenous G protein-coupled receptors by arrestins in HEK293 cells. *J Biol Chem*, **275,** 12900-8.

MUNDELL, S.J., JONES, M.L., HARDY, A.R., BARTON, J.F., BEAUCOURT, S.M., CONLEY, P.B. & POOLE, A.W. (2006a). Distinct roles for protein kinase C isoforms in regulating platelet purinergic receptor function. *Mol Pharmacol*, **70,** 1132-42.

MUNDELL, S.J., LUO, J., BENOVIC, J.L., CONLEY, P.B. & POOLE, A.W. (2006b). Distinct clathrin-coated pits sort different G protein-coupled receptor cargo. *Traffic*, **7,** 1420-31.

NANDANAN, E., CAMAIONI, E., JANG, S.Y., KIM, Y.C., CRISTALLI, G., HERDEWIJN, P., SECRIST, J.A., 3RD, TIWARI, K.N., MOHANRAM, A., HARDEN, T.K., BOYER, J.L. & JACOBSON, K.A. (1999). Structure-activity relationships of bisphosphate nucleotide derivatives as P2Y1 receptor antagonists and partial agonists. *J Med Chem*, **42,** 1625-38.

NANDANAN, E., JANG, S.Y., MORO, S., KIM, H.O., SIDDIQUI, M.A., RUSS, P., MARQUEZ, V.E., BUSSON, R., HERDEWIJN, P., HARDEN, T.K., BOYER, J.L. & JACOBSON, K.A. (2000). Synthesis, biological activity, and molecular modeling of ribose-modified deoxyadenosine bisphosphate analogues as P2Y(1) receptor ligands. *J Med Chem*, **43,** 829-42.

NELSON, G., CHANDRASHEKAR, J., HOON, M.A., FENG, L., ZHAO, G., RYBA, N.J. & ZUKER, C.S. (2002). An amino-acid taste receptor. *Nature*, **416,** 199-202.

NELSON, G., HOON, M.A., CHANDRASHEKAR, J., ZHANG, Y., RYBA, N.J. & ZUKER, C.S. (2001). Mammalian sweet taste receptors. *Cell*, **106,** 381-90.

NICHOLS, K.K., YERXA, B. & KELLERMAN, D.J. (2004). Diquafosol tetrasodium: a novel dry eye therapy. *Expert Opin Investig Drugs*, **13,** 47-54.

NIESWANDT, B. & WATSON, S.P. (2003). Platelet-collagen interaction: is GPVI the central receptor? *Blood*, **102,** 449-61.

NIITSU, Y., JAKUBOWSKI, J.A., SUGIDACHI, A. & ASAI, F. (2005). Pharmacology of CS-747 (prasugrel, LY640315), a novel, potent antiplatelet agent with in vivo P2Y12 receptor antagonist activity. *Semin Thromb Hemost*, **31,** 184-94.

NIMCHINSKY, E.A., HOF, P.R., JANSSEN, W.G., MORRISON, J.H. & SCHMAUSS, C. (1997). Expression of dopamine D3 receptor dimers and tetramers in brain and in transfected cells. *J Biol Chem*, **272,** 29229-37.

NURDEN, P., SAVI, P., HEILMANN, E., BIHOUR, C., HERBERT, J.M., MAFFRAND, J.P. & NURDEN, A. (1995). An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. *J Clin Invest*, **95,** 1612-22.

NUTTLE, L.C. & DUBYAK, G.R. (1994). Differential activation of cation channels and non-selective pores by macrophage P2z purinergic receptors expressed in Xenopus oocytes. *J Biol Chem*, **269,** 13988-96.

OAKLEY, R.H., LAPORTE, S.A., HOLT, J.A., BARAK, L.S. & CARON, M.G. (2001). Molecular determinants underlying the formation of stable intracellular G protein-coupled receptor-beta-arrestin complexes after receptor endocytosis*. *J Biol Chem*, **276,** 19452-60.

OFFERMANNS, S. (2006). Activation of platelet function through G protein-coupled receptors. *Circ Res*, **99,** 1293-304.

OH, P., MCINTOSH, D.P. & SCHNITZER, J.E. (1998). Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J Cell Biol*, **141,** 101-14.

OHTA, A. & SITKOVSKY, M. (2001). Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature*, **414,** 916-20.

OLIVIER, K.N., BENNETT, W.D., HOHNEKER, K.W., ZEMAN, K.L., EDWARDS, L.J., BOUCHER, R.C. & KNOWLES, M.R. (1996). Acute safety and effects on mucociliary clearance of aerosolized uridine 5'-triphosphate +/- amiloride in normal human adults. *Am J Respir Crit Care Med*, **154,** 217-23.

OSTROM, R.S., GREGORIAN, C. & INSEL, P.A. (2000). Cellular release of and response to ATP as key determinants of the set-point of signal transduction pathways. *J Biol Chem*, **275,** 11735-9.

OURY, C., KUIJPERS, M.J., TOTH-ZSAMBOKI, E., BONNEFOY, A., DANLOY, S., VREYS, I., FEIJGE, M.A., DE VOS, R., VERMYLEN, J., HEEMSKERK, J.W. & HOYLAERTS, M.F. (2003). Overexpression of the platelet P2X1 ion channel in transgenic mice generates a novel prothrombotic phenotype. *Blood*, **101,** 3969-76.

PAING, M.M., STUTTS, A.B., KOHOUT, T.A., LEFKOWITZ, R.J. & TREJO, J. (2002). beta - Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. *J Biol Chem*, **277,** 1292-300.

PALMER, R.K., BOYER, J.L., SCHACHTER, J.B., NICHOLAS, R.A. & HARDEN, T.K. (1998). Agonist action of adenosine triphosphates at the human P2Y1 receptor. *Mol Pharmacol*, **54,** 1118-23.

PARK, H.S. & HOURANI, S.M. (1999). Differential effects of adenine nucleotide analogues on shape change and aggregation induced by adnosine 5-diphosphate (ADP) in human platelets. *Br J Pharmacol*, **127,** 1359-66.

PARR, C.E., SULLIVAN, D.M., PARADISO, A.M., LAZAROWSKI, E.R., BURCH, L.H., OLSEN, J.C., ERB, L., WEISMAN, G.A., BOUCHER, R.C. & TURNER, J.T. (1994). Cloning and

expression of a human P2U nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc Natl Acad Sci U S A*, **91,** 13067.

PEAKMAN, M.C. & HILL, S.J. (1994). Adenosine A2B-receptor-mediated cyclic AMP accumulation in primary rat astrocytes. *Br J Pharmacol*, **111,** 191-8.

PENG, H., KUMARAVEL, G., YAO, G., SHA, L., WANG, J., VAN VLIJMEN, H., BOHNERT, T., HUANG, C., VU, C.B., ENSINGER, C.L., CHANG, H., ENGBER, T.M., WHALLEY, E.T. & PETTER, R.C. (2004). Novel bicyclic piperazine derivatives of triazolotriazine and triazolopyrimidines as highly potent and selective adenosine A2A receptor antagonists. *J Med Chem*, **47,** 6218-29.

PIERCE, K.L., PREMONT, R.T. & LEFKOWITZ, R.J. (2002). Seven-transmembrane receptors. *Nat Rev Mol Cell Biol*, **3,** 639-50.

PITCHER, J.A., FREEDMAN, N.J. & LEFKOWITZ, R.J. (1998). G protein-coupled receptor kinases. *Annu Rev Biochem*, **67,** 653-92.

PUTHENVEEDU, M.A. & VON ZASTROW, M. (2006). Cargo regulates clathrin-coated pit dynamics. *Cell*, **127,** 113-24.

QI, A.D., KENNEDY, C., HARDEN, T.K. & NICHOLAS, R.A. (2001). Differential coupling of the human P2Y(11) receptor to phospholipase C and adenylyl cyclase. *Br J Pharmacol*, **132,** 318-26.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol Rev*, **50,** 413-92.

RASSENDREN, F., BUELL, G.N., VIRGINIO, C., COLLO, G., NORTH, R.A. & SURPRENANT, A. (1997). The permeabilizing ATP receptor, P2X7. Cloning and expression of a human cDNA. *J Biol Chem*, **272,** 5482-6.

RAVI, R.G., KIM, H.S., SERVOS, J., ZIMMERMANN, H., LEE, K., MADDILETI, S., BOYER, J.L., HARDEN, T.K. & JACOBSON, K.A. (2002). Adenine nucleotide analogues locked in a Northern methanocarba conformation: enhanced stability and potency as P2Y(1) receptor agonists. *J Med Chem*, **45,** 2090-100.

RAY, K., KUNSCH, C., BONNER, L.M. & ROBISHAW, J.D. (1995). Isolation of cDNA clones encoding eight different human G protein gamma subunits, including three novel forms designated the gamma 4, gamma 10, and gamma 11 subunits. *J Biol Chem*, **270,** 21765-71.

RETTIG, J. & NEHER, E. (2002). Emerging roles of presynaptic proteins in Ca++-triggered exocytosis. *Science*, **298,** 781-5.

RHEE, S.G. (2001). Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem*, **70,** 281-312.

RHEE, S.G. & CHOI, K.D. (1992). Regulation of inositol phospholipid-specific phospholipase C isozymes. *J Biol Chem*, **267,** 12393-6.

RICKMAN, C. & DAVLETOV, B. (2003). Mechanism of calcium-independent synaptotagmin binding to target SNAREs. *J Biol Chem*, **278,** 5501-4.

ROBINSON, M.S. (2004). Adaptable adaptors for coated vesicles. *Trends Cell Biol*, **14,** 167-74.

RODRIGUES, R.J., ALMEIDA, T., RICHARDSON, P.J., OLIVEIRA, C.R. & CUNHA, R.A. (2005). Dual presynaptic control by ATP of glutamate release via facilitatory P2X1, P2X2/3, and P2X3 and inhibitory P2Y1, P2Y2, and/or P2Y4 receptors in the rat hippocampus. *J Neurosci*, **25,** 6286-95.

ROGERS, D.F. & BARNES, P.J. (2006). Treatment of airway mucus hypersecretion. *Ann Med*, **38,** 116-25.

ROLF, M.G., BREARLEY, C.A. & MAHAUT-SMITH, M.P. (2001). Platelet shape change evoked by selective activation of P2X1 purinoceptors with alpha,beta-methylene ATP. *Thromb Haemost*, **85,** 303-8.

ROLF, M.G. & MAHAUT-SMITH, M.P. (2002). Effects of enhanced P2X1 receptor Ca2+ influx on functional responses in human platelets. *Thromb Haemost*, **88,** 495-502.

ROSS, G.W., ABBOTT, R.D., PETROVITCH, H., MORENS, D.M., GRANDINETTI, A., TUNG, K.H., TANNER, C.M., MASAKI, K.H., BLANCHETTE, P.L., CURB, J.D., POPPER, J.S. & WHITE, L.R. (2000). Association of coffee and caffeine intake with the risk of Parkinson disease. *Jama*, **283,** 2674-9.

ROVATI, G.E., CAPRA, V. & NEUBIG, R.R. (2006). The Highly Conserved DRY Motif of Class A GPCRs: Beyond the Ground State. *Mol Pharmacol*.

RUGGERI, Z.M. (2002). Platelets in atherothrombosis. *Nat Med*, **8,** 1227-34.

RUGGERI, Z.M. (2003). Von Willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost*, **1,** 1335-42.

SALAHPOUR, A., ANGERS, S. & BOUVIER, M. (2000). Functional significance of oligomerization of G-protein-coupled receptors. *Trends Endocrinol Metab*, **11,** 163-8.

SANTIAGO-PEREZ, L.I., FLORES, R.V., SANTOS-BERRIOS, C., CHORNA, N.E., KRUGH, B., GARRAD, R.C., ERB, L., WEISMAN, G.A. & GONZALEZ, F.A. (2001). P2Y(2) nucleotide receptor signaling in human monocytic cells: activation, desensitization and coupling to mitogen-activated protein kinases. *J Cell Physiol*, **187,** 196-208.

SAUVONNET, N., DUJEANCOURT, A. & DAUTRY-VARSAT, A. (2005). Cortactin and dynamin are required for the clathrin-independent endocytosis of gammac cytokine receptor. *J Cell Biol*, **168,** 155-63.

SAWYNOK, J. (1998). Adenosine receptor activation and nociception. *Eur J Pharmacol*, **347,** 1-11.

SAWYNOK, J. & REID, A. (1997). Peripheral adenosine 5'-triphosphate enhances nociception in the formalin test via activation of a purinergic p2X receptor. *Eur J Pharmacol*, **330,** 115-21.

SCHACHTER, J.B. & HARDEN, T.K. (1997). An examination of deoxyadenosine 5'(alpha-thio)triphosphate as a ligand to define P2Y receptors and its selectivity as a low potency partial agonist of the P2Y1 receptor. *Br J Pharmacol*, **121,** 338-44.

SCHACHTER, J.B., LI, Q., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1996). Second messenger cascade specificity and pharmacological selectivity of the human P2Y1-purinoceptor. *Br J Pharmacol*, **118,** 167-73.

SCHEIBLER, P., PESIC, M., FRANKE, H., REINHARDT, R., WIRKNER, K., ILLES, P. & NORENBERG, W. (2004). P2X2 and P2Y1 immunofluorescence in rat neostriatal medium-spiny projection neurones and cholinergic interneurones is not linked to respective purinergic receptor function. *Br J Pharmacol*, **143,** 119-31.

SCHMIDT, C.J., THOMAS, T.C., LEVINE, M.A. & NEER, E.J. (1992). Specificity of G protein beta and gamma subunit interactions. *J Biol Chem*, **267,** 13807-10.

SCHULTE AM ESCH, J., 2ND, SEVIGNY, J., KACZMAREK, E., SIEGEL, J.B., IMAI, M., KOZIAK, K., BEAUDOIN, A.R. & ROBSON, S.C. (1999). Structural elements and limited proteolysis of CD39 influence ATP diphosphohydrolase activity. *Biochemistry*, **38,** 2248-58.

SHENG, M. & SALA, C. (2001). PDZ domains and the organization of supramolecular complexes. *Annu Rev Neurosci*, **24,** 1-29.

SHENOY, S.K., MCDONALD, P.H., KOHOUT, T.A. & LEFKOWITZ, R.J. (2001). Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science*, **294,** 1307-13.

SHIH, M., LIN, F., SCOTT, J.D., WANG, H.Y. & MALBON, C.C. (1999). Dynamic complexes of beta2-adrenergic receptors with protein kinases and phosphatases and the role of gravin. *J Biol Chem*, **274,** 1588-95.

SHIH, S.C., KATZMANN, D.J., SCHNELL, J.D., SUTANTO, M., EMR, S.D. & HICKE, L. (2002). Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. *Nat Cell Biol*, **4,** 389-93.

SHINOZAKI, Y., KOIZUMI, S., ISHIDA, S., SAWADA, J., OHNO, Y. & INOUE, K. (2005). Cytoprotection against oxidative stress-induced damage of astrocytes by extracellular ATP via P2Y1 receptors. *Glia*, **49,** 288-300.

SHNEYVAYS, V., LESHEM, D., ZINMAN, T., MAMEDOVA, L.K., JACOBSON, K.A. & SHAINBERG, A. (2005). Role of adenosine A1 and A3 receptors in regulation of cardiomyocyte homeostasis after mitochondrial respiratory chain injury. *Am J Physiol Heart Circ Physiol*, **288,** H2792-801.

SHNEYVAYS, V., ZINMAN, T. & SHAINBERG, A. (2004). Analysis of calcium responses mediated by the A3 adenosine receptor in cultured newborn rat cardiac myocytes. *Cell Calcium*, **36,** 387-96.

SIMON, J., WEBB, T.E., KING, B.F., BURNSTOCK, G. & BARNARD, E.A. (1995). Characterisation of a recombinant P2Y purinoceptor. *Eur J Pharmacol*, **291,** 281-9.

SIMONS, K. & TOOMRE, D. (2000). Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol*, **1,** 31-9.

SMITH, T.M. & KIRLEY, T.L. (1998). Cloning, sequencing, and expression of a human brain ecto-apyrase related to both the ecto-ATPases and CD39 ecto-apyrases1. *Biochim Biophys Acta*, **1386,** 65-78.

SMITH, T.M. & KIRLEY, T.L. (1999). Glycosylation is essential for functional expression of a human brain ecto-apyrase. *Biochemistry*, **38,** 1509-16.

SOLLE, M., LABASI, J., PERREGAUX, D.G., STAM, E., PETRUSHOVA, N., KOLLER, B.H., GRIFFITHS, R.J. & GABEL, C.A. (2001). Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem*, **276,** 125-32.

SOMERS, G.R., HAMMET, F.M., TRUTE, L., SOUTHEY, M.C. & VENTER, D.J. (1998). Expression of the P2Y6 purinergic receptor in human T cells infiltrating inflammatory bowel disease. *Lab Invest*, **78,** 1375-83.

SORENSEN, C.E. & 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,** 32925-32.

SOUSLOVA, V., CESARE, P., DING, Y., AKOPIAN, A.N., STANFA, L., SUZUKI, R., CARPENTER, K., DICKENSON, A., BOYCE, S., HILL, R., NEBENUIS-OOSTHUIZEN, D., SMITH, A.J., KIDD, E.J. & WOOD, J.N. (2000). Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X3 receptors. *Nature*, **407,** 1015-7.

SPYCHALA, J., ZIMMERMANN, A.G. & MITCHELL, B.S. (1999). Tissue-specific regulation of the ecto-5'-nucleotidase promoter. Role of the camp response element site in mediating repression by the upstream regulatory region. *J Biol Chem*, **274,** 22705-12.

SROMEK, S.M. & HARDEN, T.K. (1998). Agonist-induced internalization of the P2Y2 receptor. *Mol Pharmacol*, **54,** 485-94.

STEWART, M., STEINIG, A.G., MA, C., SONG, J.P., MCKIBBEN, B., CASTELHANO, A.L. & MACLENNAN, S.J. (2004). [3H]OSIP339391, a selective, novel, and high affinity antagonist radioligand for adenosine A2B receptors. *Biochem Pharmacol*, **68,** 305-12.

SUTHERLAND, E.W. & RALL, T.W. (1958). Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J Biol Chem*, **232,** 1077-91.

SZENTMIKLOSI, A.J., UJFALUSI, A., CSEPPENTO, A., NOSZTRAY, K., KOVACS, P. & SZABO, J.Z. (1995). Adenosine receptors mediate both contractile and relaxant effects of adenosine in main pulmonary artery of guinea pigs. *Naunyn Schmiedebergs Arch Pharmacol*, **351,** 417-25.

TANG, W.J. & GILMAN, A.G. (1991). Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. *Science*, **254,** 1500-3.

TOKUYAMA, Y., HARA, M., JONES, E.M., FAN, Z. & BELL, G.I. (1995). Cloning of rat and mouse P2Y purinoceptors. *Biochem Biophys Res Commun*, **211,** 211-8.

TRACEY, W.R., MAGEE, W., MASAMUNE, H., OLEYNEK, J.J. & HILL, R.J. (1998). Selective activation of adenosine A3 receptors with N6-(3-chlorobenzyl)-5'-N-methylcarboxamidoadenosine (CB-MECA) provides cardioprotection via KATP channel activation. *Cardiovasc Res*, **40,** 138-45.

TRAN, T.M., FRIEDMAN, J., QUNAIBI, E., BAAMEUR, F., MOORE, R.H. & CLARK, R.B. (2004). Characterization of agonist stimulation of cAMP-dependent protein kinase and G protein-coupled receptor kinase phosphorylation of the beta2-adrenergic receptor using phosphoserine-specific antibodies. *Mol Pharmacol*, **65,** 196-206.

TSUDA, M., UENO, S. & INOUE, K. (1999). Evidence for the involvement of spinal endogenous ATP and P2X receptors in nociceptive responses caused by formalin and capsaicin in mice. *Br J Pharmacol*, **128,** 1497-504.

TULAPURKAR, M.E., ZUNDORF, G. & REISER, G. (2006). Internalization and desensitization of a green fluorescent protein-tagged P2Y nucleotide receptor are differently controlled by inhibition of calmodulin-dependent protein kinase II. *J Neurochem*, **96,** 624-34.

VALERA, S., HUSSY, N., EVANS, R.J., ADAMI, N., NORTH, R.A., SURPRENANT, A. & BUELL, G. (1994). A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. *Nature*, **371,** 516-9.

VAN GIEZEN, J.J. & HUMPHRIES, R.G. (2005). Preclinical and clinical studies with selective reversible direct P2Y12 antagonists. *Semin Thromb Hemost*, **31,** 195-204.

VASCONCELOS, E.G., FERREIRA, S.T., CARVALHO, T.M., SOUZA, W., KETTLUN, A.M., MANCILLA, M., VALENZUELA, M.A. & VERJOVSKI-ALMEIDA, S. (1996). Partial purification and immunohistochemical localization of ATP diphosphohydrolase from

Schistosoma mansoni. Immunological cross-reactivities with potato apyrase and Toxoplasma gondii nucleoside triphosphate hydrolase. *J Biol Chem*, **271,** 22139-45.

VOLPINI, R., COSTANZI, S., VITTORI, S., CRISTALLI, G. & KLOTZ, K.N. (2003). Medicinal chemistry and pharmacology of A2B adenosine receptors. *Curr Top Med Chem*, **3,** 427-43.

WALDO, G.L., CORBITT, J., BOYER, J.L., RAVI, G., KIM, H.S., JI, X.D., LACY, J., JACOBSON, K.A. & HARDEN, T.K. (2002). Quantitation of the P2Y(1) receptor with a high affinity radiolabeled antagonist. *Mol Pharmacol*, **62,** 1249-57.

WALDO, G.L. & HARDEN, T.K. (2004). Agonist binding and Gq-stimulating activities of the purified human P2Y1 receptor. *Mol Pharmacol*, **65,** 426-36.

WANG, L.K. & SHUMAN, S. (2002). Mutational analysis defines the 5'-kinase and 3'-phosphatase active sites of T4 polynucleotide kinase. *Nucleic Acids Res*, **30,** 1073-80.

WANG, T.F. & GUIDOTTI, G. (1998). Golgi localization and functional expression of human uridine diphosphatase. *J Biol Chem*, **273,** 11392-9.

WAUGH, M.G., CHALLISS, R.A., BERSTEIN, G., NAHORSKI, S.R. & TOBIN, A.B. (1999). Agonist-induced desensitization and phosphorylation of m1-muscarinic receptors. *Biochem J*, **338 (Pt 1),** 175-83.

WEBB, T.E., SIMON, J., KRISHEK, B.J., BATESON, A.N., SMART, T.G., KING, B.F., BURNSTOCK, G. & BARNARD, E.A. (1993). Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett*, **324,** 219-25.

WEISS, S.M., BENWELL, K., CLIFFE, I.A., GILLESPIE, R.J., KNIGHT, A.R., LERPINIERE, J., MISRA, A., PRATT, R.M., REVELL, D., UPTON, R. & DOURISH, C.T. (2003). Discovery of nonxanthine adenosine A2A receptor antagonists for the treatment of Parkinson's disease. *Neurology*, **61,** S101-6.

WILCOX, C.S., WELCH, W.J., SCHREINER, G.F. & BELARDINELLI, L. (1999). Natriuretic and diuretic actions of a highly selective adenosine A1 receptor antagonist. *J Am Soc Nephrol*, **10,** 714-20.

WOLFF, S.C., QI, A.D., HARDEN, T.K. & NICHOLAS, R.A. (2005). Polarized expression of human P2Y receptors in epithelial cells from kidney, lung, and colon. *Am J Physiol Cell Physiol*, **288,** C624-32.

XU, K., BASTIA, E. & SCHWARZSCHILD, M. (2005). Therapeutic potential of adenosine A(2A) receptor antagonists in Parkinson's disease. *Pharmacol Ther*, **105,** 267-310.

YAMAUCHI, J., NAGAO, M., KAZIRO, Y. & ITOH, H. (1997). Activation of p38 mitogen-activated protein kinase by signaling through G protein-coupled receptors. Involvement of Gbetagamma and Galphaq/11 subunits. *J Biol Chem*, **272,** 27771-7.

YANG, J., WU, J., JIANG, H., MORTENSEN, R., AUSTIN, S., MANNING, D.R., WOULFE, D. & BRASS, L.F. (2002). Signaling through Gi family members in platelets. Redundancy and specificity in the regulation of adenylyl cyclase and other effectors. *J Biol Chem*, **277,** 46035-42.

YOSHIOKA, K., HOSODA, R., KURODA, Y. & NAKATA, H. (2002). Hetero-oligomerization of adenosine A1 receptors with P2Y1 receptors in rat brains. *FEBS Lett*, **531,** 299-303.

ZABLOCKI, J.A., WU, L., SHRYOCK, J. & BELARDINELLI, L. (2004). Partial A(1) adenosine receptor agonists from a molecular perspective and their potential use as chronic ventricular rate control agents during atrial fibrillation (AF). *Curr Top Med Chem*, **4,** 839-54.

ZAMBROWICZ, B.P., TURNER, C.A. & SANDS, A.T. (2003). Predicting drug efficacy: knockouts model pipeline drugs of the pharmaceutical industry. *Curr Opin Pharmacol*, **3,** 563-70.

ZHENG, J.H. & CHEN, J. (2000). Modulatory roles of the adenosine triphosphate P2x-purinoceptor in generation of the persistent nociception induced by subcutaneous bee venom injection in the conscious rat. *Neurosci Lett*, **278,** 41-4.

ZHOU, Q.Y., LI, C., OLAH, M.E., JOHNSON, R.A., STILES, G.L. & CIVELLI, O. (1992). Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proc Natl Acad Sci U S A*, **89,** 7432-6.

ZHU, Y. & KIMELBERG, H.K. (2004). Cellular expression of P2Y and beta-AR receptor mRNAs and proteins in freshly isolated astrocytes and tissue sections from the CA1 region of P8-12 rat hippocampus. *Brain Res Dev Brain Res*, **148,** 77-87.

ZHU, Y. & KIMELBERG, H.K. (2001). Developmental expression of metabotropic P2Y(1) and P2Y(2) receptors in freshly isolated astrocytes from rat hippocampus. *J Neurochem*, **77,** 530-41.

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

ZIMMERMANN, H. & BRAUN, N. (1999). Ecto-nucleotidases--molecular structures, catalytic properties, and functional roles in the nervous system. *Prog Brain Res*, **120,** 371-85.