ROLE OF THE $P2Y_1$ RECEPTOR IN PLATELET ACTIVATION

Tasha Nalywajko Blatt

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Approved by:

Robert Nicholas

Kendall Harden

Wolfgang Bergmeier

Nigel Mackman

Leslie Parise

Bryan Roth

ABSTRACT

Tasha Nalywajko Blatt: Role of the $P2Y_1$ receptor in platelet activation
(Under the direction of Robert Nicholas)

Understanding and manipulating thrombosis and blood hemostasis is critical for the effective treatment of patients at risk for heart attack and stroke. ADP is an important modulator of platelet function and vascular tone that acts by binding to and activating the $P2Y_1$ and $P2Y_{12}$ receptors. Deletion or inhibition of either receptor results in nearly a total loss of ADP-promoted aggregation. Interestingly, the $P2Y_1$ receptor desensitizes extremely rapidly, with a half-life of approximately 18 sec, whereas the $P2Y_{12}$ receptor continuously signals minutes after initial activation. We hypothesized that the rapid desensitization of the $P2Y_1$ receptor in platelets prevents excess thrombosis and unwanted aggregation at lower concentrations of ADP. We have used *ex vivo* platelet activation experiments to demonstrate that the observed desensitization is specific to the $P2Y_1$ receptor compared to other Gq-coupled receptors in platelets and is recapitulated in mouse platelets. We focused on serine and threonine residues on the C-terminus because of the involvement of various Ser and Thr phosphorylation on the regulation of the $P2Y_1$ receptor in cell culture systems. Using an optimized viral transduction model to introduce variants of the $P2Y_1$ receptor into bone marrow, we show preliminary data suggesting that mutating multiple serine and threonine residues in the C-terminus of the $P2Y_1$ receptor ("340-0P") results in prolonged activation of platelets in the absence of the $P2Y_{12}$ receptor pathway, thus eliminating the observed desensitization in the wild type $P2Y_1$ receptor. Furthermore, creation of a knock-in mouse for the 340-0P variant of the $P2Y_1$ receptor revealed a loss of

desensitization in platelets upon ADP stimulation in 340-0P heterozygous mice. These data suggest that the loss of Ser and Thr residues on the C-terminus of the $P2Y_1$ receptor nullifies the observed desensitization and provides insights regarding the physiological relevance of this process in platelets.

To my husband, who never gave up on me through the good and the bad.
To my son, who had to share Mommy with this project.
Thank you both for your love and laughter.

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LIST OF ABBREVIATIONS

5'UTR – Five prime untranslated region

aa – Amino acid

AAC – ATP-ADP carrier

AC – Adenylate cyclase

ADP – Adenosine diphosphate

AMP – Adenosine monophosphate

AP2 – Adaptor protein 2

Arr – Arrestin

ATP – Adenosine triphosphate

β2AR – Beta-adrenergic receptor 2

BSA – Bovine serum albumin

CAD – Coronary artery disease

cAMP – Cyclic adenosine monophosphate

CD – Cluster of differentiation

cGMP – Cyclic guanidine monophosphate

CML – Chronic myeloid leukemia

CREB – Cyclic AMP response element-binding protein

CTX – Cholera toxin

CVD – Cardiovascular disease

DAG – Diacylglycerol

DMS – Demarcation membrane system

DNA – Deoxyribonucleic acid

DTS – Dense tubular system

DTT – Dithiothreitol

ECM – Extracellular matrix

EGF – Epithelial growth factor

eNOS – Epithelial nitric oxide synthase

ER – Endoplasmic reticulum

ERK – Extracellular signal-regulated kinase

F – Factor

FBS – Fetal bovine serum

G protein - Guanosine nucleotide-binding proteins

GAP – GTPase activating protein

GDP – Guanosine diphosphate

GEF – Guanine nucleotide exchange factor

GFP – Green fluorescent protein

GIRK – G protein inward rectifying potassium

GP - Glycoprotein

GPCR – G protein-coupled receptor

GRK – G protein receptor kinase

GTP – Guanosine triphosphate

HA – Hemagglutinin

HTS – High throughput screen

IL - Interleukin

IP_3 – Inositol 1,4,5-trisphosphate

LDL – Low density lipoprotein

LH – Lutenizing hormone

LPA – Lysophosphatidic acid

MAPK – Mitogen-activated protein kinase

MI – Myocardial infarction

NCS – Newborn calf serum

NO – Nitric oxide

NSAID – Non-steroidal anti-inflammatory drug

OCS – Open canalicular system

PAR – Protease-activated receptor

PBS – Phosphate buffered saline

PDGF – Platelet-derived growth factor

PGH_2 – Prostaglandin H2

PGI_2 – Prostacyclin

PGIS – Prostacyclin synthase

PI3K – Phosphatidylinositide 3-kinase

PIP_2 – Phosphatidylinositol 4,5-bisphosphate

PKA – Protein kinase A

PKC – Protein kinase C

PLC – Phospholipase C

P/S – Penicillin/streptomycin

PTX – Pertussis toxin

RGS – Regulator of G protein signaling

RIAM – Rap1-GTP-interacting adaptor molecule

RNA – Ribonucleic acid

SCF – Stem cell factor

sGC – Soluble guanylyl cyclase

SNP – Single nucleotide polymorphism

TF – Tissue factor

TM – Transmembrane region

TNFα – Tumor necrosis factor alpha

Tpo – Thrombopoietin

UDP – Uridine diphosphate

UTP – Uridine triphosphate

VEGF – growth factor

vWF – von Willebrand Factor

WT – Wild type

CHAPTER 1: A LITERARY REVIEW OF G-PROTEIN COUPLED RECEPTORS, NUCLEOTIDE SIGNALING AND PLATELET BIOLOGY

G-protein coupled receptors

Introduction

G-protein coupled receptors (GPCRs) are members of a superfamily of membrane-bound proteins that comprise approximately 4% of the protein coding sequence of human genome (Bjarnadóttir et al., 2006). Their primary function is to transduce extracellular signals to intracellular activity through various effectors. The nomenclature of GPCRs is derived from their coupling to heterotrimeric guanosine nucleotide-binding proteins (G proteins) within the cell, which are responsible for initiating distinct intracellular signaling pathways upon GPCR activation. The GPCR field was born out of the discovery of an entity that was able to transmit a signal from an extracellular hormone to the intracellular activation of liver phosphorylase through an intermediary factor (Berthet et al., 1957), which was later identified as cyclic AMP (cAMP) (Sutherland and Rall, 1958). Since their discovery, GPCRs have been classified, cloned, and crystallized in what is now a broad field of study that transcends many areas of basic and clinical research.

GPCR structure and function

Structure

GPCRs are defined structurally as having an extracellular N-terminus, 7 transmembrane domains, and an intracellular C-terminus. Between the transmembrane domains are three

extracellular loops and three intracellular loops of various lengths that contribute to receptor function. The majority of GPCRs fall into one of five families: i) the *Rhodopsin* family, which is activated by small molecules and constitutes the majority of GPCRs (including all olfactory receptors); ii) the *Secretin* and iii) *Adhesion* families, which are activated by larger peptide hormones and extracellular matrix (ECM) proteins, respectively; iv) the *Glutamate* family, which has a large N-terminus that binds to the endogenous ligand; and v) the *Frizzled/Taste2* family (Lagerström and Schiöth, 2008). The first crystal structure of a GPCR was of rhodopsin (Palczewski et al., 2000); since then more than 100 crystal structures have been published, including the first structure of a GPCR in an active conformation (Rasmussen et al., 2011). Despite the extent of this superfamily, there are still over 120 "orphan receptors" that were identified from the Human Genome project at the turn of the millennium but whose ligand and function remain unknown (Tang et al., 2012).

Canonical function

When the GPCR is inactive, nearby complexes of heterotrimeric G proteins comprising a Gα, Gβ, and Gγ subunit are at a resting state. The Gα subunit interacts with the GPCR as well as with the Gβ and Gγ subunits, the latter two of which form a stable complex and are not observed separately. To allow for localization of the G proteins to the plasma membrane, the Gα subunits are either myristoylated or palmitoylated at the N-terminus, and the Gγ subunit is prenylated at the C-terminus (Wedegaertner et al., 1995); the Gβ subunit does not require lipidation because of its strong affinity to the Gγ subunit.

Canonical GPCR function occurs by binding an endogenous ligand to the orthosteric site on the extracellular surface of the receptor, which causes a conformational change in the receptor structure. This change is sufficient to allow the receptor to serve as a guanine nucleotide

exchange factor (GEF), which promotes exchange of GDP for GTP in the Gα subunit of the heterotrimeric G protein complex. This nucleotide exchange allows both the Gα and Gβγ subunits to dissociate from the receptor. When GTP-bound, the Gα subunit activates downstream effectors and initiates a specific signaling cascade based on the subunit type, which will be discussed later, and the cell type. The Gα subunit has intrinsic GTPase activity, and therefore catalyzes the hydrolysis of GTP to GDP by reducing the energy required for hydrolysis; this step is aided by regulators of G-protein signaling (RGS), which act as GTPase-activating proteins (GAPs) to increase the rate of hydrolysis (De Vries et al., 1995; Druey et al., 1996). The Gα subunit then rebinds to Gβγ subunits, and the complex reassociates with the GPCR when the ligand is released from the extracellular binding pocket, thus returning the system to its original inactive state. This cycle of activation is summarized in Figure 1-1.

Heterotrimeric G protein signaling

Members of the GPCR superfamily are primarily characterized based on the Gα subunit of the heterotrimeric G protein to which they couple. There are 4 primary classes of Gα subunits based on their sequence homology comprising 20 different proteins. The canonical downstream effectors of these four families are illustrated in Figure 1-2. Additionally, there are 5 Gβ subunits and 12 Gγ subunits that have several functions outside of complexing with Gα.

Gαs and Gαi

The Gαs and Gαi families act on the same target protein, albeit in an opposing fashion. The Gαs family is comprised of $G\alpha_s$ and $G\alpha_{olf}$. Activation of $G\alpha_s$ increases the activity of adenylyl cyclase (AC), which converts ATP to cAMP. Increased cAMP levels result in the activation of protein kinase A (PKA); some of the downstream effectors of PKA include the activation of the transcription factors nuclear factor kappa B (NFκB) (Zhong et al., 1997) and

cyclic AMP response element-binding protein (CREB) (Parker et al., 1996) to influence transcriptional changes in the cell, glycolytic enzymes in glucose production, and ion channels to increase permeability. The $G\alpha_{olf}$ subunit has high homology to $G\alpha_s$ and activates AC in a similar manner (Jones and Reed, 1989), but its expression is limited to olfactory sensory neurons, where it couples to olfactory GPCRs.

It was discovered that cholera toxin (CTX) acts by ADP-ribosylating $G\alpha_s$, which inhibits the GTPase catalytic activity of the protein and prevents the GTP from being hydrolyzed (Moss and Vaughan, 1979). This in turn decouples the heterotrimeric G protein complex from GPCR regulation and results in sustained intracellular cAMP levels.

The $G\alpha_i$ family includes $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_z$, and $G\alpha_t$. The majority of the proteins in this class act by inhibiting AC activity, which results in a reduction of cAMP levels. The $G\alpha_i$ isoforms are ADP-ribosylated by pertussis toxin (PTX) in a manner similar to that of cholera toxin with $G\alpha_s$. However, ADP-ribosylation of $G\alpha_i$ prevents the interaction of the subunit with the GPCR, thus preventing GEF-mediated GDP-GTP exchange and blocking activation of the pathway, which results in increased levels of intracellular cAMP (Burns, 1988). Interestingly, $G\alpha_z$ is able to inhibit AC but is insensitive to PTX (Wong et al., 1992). Although it shares the most homology with other members of the $G\alpha_i$ family, $G\alpha_t$ couples exclusively to rhodopsin and activates phosphodiesterase 6 (PDE6), which converts cyclic GMP (cGMP) to 5'-GMP to regulate Na^+/Ca^{++} channels and hyperpolarize of the cell (Fung et al., 1981). This pathway allows for the transduction of light signals to the brain.

Gαq

The $G\alpha_q$ family includes $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$. Upon activation, these proteins activate phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2)

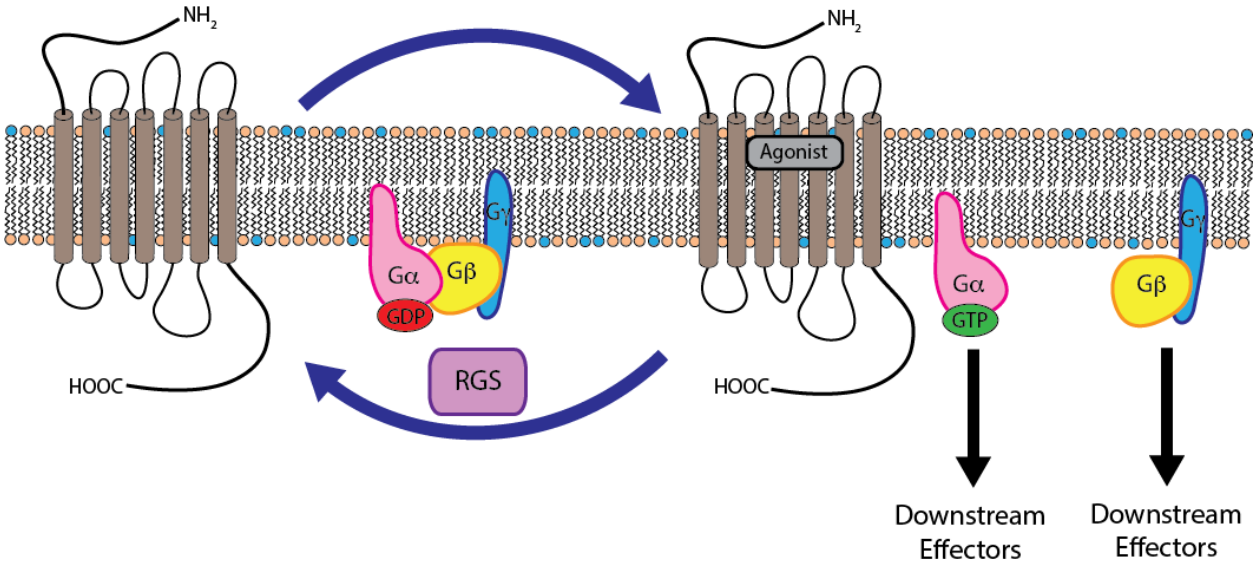

Figure 1-1. Canonical activation and inactivation of GPCR signaling.

Binding of an agonist causes the GPCR to change conformation and promote exchange of GTP for GDP on the associated Gα subunit. This causes separation from Gβγ, which allows for interaction with downstream effectors for both Gα and Gβγ. RGS proteins act as GAPs for the hydrolysis of the GTP bound to Gα; this promotes reassociation of the heterotrimeric G protein complex with the inactive GPCR.

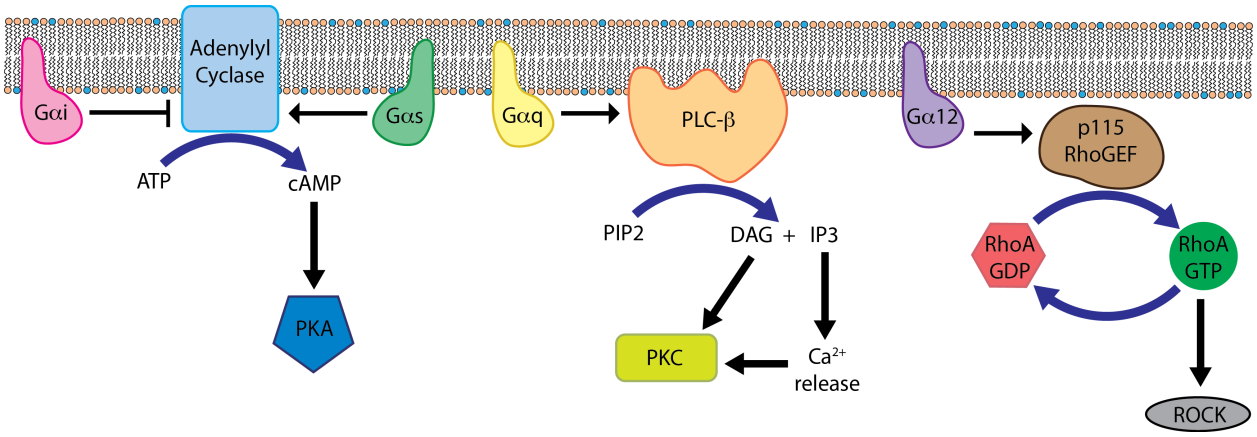

Figure 1-2. Traditional downstream effectors of various Gα subunits.

into diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG is a second messenger that activates protein kinase C (PKC), whereas IP_3 binds to IP_3 receptors in the endoplasmic or sarcoplasmic reticulum and induces the release of Ca^{2+} into the cytoplasm of the cell. PKC activation leads to modulation of several downstream effectors, e.g. increased extracellular-signal-regulated kinase (ERK) activity and β-catenin degradation (Druey et al., 1996; Gwak et al., 2006). Interestingly, several PLC isoforms have been shown to have GAP activity on $G\alpha_q$, thus serving as negative feedback, preventing aberrant activation, and providing exquisite control of PLC activation. More recent studies have discovered $G\alpha_q$ is capable of activating RhoGEFs such as p63, Kalirin and Trio (Lutz et al., 2005; Schmidt and Debant, 2014).

Gα12/13

The $G\alpha_{12/13}$ subunits signal through several RhoGEFs, including p115-RhoGEF and PDZ-RhoGEF, to activate the small G protein RhoA and influence cytoskeletal changes within the cell (Fukuhara et al., 1999; Hart et al., 1998). Many GPCRs that couple to $G\alpha_{12/13}$ also couple to other Gα subunits (Riobo and Manning, 2005), suggesting that these Gα subunits are more promiscuous in their preferred coupling and aid in tissue-specific signaling.

Gβγ

Although originally thought to be secondary to Gα protein signaling, the Gβγ dimer activates distinct downstream pathways independent of the Gα subunit. The most well characterized pathway is activation of inward-rectifier potassium channels (GIRKs), which are found primarily in the nervous system and heart, to cause hyperpolarization of the cell. The Gβγ complex has also been shown to recruit G-protein receptor kinase 2 (GRK2) to the plasma membrane (Li et al., 2003), and indirectly activate the mitogen-activated protein kinase (MAPK) pathway via Src family kinases (Luttrell et al., 1996). Gβγ is also capable of activation multiple

PLC-β family members (Camps 1992), though the affinity for PLC-β is lower for Gβγ compared to $G\alpha_q$.

Taken together, the multiple combinations of Gα and Gβγ proteins with the hundreds of GPCRs and tissue-specific downstream effectors result in nearly limitless possibilities and fine-tuning of outside-in signaling.

GPCR regulation

Receptor desensitization

One of the most rapid forms of regulation of GPCRs is phosphorylation of the receptor. Upon ligand binding and G-protein dissociation, many GPCRs are phosphorylated on their intracellular regions by G-protein receptor kinases (GRKs). These phosphorylated residues are binding sites for arrestins (described below), which prevents reassociation and subsequent reactivation of the G-protein pathway. This phenomenon referred to as homologous desensitization, which is defined by the inability of the receptor to signal to the downstream G proteins despite the continued presence of the receptor agonist. These GRKs are serine/threonine kinases that primarily phosphorylate target residues on the C-termini of the activated GPCR; an exception to this rule is that receptors with large 3rd intracellular loops, e.g. muscarinic receptors, become phosphorylated in the loop and not the C-terminus. The first GRKs were described as phosphorylating rhodopsin (GRK1) and the β_2-adrenergic receptor (GRK2) only after agonist stimulation (Benovic et al., 1986; Shichi and Somers, 1978). Since then, a total of 7 GRKs have been identified, with GRK2, GRK3, GRK5, and GRK6 as the predominant kinases expressed in the majority of tissues. GRK1 and GRK7 are solely expressed in regions of the eye.

Aside from their activities as second messengers and downstream effectors of Gα signaling, PKA and PKC have been shown to phosphorylate GPCRs on intracellular loops and

C-termini as a form of negative feedback (Benovic et al., 1985; Raymond, 1991). This differs from GRK-mediated desensitization in that other receptors, i.e. those that were not activated, can display desensitization to their cognate agonists, which is defined as heterologous desensitization.

Arrestin binding

Upon phosphorylation, one of two events can occur: the phosphates can be removed by phosphatases to allow the receptor to regain its signaling capabilities, or regulatory proteins called arrestins can bind to the phosphorylated residues and serve as a physical disruptor to the binding motif of the GPCR, preventing any interaction with the heterotrimeric G-protein complex. Arrestin 1 (Arr1), also known as visual arrestin, is expressed in the eye and binds to the phosphorylated C-terminal residues on rhodopsin, whereas β-arrestin 1 and β-arrestin 2 (Arr2 and Arr3, respectively) are expressed ubiquitously throughout the organism. Arr2 and Arr3 bind to many phosphorylated GPCRs in a sequence non-specific manner; these proteins interact with several intracellular regions of the GPCR (provided they are phosphorylated), including the third intracellular loop (Dohlman et al., 1987). Furthermore, binding of the β-arrestins is dependent on the phosphorylation state of the GPCR, suggesting that this regulation occurs only when the GPCR is activated (Vilardaga et al., 2003).

Receptor internalization

Aside from the rapid regulation of desensitization of GPCRs, another common and more elaborate form of regulation is receptor internalization. Internalization can be independent of desensitization and involves the physical removal of the GPCR from the plasma membrane surface into the cell via a process known as endocytosis. This relocation prevents activation of those GPCRs by ligands on the surface; however, some studies suggest that activated signaling

cascades can continue after endocytosis (Calebiro et al., 2010; Kotowski et al., 2011; Wehbi et al., 2013). There are two common types of endocytosis: clathrin-dependent and clathrin-independent, each of which is described below.

Clathrin-dependent internalization

Clathrin is a heterodimeric protein consisting of a heavy chain (approximately 180 kDa) and a light chain (30-40 kDa), the latter of which associates with the C-terminal region of the heavy chain (Fotin et al., 2004). The basic functional clathrin unit is comprised of a trimer of dimers, in which the three heavy chains associate at their C-termini (residues 1488-1587) (Blank and Brodsky, 1986) and form a "triskelion" shape. These trimers are the basis for the lattice structure that forms clathrin-coated pits, which are the precursor to an endocytic vesicle.

Also required for clathrin-mediated endocytosis is adaptor protein 2 (AP2), a tetramer with 4 unique subunits (α, α2, β2 and μ2). This protein serves as a scaffold between clathrin and targeted proteins in the plasma membrane, as clathrin itself is unable to associate with the lipid bilayer. The β2 subunit contains a specific dileucine motif (LLNLD) that can bind to the N-terminus of the clathrin heavy chain (ter Haar et al., 2000). Various regions of the other AP2 subunits are able to bind to the intracellular domains of membrane-associated proteins, thus linking the target proteins with clathrin. As more AP2 binds to the target proteins, there is an increase in the size of the clathrin lattice, which stabilizes the membrane into a rounded pit. As a "neck" is created at the invagination of the vesicle, the GTPase dynamin is recruited to the site (van der Bliek et al., 1993) and catalyzes the "pinching" of the invaginated vesicle to separate it from the plasma membrane (Zhang et al., 1996). Once the vesicle is separated, the clathrin coating destabilizes and dissociates from the lipid bilayer of the vesicle, which allows the vesicle to fuse with early endosomes.

Although the binding of Arr2 and Arr3 to the phosphorylated GPCR serves to temporary block signaling, these proteins bind both clathrin and AP2 to facilitate clathrin-dependent internalization of receptors. Upon binding of β-arrestins to the GPCR, the C-terminus of β-arrestin becomes exposed (Gurevich and Gurevich, 2006) and binds to clathrin through a clathrin binding motif [LIEΦ(D/E)] (Goodman et al., 1996). More importantly, it was shown that Arr2 and Arr3 bind to the β2 subunit of AP2 and serve as a scaffold between the GPCR and the AP2-clathrin complex (Laporte et al., 1999). Thus, β-arrestins are play a central role in clathrin-mediated endocytosis of GPCRs.

Clathrin-independent internalization

Clathrin-independent internalization of plasma membrane proteins occurs through dense hydrophobic regions within the membrane known as lipid rafts. These rafts are enriched with cholesterol and sphingolipids and are considered to be less fluid than the rest of the plasma membrane. A subset of lipid rafts known as caveolae are 50-80 nm invaginations that are intracellularly surrounded by caveolin. Endocytosis of caveolae is stimulated by both Src and PKC-α activity (Sharma et al., 2004) as well as actin polymerization and recruitment of dynamin (Pelkmans et al., 2002). Upon internalization, these caveolae can be sorted into either pre-early endosomes known as caveosomes, which are unique to caveolae (Pelkmans et al., 2001), or directly to early endosomes.

Another common form of internalization is macropinocytosis. This dynamin-independent process is driven by Rho GTPase activation and reorganization of the actin cytoskeleton to form protrusions. Macropinocytosis is both non-specific and non-absorptive, as it engulfs an area of extracellular fluid to internalize free-floating particles and nutrients. Once internalized, these vesicles enter into the endosome pathway, where they fuse with lysosomes to metabolize the

extracellular particles. As this process is less organized, macropinocytosis uses very little energy. However, pathogens such as Ebola virus (Shimojima et al., 2006) and *Salmonellae* bacteria (Alpuche-Aranda et al., 1994) have taken advantage of this uptake mechanism to infect target cells.

Receptor Trafficking

Upon fusion with early endosomes, GPCRs are subjected to one of two fates: recycling to the plasma membrane (resensitization) or degradation by either lysosomes or proteasomes (downregulation). These fates are influenced by the C-terminal tail region of the GPCRs and their capacity to interact with β-arrestins. Class A GPCRs have a less stable interaction with β-arrestins and are generally recycled back to the surface; however, class B GPCRs have a stronger association with β-arrestins and remain in the endosomes (Anborgh et al., 2000).

Other protein modifications and interactions also impact the fate of internalized GPCRs. There exist several recycling sequences in the C-terminal tail of GPCRs to promote their return to the plasma membrane. The most common of these sequences is a 4 amino acid (aa) motif on the distal C-terminus that binds to post synaptic density 95/Drosophila large disc tumor suppressor/zonula occludens-1 (PDZ) domains; these motifs have been observed on both the β2-adrenergic receptor (β2AR) (Gage et al., 2005) and the luteinizing hormone (LH) receptor (Galet et al., 2004). Conversely, the ubiquitination of GPCRs shifts their trafficking towards proteasome-dependent degradation and lysosomes and has been shown to occur with β2AR (Shenoy et al., 2001), protease-activated receptor 2 (PAR2) (Jacob et al., 2005), and the platelet-activating factor receptor (PAFR) (Dupré et al., 2003). For the β2AR, this ubiquitination requires both β-arrestin binding and GRK phosphorylation (Shenoy et al., 2001).

Non-canonical signaling

Over the past two decades, new evidence has emerged regarding GPCR signaling outside of the traditional heterotrimeric G protein complex. The majority of these studies involve MAPK pathway activation via β-arrestin binding to both activated and quiescent GPCRs. MAPK pathways are characterized by their activation of transcription factors such as CREB and c-Myc, which promote gene expression and cell proliferation.

When β-arrestin is bound to the activated angiotensin II type 2 receptor (AT_2), it prevents the translocation of the MAPK c-Jun N-terminal kinase (JNK) to the nucleus (McDonald et al., 2000); a similar mechanism was observed in the sequestration of ERK in the cytosol upon β-arrestin binding to activated PAR2 (DeFea et al., 2000). Conversely, β-arrestin 2 is able to interact with the vasopressin receptors V1a and V2 without agonist stimulation and initiate clathrin-mediated internalization of the receptor. Furthermore, β-arrestin 2 binding and internalization of the receptors is sufficient to promote ERK phosphorylation (Terrillon and Bouvier, 2004). It has been shown that more stable arrestin interactions with the intracellular regions of GPCRs are correlated with increased MAPK pathway activation as measured by the levels of phosphorylated ERK1/2 (Tohgo et al., 2003). Thus, the GPCR signaling field has expanded dramatically to determine the mechanisms that control canonical versus non-canonical signaling.

GPCRs as therapeutic targets

Because of the ability of GPCRs to transduce extracellular signaling to intracellular changes, these proteins have been utilized as drug targets to treat various diseases and illnesses. The pharmaceutical industry implemented high-throughput screening (HTS) techniques to test millions of compounds against an array of GPCRs to identify novel chemical structures that

could be further developed into clinically viable drugs. Approximately 30% of all FDA-approved drugs target canonical GPCR signaling pathways (Lee et al., 2014), with 18% of the drugs approved in 2012 alone targeting GPCRs either directly or indirectly (Jarvis, 2013). However, like many drug treatments, GPCR-targeted therapies are not without their drawbacks. Variable tissue expression profiles can result in off-target side effects; (insert example here regarding original and off target effects of GPCR-targeted drug). Also, single-nucleotide polymorphisms (SNPs) have been found to influence drug effectiveness between patients, resulting in subpopulations that are unresponsive to certain therapies. However, recent scientific advances have allowed for fewer adverse drug reactions and increased specificity to the targeted disease.

Biased agonism

Originally, GPCR dogma stated that there existed a single binding site for either an agonist or an antagonist on a receptor that could promote or block, respectively, the propagation of a single downstream signal. However, one study showed that the recruitment of β-arrestin upon activation of the β2AR caused the formation of Src kinase complexes with the β2 receptor, which was dependent on the presence of β-arrestin (Luttrell et al., 1999). Since this discovery, β-arrestin recruitment has been linked to cAMP degradation (Perry et al., 2002), inhibition of NF-κB (Witherow et al., 2004), and JNK (DeFea et al., 2000) and Akt activation (Beaulieu et al., 2005; Beaulieu et al., 2004). Another example that has garnered attention is the bias at the μ-opioid receptor: β-arrestin recruitment has been shown to be responsible for the adverse effects of opioid usage in patients undergoing pain management, such as constipation, nausea, respiratory depression and drug abuse (Violin et al., 2014).

The increasing evidence supporting non-canonical GPCR signaling has contributed to the theory of biased agonism; that is, certain agonists can shift the downstream signaling fate from

the receptor towards either traditional heterotrimeric G protein effectors or β-arrestin-dependent effectors. By developing drugs that are biased towards one pathway, the negative off-target side effects could be reduced.

Allosteric modulation

The majority of clinically available GPCR-targeted drugs function by binding to the orthosteric site of the receptor to directly influence GPCR activity. However, the past two decades have seen a shift in focus from identifying ligands that bind to the orthosteric site to those that bind to allosteric sites. Allostery is the ability of a compound to bind to a receptor without directly influencing signaling; however, the binding of an allosteric ligand can either promote or hinder the receptor's natural ability to interact with a ligand at the orthosteric site, as well as influence the potency of said ligand. Two examples of altered ligand interactions in the native binding site include a positive allosteric modulator of the M1 muscarinic receptor (Marlo et al., 2009) and a negative allosteric modulator of the prostaglandin D2 receptor CRTH2 (Mathiesen et al., 2005). In conjunction with their cognate agonists, these modulators either enhance or inhibit the responsiveness of their respective receptors; however, when they are bound in the absence of the native ligand, the receptors do not elicit a response. Allosteric ligands are becoming increasingly popular in drug discovery because of their high specificity, reduced off-target effects, and wider array of binding targets on the extracellular regions of GPCRs.

Genome-based drug targeting

Another practice that is becoming more common is the sequencing of patient genomes prior to initiating treatment. Certain drugs have been shown to be less effective in patients carrying mutations either in the target GPCR or in the cytochrome enzymes responsible for

metabolizing the prodrug into a viable compound. This allows for patients to not only receive the best care possible but also prevent wasted time and money on ineffective drug treatments. The majority of targeted therapies either in practice or in development focus on cancer treatments, as the heterogeneity of cancer subtypes render a large percentage of possible drug treatments either partially or completely ineffectual (Barretina et al., 2012).

Purinergic Signaling

History

The year 1929 was important for the molecule adenosine triphosphate (ATP) for two reasons: first, the existence of the ATP molecule was discovered independently by two scientists – Karl Lohmann (Lohmann, 1929) and Cyrus Fiske & Yellapragada SubbaRow (Fiske and Subbarow, 1929); and second, Drury and Szent-Gyorgyi reported the physiological effects of adenine compounds on guinea pig hearts, in which they observed a rapid but reversible decrease in the heart rate (Drury and Szent-Györgyi, 1929). Though they could have not known it at the time, one of those adenine compounds was ATP.

Over the course of the 20th century, adenosine and adenosine-derived molecules were shown to exert effects on the heart (Honey et al., 1930), vasculature (Bennet and Drury, 1931; Scott et al., 1965), intestines & uterus (Mihich et al., 1954) nervous system (Holton, 1959), lung (Bianchi et al., 1963), platelets (Born, 1962) and brain (Galindo et al., 1967). Furthermore, Berne discovered that systemic administration of adenosine caused an increase in coronary blood flow, suggestive of vasodilation, which was rapidly reversed (Berne, 1963).

However, while many scientists who studied ATP focused on its importance as the energy source of the cell in virtually all organisms, there was mounting evidence that adenosine and adenosine-derived molecules (especially ATP) were involved in cellular signaling events.

These studies culminated in the purinergic hypothesis put forth by Geoffrey Burnstock, who suggested that there existed proteins with the capability to bind ATP and other purinergic molecules to induce signaling (Burnstock et al., 1972). This hypothesis was borne out of Pamela Holton's publication in 1959, which stated that ATP was a neurotransmitter in the great auricular nerve in rabbits (Holton, 1959). Unfortunately, the neurotransmitter field highly criticized this theory and ridiculed Burnstock for 2 decades because of ATP's well-established role in energy production (Burnstock, 2012). However, with overwhelming evidence published during the 1970s and 1980s, Burnstock's hypothesis was finally accepted, and the field of purinergic signaling was born.

ATP synthesis

ATP is comprised of adenosine (the nucleobase adenine attached to a ribose sugar) with three phosphates daisy-chained via pyrophosphate bonds on the 5' carbon of the ribose. ATPases and pyrophosphatases can hydrolyze one or more of the phosphate groups to form adenosine diphosphate (ADP) or adenosine monophosphate (AMP), both of which are also involved in purine signaling. Another critical member of this family of molecules is cyclic AMP (cAMP), which is formed from ATP by adenylyl cyclase and produces pyrophosphate as a byproduct.

The majority of ATP in humans is produced in two of the three stages of glucose metabolism: glycolysis and oxidative phosphorylation via the electron transport chain in the mitochondria; the substrates used in the electron transport chain are derived from the third stage of glucose metabolism, the citric acid cycle. The latter produces the largest percentage of ATP, thus ATP exists in millimolar concentrations inside the mitochondria (compared to extracellular nanomolar concentrations) The ATP-ADP carrier (AAC) is an antiporter that pumps ATP out of the mitochondria in exchange for ADP, thus accounting for the high concentrations of ATP in

the cytoplasm (Pfaff et al., 1965). The body produces approximately its entire weight of ATP in a single day (Törnroth-Horsefield and Neutze, 2008); however, its prolific requirements result in approximately 250 grams existing at any given time.

Nucleotide transport and release

As with any signaling molecule, the transport and release of ATP is critical to effectively communicate a cell's requirements. Cytoplasmic ATP is primarily used for its communicative capacity along with its energy release for the molecules that require it. The methods by which ATP is either released or transported are passive release, exocytosis, active release, and cell lysis. Although less prevalent, signaling with uridine triphosphate (UTP) and other derivatives is also subjected to similar methods of release and regulation.

Passive release

Passive release involves the movement of nucleotides down their gradient through open channels. Here, we describe three such channels and their role in nucleotide release.

Gap junctions

One such channel is the gap junction, which is comprised of a connexin heterohexamer (also referred to as a 'hemichannel') connecting two adjacent cells. These gap junctions allow for the diffusion of small molecules throughout a cell monolayer, such as epithelial cells in the kidneys or intestines. ATP was observed to move through gap junctions when connexins were introduced into C6 cells, an astrocytoma culture line that does not natively express any of the connexin proteins. When transduced, ATP levels were increased within the cells (Cotrina et al., 2000). Direct evidence of ATP movement across gap junctions was discovered using membrane patch-clamp to measure the permeability of ATP (Kang et al., 2008).

Pannexins

Another passive ATP release mechanism is via diffusion through plasma membrane pannexin channels, which are hexamers of pannexin subunits. Pannexins form gap junctions in invertebrates but serve as channels linking the cytoplasm to the extracellular space in vertebrates. The involvement of pannexins in ATP release was discovered when *Xenopus* oocytes were injected with mRNA encoding human pannexin. These oocytes exhibited increased ATP release compared to control oocytes (Bao et al., 2004). Pannexins are also sensitive to mechanical stresses, including shear stress, osmotic pressure and liquid flow.

Active release

Exocytosis

Exocytosis is the fusion of intracellular membrane vesicles with the plasma membrane to release the vesicular contents into the extracellular space. One of the most well established systems of exocytosis is the release of neurotransmitters into the synapses of neurons. Prior to release, the vesicles must be loaded with high concentrations of molecules. The vesicular nucleotide transporter 1 (VNUT-1) actively concentrates ATP within secretory vesicles of astrocytes (Oya et al., 2013), T-cells (Tokunaga et al., 2010), and pancreatic cells (Geisler et al., 2013). VNUT-1 has also been shown to contribute to vesicle loading in platelets (Hiasa et al., 2014) and lung airway epithelial cells (Okada et al., 2013); the latter study further showed that ATP release was secondary to protein secretion into the lung mucosa.

Physical stress and lysis

Physical influences also affect nucleotide release. Electrical signals and myofibrillar contractions cause ATP release, and cell lysis due to apoptosis or other pathways results in a local increase of free ATP because of the high concentration of ATP within cells.

External nucleotide metabolism

Nucleotides are degraded by a family of enzymes referred to as ecto-nucleotidases. These enzymes break down ATP to ADP, AMP, and eventually adenosine (Zimmermann, 2001). The largest subfamily of this group is the nucleoside diphosphohydrolases, also known as NTPDases. There are 8 members of this family that exhibit different affinities for either the triphosphate or the diphosphate. The catalytic domain of diphosphohydrolases is either extracellular (i.e., membrane bound) or organelle bound (Robson et al., 2006). A highly characterized NTPDase is CD39, which is expressed on smooth muscle and endothelial cells (Kaczmarek et al., 1996). CD39 function with regard to platelet activity will be described later.

The other two types of ecto-nucleotidases are the nucleotide pyrophosphatase/ photodiesterases (NPPs) and ecto-5'-nucleotidase. There are 7 members of the NPP family, but only 3 are capable of acting on nucleotides; the other 4 prefer phospholipid substrates (Vollmayer et al., 2003). Ecto-5'-nucleotidase, also known as CD71, hydrolyzes AMP into adenosine, which can act on its own receptors, be phosphorylated to reform AMP via adenosine kinase (Spychala et al., 1996), or deaminated to form inosine via adenosine deaminase (Blackburn et al., 1996).

Purinergic receptors

P1 receptors

There are four P1 receptors that are activated by adenosine, which are classified based on their G protein coupling. The Gαi-coupled receptors A_1- and A_3-adenosine decrease cAMP levels by inhibiting adenylyl cyclase. The A_1-adenosine receptor is primarily expressed in the central nervous system and on smooth muscle cells. Activation of the A_1-adenosine receptor has been shown to decrease heart rate (Olsson and Pearson, 1990), protect against ischemia (Matherne et

al., 1997) and inhibit neurotransmitter release (Hu and Li, 1997; Santicioli et al., 1993). Activation of the A_1-adenosine receptor in airway epithelial cells under inflammatory conditions results in increased mucosal secretion and hyperresponsiveness to airflow (Ponnoth et al., 2010), which are two main contributors to asthmatic conditions. A_1-adenosine receptor inhibitors have been shown to reduce Ca^{2+} signaling in human bronchial smooth muscle cells (Ethier and Madison, 2006), indicating their potential as a therapeutic target for asthma patients. More recent evidence suggests that A_1 forms a heterodimer with the D_1 dopamine receptor to inhibit neurotransmitter release in the mesocorticolimbic system (Fuxe et al., 2007), though this is still controversial.

The A_3-adenosine receptor is expressed in the lung, kidney, heart, liver, eyes, and neutrophils (Jacobson and Gao, 2006). The receptor is cardioprotective under ischemic conditions (Ge et al., 2006), but excessive signaling can result in cardiomyopathy (Black et al., 2002). The A_3-adenosine receptor can also reduce superoxide anion levels and suppress TNF-α release under inflammatory conditions (Gessi et al., 2002). With the prevalence of secondary signaling cascades in GPCRs emerging, evidence suggests that the A_3 receptor can also activate the MAPK pathway (Matot et al., 2006; Neary et al., 1998).

The other two P1 receptors, A_{2A} and A_{2B}, couple to the Gαs pathway. The A_{2A} receptor is the most extensively studied of the four P1 receptors due to its ubiquitous tissue distribution; in fact, crystal structures with a bound agonist (Lebon et al., 2011) and antagonist (Jaakola et al., 2008) are available. Though the highest levels are found in the immune system and brain (Fredholm et al., 2001), these receptors also are expressed on coronary smooth muscle cells, endothelial cells, monocytes and macrophages. Activation of the A_{2A} receptor promotes neurotransmitter release in the peripheral nervous system (Gonçalves and Queiroz, 1996),

endothelial proliferation (Sexl et al., 1997), vasodilation (Conti et al., 1993), (Belardinelli et al., 1998), and TNF-α production (McColl et al., 2006). The involvement of A_{2A} receptors with neurodegenerative and cognitive disorders has become more apparent in recent years – Fuxe and colleagues reported that the A_{2A} receptor dimerized with the D_2 dopamine receptor and reduced D_2 signaling in the indirect pathway *in vivo* (Fuxe et al., 2007), whereas A_{2A} antagonists have been shown to protect against Parkinsons disease "off" time in patients (LeWitt et al., 2008).

The A_{2B} receptor is not as widely expressed as the A_{2A} receptor but is still found in airway smooth muscle cells, fibroblasts, platelets, intestinal epithelial cells and glial cells. A_{2B} receptor activation has been shown to exert a protective effect in reperfused ischemic tissues (Kuno et al., 2007; Methner et al., 2010) and increase the release of anions (e.g., Cl^- and HCO_3^-) to induce water release in the intestines (Ham et al., 2010; Strohmeier et al., 1995). The A_{2B} receptor has also been shown to promote bronchoconstriction in airway epithelial cells (Zhong et al., 2004), which provides another nucleotide-related target for asthma therapies.

P2X receptors

The ionotropic P2X family of receptors is comprised of ligand-gated cation channels whose native ligand is ATP. Each individual member of this family has a short N-terminus (18-20 amino acids) and two transmembrane domains. Because of this, multimers are required to form a functioning channel – the most stable configuration has been shown to be trimers based on crystal structure data (Gonzales et al., 2009; Kawate et al., 2009). These channels regulate the flow of positive ions into the cell and are not highly selective with regard to the ion, though there appears to be preference to Ca^{2+} ions over Na^+ and K^+ (Egan and Khakh, 2004).

There are seven subunits within this family (X_1-X_7) that can form either homotrimers or heterotrimers, with the exception of the X_6 subunit, which can only form heterotrimers with other

family members, and the X_7 subunit, which can only form homotrimers. The known stable multimer combinations are $P2X_2/X_3$, $P2X_4/X_6$, $P2X_1/X_5$, $P2X_2/X_6$, $P2X_1/X_2$ and $P2X_1/X_4$ (Burnstock, 2007). The family as a whole is expressed in nearly every tissue type, but the distribution is highly variable. Because of this widespread expression, ATP dysregulation can cause mild to severe pathogenesis in cation-related signaling.

P2Y receptors

The metabotropic P2Y family of receptors is comprised of GPCRs whose native ligands are either nucleotides or nucleotide variants: ATP, ADP, UTP, UDP, and UDP-glucose. The majority of these genes were cloned during the 1990s; several cloned receptors were either later shown not to respond to nucleotides or were species homologues of previously identified human receptors. This resulted in the disjointed numbering of the receptors and the absence of $P2Y_3$, $P2Y_5$, $P2Y_7$, $P2Y_8$, $P2Y_9$ and $P2Y_{10}$ within the nomenclature. These receptors share between 20-50% sequence homology with each other, primarily in transmembrane domains 3, 6, and 7 (Erb et al., 1995), suggesting that these regions are involved in ligand binding – this became evident when the crystal structures of two P2Y receptors ($P2Y_{12}$ and $P2Y_1$) were solved, as the largest shifts were observed on TM6 and TM7 upon agonist binding (Figure 1-3). The P2Y family is broken up into two subfamilies based on relative homology and the downstream G-alpha signaling: the $P2Y_1$-like receptors, and the $P2Y_{12}$-like receptors.

$P2Y_1$-like receptors

This subfamily of receptors is coupled to the Gαq family of signaling pathways. The sections below provide a brief description of the expression profiles, pharmacological selectivities, and physiological activities of each receptor.

$P2Y_1$ receptor

The $P2Y_1$ receptor was first cloned from a chick brain in 1993 (Webb et al., 1993). Its natural cognate agonist is ADP (K_i 0.92 μM), with ATP having both a lower affinity (K_i 17.7 μM) (Waldo 2004) and acting as a partial agonist at low levels of receptor reserve. The receptor is expressed in a wide number of animals, including cow (Henderson et al., 1995), turkey (Filtz et al., 1994), frog (Cheng et al., 2003), mouse (Tokuyama et al., 1995) and humans (Janssens et al., 1996; Léon et al., 1996). In humans, the receptor is widely distributed among various tissues such as skeletal muscle, kidney, heart, platelets and liver (Ayyanathan et al., 1996), with the highest levels of mRNA message found in the prostate, placenta and multiple brain regions (Moore et al., 2000a; Moore et al., 2000b). In platelets, the $P2Y_1$ receptor contributes to nucleotide-induced platelet activation in conjunction with the $P2Y_{12}$ receptor. The receptor also promotes prostaglandin release and muscle relaxation in skeletal muscle tissues.

$P2Y_1$ knockout mice were developed by two independent research groups in the late 1990s (Fabre et al., 1999; Léon et al., 1999). Both of these labs reported that mice lacking the $P2Y_1$ receptor had defects in platelet activation. Although initial studies showed no developmental or survival deformities, these knock-out mice have reduced pain responses in a model of nociceptive hyperalgesia (Malin and Molliver, 2010) and lower inflammation responses in the vasculature (Zerr et al., 2011). Global loss of $P2Y_1$ receptor expression also contributes to decreased bone density (Orriss et al., 2011) and increased glucose levels and weight gain compared to wild-type mice (Léon et al., 2005). All of these observations have been repeated and confirmed with the use of $P2Y_1$ receptor-specific antagonists.

There are several synthesized compounds that selectively bind at the $P2Y_1$ receptor. Members of the MRS series of antagonists, including MRS2179 (Nandanan et al., 1999),

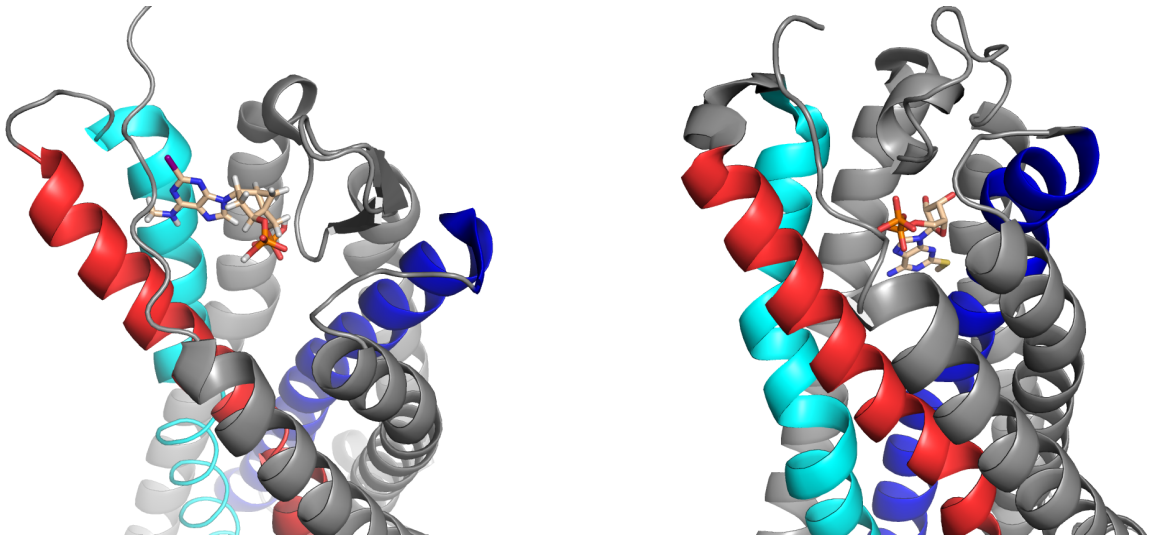

Figure 1-3. Crystal structures of the $P2Y_1$ and $P2Y_{12}$ receptor.

A, Structure of the $P2Y_1$ receptor bound to the antagonist MRS2500. **B**, Structure of the $P2Y_{12}$ receptor bound to the agonist 2MeSADP. Red, TM7; Cyan, TM6; Blue, TM3; stick, structure of respective molecule.

MRS2279 and MRS2500 (Waldo et al., 2002), are highly selective for the $P2Y_1$ receptor and show increasing affinity, with MRS2500 having a K_i less than 1 nM (Ohno et al., 2004). A selective agonist, MRS2365, was also developed with sub-nanomolar affinity to the $P2Y_1$ receptor (Chhatriwala et al., 2004). The availability of these high affinity methanocarbo ring-based compounds allowed for the $P2Y_1$ receptor crystal structure to be solved (Zhang et al., 2015). Surprisingly, it was observed that two agonist binding sites exist on the receptor: the first within the predicted binding pocket near TM6 and TM7, and a second on the exterior of the receptor.

$P2Y_2$ receptor

The $P2Y_2$ receptor was cloned from multiple species in the mid 1990s (Lustig et al., 1993; Parr et al., 1994; Rice et al., 1995). It is activated equipotently by the nucleotide triphosphates ATP and UTP but has a much lower affinity for either of the corresponding diphosphates ADP and UDP (Lazarowski et al., 1995). The receptor is expressed primarily in epithelial, endothelial, and smooth muscle cells, but also in skeletal muscle, heart, lung, lymphocytes and macrophages (Bowler et al., 1995; Ho et al., 1995; Kirischuk et al., 1995; Rice et al., 1995). In polarized epithelial cells, the $P2Y_2$ receptor is expressed on the apical surface and responds to nucleoside triphosphates released into the lumen of organs that are lined with epithelial cells (Wolff et al., 2005). Some of the common functions of the $P2Y_2$ receptor are the release of prostaglandins and nitric oxide from endothelial tissues (Welch et al., 2003; Xing et al., 1999), increased proliferation (Burnstock, 2007), and increased anion transport in epithelial tissues (Leipziger, 2003).

$P2Y_2$ receptor knock-out mice exhibit a loss of Cl^- secretion in airway epithelia, which results in increased mucosal thickness (Cressman et al., 1999). This phenotype has allowed for

the $P2Y_2$ knockout mouse to serve as a mouse model for cystic fibrosis, a disease in which the anion channel CFTR is dysfunctional and prevents the release of Cl^- into the airway mucosa. These mice also have decreased bone density due to lower osteoblast activity (Hoebertz et al., 2002) and more rapid kidney deterioration (Potthoff et al., 2013; Zhang et al., 2012); the latter condition is possibly due to increased sodium and water reabsorption (Rieg et al., 2007).

Currently, there are a few agonists selective to the $P2Y_2$ receptor that could serve as a lynchpin to future cystic fibrosis treatments. The agonist INS37217 has been shown to increase Cl^- and water secretion in human airway epithelia, thereby promoting mucosal clearance (Yerxa et al., 2002). One $P2Y_2$ receptor agonist (4-thio-β,γ-difluoromethylene-UTP) was shown to be approximately 50-fold more selective for the $P2Y_2$ receptor compared to $P2Y_4$ and $P2Y_6$ receptors, which also are activated by UTP (El-Tayeb et al., 2011).

$P2Y_4$ receptor

The human $P2Y_4$ receptor was cloned and sequenced in 1995 (Communi et al., 1995), with rat (Webb et al., 1998) and mouse (Lazarowski et al., 2001) following soon after. In humans, rodents, and other mammals, UTP is the primary native ligand (EC_{50} = 0.55 μM). Interestingly, ATP is a naturally occurring competitive antagonist (Kennedy et al., 2000), but is a full agonist at rodent $P2Y_4$ receptors (Kennedy et al., 2000). The $P2Y_4$ receptor is expressed in placenta, intestine, lung, leukocytes and human umbilical vein endothelial cells (HUVECs), with the intestine having the highest levels of expression (Moore et al., 2001). Most studies involving the $P2Y_4$ receptor focus on its involvement in anion release in intestinal epithelia, in conjunction with the $P2Y_2$ receptor (Matos et al., 2005). In polarized epithelial cells, the $P2Y_4$ receptor (along with the $P2Y_2$ receptor) is expressed solely on the apical membranes (Wolff et al., 2005).

Regarding $P2Y_4$ knockout mice, there is less data available compared to the $P2Y_1$ and $P2Y_2$ knockout mice. Studies have shown that loss of $P2Y_4$ receptors result in reduced chloride secretion in the intestinal epithelium (Robaye et al., 2003) and colon (Ghanem et al., 2005), as well as a decreased instance of cardiac hypertrophy and overall smaller heart size via downregulation of endothelin-1 activity (Horckmans et al., 2015; Horckmans et al., 2012)

Because of their high sequence identity (~50%), similar expression patterns, and endogenous ligands, it has been difficult to differentiate between $P2Y_2$ and $P2Y_4$ activity within the intestinal lumen. However, the compound iso-CMP was shown to be approximately 20-fold more selective for the $P2Y_4$ receptor compared to $P2Y_2$ and $P2Y_6$ receptors (El-Tayeb et al., 2011). The Jacobson group synthesized three compounds with sub-micromolar EC_{50} values (MRS4062, MRS2927, and N4-phenylethoxy-CTP) that showed at least one log order of selectivity of the $P2Y_4$ receptor over the $P2Y_2$ receptor (Maruoka et al., 2011).

$P2Y_6$ receptor

The $P2Y_6$ receptor has been cloned from humans (Communi et al., 1996), mice (Lazarowski et al., 2001) and rats (Nicholas et al., 1996), and is preferentially activated by UDP (EC_{50} = 0.3 μM) and UTP (EC_{50} = 6 μM) compared to adenosine nucleotides. The receptor is expressed in vascular smooth muscle, gall bladder, placenta, leukocytes, and epithelial cells (Chang et al., 1995; Somers et al., 1999). $P2Y_6$ receptor activation promotes cell growth (Hou et al., 2002) and vasoconstriction (Mitchell et al., 2012) in smooth muscle cells and exerts an anti-apoptotic effect against TNF-α signaling in an astrocytoma cell line (Kim et al., 2003). This receptor is also involved in anion release in several tissues (Köttgen et al., 2003; Lazarowski and Boucher, 2001), but the activity is not as pronounced as either the $P2Y_2$ or $P2Y_4$ receptors. More recent physiological data suggests that the $P2Y_6$ receptor contributes to the pro-inflammatory

responses in macrophages (Garcia et al., 2014) and microglia (Koizumi et al., 2007; Quintas et al., 2014). Regarding its regulation, the $P2Y_6$ receptor is slow to desensitize and internalize compared to the other P2Y receptors (Brinson and Harden, 2001).

A $P2Y_6$ receptor knockout mouse was developed in 2008 (Bar et al., 2008), in which the macrophage UDP response, in particular UDP-promoted interleukin release, was lost. These mice also exhibited a lack of aortic vasoconstriction and endothelial-induced relaxation, which was dependent on the tissue studied. $P2Y_6$ receptor activity has also been shown to be involved in bone maintenance, as $P2Y_6^{-/-}$ knockout mice showed reduced bone mass (Orriss et al., 2011).

Regarding synthetic compounds targeted to the $P2Y_6$ receptor, the thiol derivative UDPβS shows high selectivity for $P2Y_6$ compared to the $P2Y_2$ and $P2Y_4$ receptors (Malmsjö et al., 2000). Interestingly, three compounds (MRS2567, MRS2575 and MRS2578) had sub-micromolar IC_{50} values and were shown to insurmountably eliminate the protective effect against apoptosis (Mamedova et al., 2004), but it is unclear whether these compounds are selective to the $P2Y_6$ receptor.

$P2Y_{11}$ receptor

The $P2Y_{11}$ receptor was first cloned in 1997 and is unique for two reasons: 1) it is the only P2Y receptor to contain an intron within its mRNA (Communi et al., 2001b), and 2) its natural ligand, ATP, can activate both Gαq and Gαs pathways (Communi et al., 1997) with varying EC_{50} values (3.6 μM and 62.4 μM, respectively) (Qi et al., 2001). However, this receptor is less well understood because there is no homolog expressed in rodents, and the EC_{50} values of endogenous and synthetic agonists are dependent on the cell in which the receptor is studied (Communi et al., 1999). Expression of $P2Y_{11}$ receptor mRNA is observed in spleen, intestine, and immunocytes (Communi et al., 1997). Though this receptor can function to regulate ion

secretion similar to its other subfamily members (Nguyen et al., 2001), there is more research focused on $P2Y_{11}$ receptor activity in inflammation and leukocyte signaling. $P2Y_{11}$ receptor activity has been attributed to the differentiation and maturation of leukocytes (Wilkin et al., 2001) and neutrophils, as well as immunosuppression in dendritic cells (Chadet et al., 2015). The $P2Y_{11}$ receptor has also been shown to be involved in cytokine regulation, either via inhibition of IL-12 production, stimulation of IL-10 production, or changes in chemokine receptor expression levels (la Sala et al., 2002; Wilkin et al., 2002).

$P2Y_{12}$-like receptors

This subfamily of receptors is coupled to the Gαi family of signaling pathways. Below we provide a brief description of the expression, agonist and antagonist profiles, and physiological activity of this subfamily.

$P2Y_{12}$ receptor

$P2Y_{12}$ receptors in human, mouse and rat were cloned by multiple groups in 2001 (Foster et al., 2001; Hollopeter et al., 2001; Nicholas, 2001; Zhang et al., 2001), although its existence was first considered in platelets over two decades earlier (Cooper and Rodbell, 1979). At the time, it was classified as the P2cyc receptor due to its coupling to adenylyl cyclase activity, which was uncommon for P2Y receptors at the time. The endogenous ligand of the $P2Y_{12}$ receptor is ADP (EC_{50} = 0.061 μM) (Zhang et al., 2001), with high concentrations of ATP acting as an antagonist (Park and Hourani, 1999). The $P2Y_{12}$ receptor was the first of the P2Y receptors to be successfully crystallized bound to both an agonist (Zhang et al., 2014a) and an antagonist (Zhang et al., 2014b). These two structures supported studies suggesting that ligands activate the receptor by interacting to TM6 and TM7.

The $P2Y_{12}$ receptor is expressed primarily in platelets and neural tissues, particularly in microglia (Sasaki et al., 2003; Zhang et al., 2001). Its activity in platelets will be further discussed below, but there are other reports regarding its involvement in the formation of processes and polarization in microglia via chemotaxis (Koizumi et al., 2013), as well as in neuropathic pain development (Ohsawa et al., 2007).

The $P2Y_{12}$ knockout mouse was developed shortly after its successful cloning, in which a distinctive loss of platelet aggregation was observed (Andre et al., 2003; Foster et al., 2001). More recent studies involving these knockout mice have shown that the loss of $P2Y_{12}$ receptor results in decreased osteoclast activity and bone loss (Su et al., 2012; Syberg et al., 2012), increased protection against brain ischemia (Webster et al., 2013), and reduced cytokine production and neuropathic pain (Horváth et al., 2014).

Although there are several synthetic agonists that activate the $P2Y_{12}$ receptor (e.g., 2-MeSADP, ATPγS), none of these are highly selective for the $P2Y_{12}$ receptor. However, the limited tissue profile of this receptor, combined with its importance in platelet aggregation, has led to the development of clinically available $P2Y_{12}$-selective antagonists, many of which were developed before the receptor was cloned. These prodrugs, termed thienopyridines, are metabolized to a form that covalently reacts with the $P2Y_{12}$ receptor to irreversibly inhibit its activity (Gachet et al., 1992). Ticlopidine was the first of these prodrugs developed, but the second generation drugs clopidogrel and prasugrel are part of the current gold standard of anti-platelet therapies. More recently, the non-competitive antagonist ticagrelor has been touted as a possible replacement for clopidogrel and prasugrel because of its reversible nature (van Giezen et al., 2009).

$P2Y_{13}$ receptor

The $P2Y_{13}$ receptor was first identified after the human genome sequencing project was completed owing to its homology to the $P2Y_{12}$ receptor and was successfully cloned from human (Communi et al., 2001a), mouse (Zhang et al., 2002) and rat (Fumagalli et al., 2004). ADP (EC_{50} = 0.06 μM), ATP (EC_{50} = 0.26 μM), and the diadenosine polyphosphate Ap3A (EC_{50} 0.072 μM) act as agonists at this receptor. $P2Y_{13}$ receptor mRNA is expressed in the spleen, leukocytes, bone marrow, liver and brain (Communi et al., 2001a). Activation of the $P2Y_{13}$ receptor promotes a neuroprotective effect in astrocytes (Ortega et al., 2011) and granule neurons (Pérez-Sen et al., 2015), as well as an anti-apoptotic effect in neurons (Voss et al., 2014) and pancreatic beta cells (Tan et al., 2013).

The $P2Y_{13}$ knockout mouse presents with decreased levels of high-density lipoprotein (HDL) and fatty acids within the plasma (Blom et al., 2010), suggesting the receptor's involvement in sterol transport and/or metabolism. This was further established in an atherosclerotic mouse model, whereby loss of $P2Y_{13}$ receptor expression in these mice increased plaque development and reduced HDL levels in the plasma and fecal matter (Lichtenstein et al., 2015). One developmental defect observed in the $P2Y_{13}$ knockout mice was an increase in bone mass in young and adolescent mice, which was completely reversed to significant bone loss in adult mice (Wang et al., 2014).

There are no known selective agonists to the $P2Y_{13}$ receptor; however, the selective antagonist MRS2211 (IC_{50} = 1 μM) was synthesized and shown to have approximately 20-fold selectivity for the $P2Y_{13}$ receptor compared to $P2Y_1$ and $P2Y_{12}$ receptors.

$P2Y_{14}$ receptor

The $P2Y_{14}$ receptor was first cloned in 2000 (Chambers et al., 2000) but was not officially added to the P2Y family until 2003. It is active by UDP but is unique compared to other P2Y receptors in that it is also activated by UDP sugars (Harden et al., 2010). Of the 4 known UDP sugars that activate the $P2Y_{14}$ receptor, only UDP-glucose (EC_{50} 0.08 μM) has been shown to be released extracellularly, likely as a result of fusion to the plasma membrane of protein secretory vesicles (Lazarowski et al., 2003). The $P2Y_{14}$ receptor is widely expressed in humans, including the placenta, intestine, brain, spleen, lung, and bone marrow, with higher mRNA levels found in neutrophils, glial cells and lymphocytes (Moore et al., 2003). Receptor activation has been linked to chemotaxis in both hematopoietic progenitors (Lee et al., 2003) and mature neutrophils (Barrett et al., 2013; Sesma et al., 2012). $P2Y_{14}$ receptor signaling is also involved in the release of pro-inflammatory cytokines (Barrett et al., 2013; Müller et al., 2005).

Data from recently created $P2Y_{14}$ knockout mice show increased release of TNF-α from astrocytes (Kinoshita et al., 2013) and increased bone marrow senescence (Cho et al., 2014) in these animals. Loss of this receptor also results in increased protection from radiation-induced stress of developing embryos *in utero* (Kook et al., 2013) and reduced glucose tolerance and insulin release (Meister et al., 2014).

Currently there are two synthetic agonists with high selectivity to the $P2Y_{14}$ receptor – MRS2802 (EC_{50} 0.05 μM) and MRS2907 (EC_{50} 0.05 μM) (Carter et al., 2009). A highly potent and selective synthetic antagonist, PPTN (IC_{50} 0.008 μM), has been crucial for studying $P2Y_{14}$ receptor activity (Barrett et al., 2013).

Purinergic signaling in platelets

GPCR-based activation of platelets is dependent on the Gα12/13 pathway, the Gαq pathway, and the Gαi pathway. Within platelets, four proteins from the purinergic family are expressed to either promote or inhibit this platelet activation. The A_{2B} receptor is expressed on the platelet plasma membrane and binds adenosine to activate adenylyl cyclase, which increases intracellular cAMP concentrations to keep the platelet in a quiescent state.

$P2Y_1$ and $P2Y_{12}$ receptors are activated by ADP, with ADP being more potent at the $P2Y_1$ receptor compared to the $P2Y_{12}$ receptor. $P2Y_1$ receptor activation results in Gαq activation, formation of IP_3 and DAG, and the subsequent release of Ca^{2+} from the calcium stores. The 3-log shift in intracellular calcium concentration contributes to both microfilament rearrangement in shape change and increases in Rap1B signaling. The $P2Y_{12}$ receptor inhibits adenylyl cyclase activity via $G\alpha i_2$ signaling, resulting in a decrease of inhibitory cAMP levels. When either the $P2Y_1$ or $P2Y_{12}$ receptor is activated, the platelet does not fully convert to an activated state; it is only upon simultaneous activation of these two P2Y receptors that ADP-induced integrin αIIbβ3 activation can occur. This integrin activation is the hallmark of activated platelets. Though not a major player, ATP binding to $P2X_1$ homotrimers on the platelet membrane causes an influx of Ca^{2+} into the cytoplasm, which contributes to shape change. The following section describes the activation and signaling of platelets in more detail.

There are few cases of polymorphisms of the $P2Y_1$ and $P2Y_{12}$ receptor affecting platelet function in humans. The only known dimorphism of the $P2Y_1$ receptor was identified by Hetherington et al, in which the affected individuals showed an increase in ADP-induced platelet activation (Hetherington et al., 2005). There have been far more recorded instances of congenital $P2Y_{12}$ defects and polymorphisms. All of these individuals either have reduced or no ADP-

induced platelet activation, with varying penetrance of the bleeding phenotype. These cases are summarized in more detail by Marco Cattaneo (Cattaneo, 2011).

Platelet Biology

Introduction

The platelet is a unique manifestation of the mammalian cardiovascular system. Although there were earlier reports, platelet discovery is officially credited to Guilio Bizzozero in 1881, who described "small plates" of cells that would attach to sites of vessel wall injury and form clumps (Bizzozero, 1881; Bizzozero, 1882). Building on these results, William Osler (who identified platelets nearly 10 years earlier but was uncertain as to their origin) described the first "plaques" in thrombotic diseases as containing platelets (Osler, 1886). However, it wasn't until 1906 that James Wright discovered megakaryocytes, the cells from which platelets are born (Wright, 1906).

Believed to be evolutionarily derived from the amebocytes of invertebrates, platelets are anuclear fragments of cytoplasm from megakaryocytes and primarily serve as a hemostatic mechanism at sites of vessel injury to prevent blood loss and initiate the repair of the damaged vessel. However, the platelet is more than a glorified plug, as we will discuss below.

Thrombopoiesis

All blood cells are formed from totipotent hematopoietic stem cells, which express cluster of differentiation 34 (CD34) and CD41 (integrin αIIb) on their surfaces. Stem cells are exposed to interleukin 3 (IL-3), IL-6, IL-11 and stem cell factor (SCF) to maintain their pluripotency. Upon exposure to thrombopoietin (Tpo) along with these other cytokines, the stem cells shift towards the formation of burst-forming cells, which give rise to promegakaryoblasts. Continuous exposure to Tpo and IL-11 along with a loss of IL-3, IL-6 and SCF exposure

promotes the formation of a megakaryoblast, followed by a promegakaryocyte and eventually a mature megakaryocyte. The maturation process is marked by a loss of CD34 expression and an increase of CD61 expression (integrin β3); the co-expression of CD41 and CD61 is the hallmark of platelet identification. The process from burst-forming cell to megakaryocyte takes approximately 1 week; a single burst-forming cell can produce 40-500 megakaryocytes (Briddell et al., 1989).

As an immature megakaryocyte, the cell undergoes endomitosis; that is, the chromosomes undergo mitosis but the cell does not execute cytokinesis. This increases the size of the cell (50-100 μm diameter; up to 150 μm) as well as the amount of DNA within the megakaryocyte. This polyploid cell has an average of 16N (Odell et al., 1970), but megakaryocytes are capable of existing with 64N. It is believed that this massive increase in DNA is required to produce higher levels of protein for the eventual platelets (Raslova et al., 2003).

Megakaryocyte maturation is signified by the presence of alpha granules and the demarcation membrane system (DMS) (Yamada, 1957); however, not much is known about the function of the DMS. It was hypothesized that the DMS was involved in the release of platelets from the megakaryocyte, but the widely held theory today is that platelets are borne from the formation of proplatelets (Becker and De Bruyn, 1976; Schmitt et al., 2001; Thiery and Bessis, 1956). The megakaryocyte forms long protrusions from its cell body referred to as proplatelets that are akin to beads on a string. These projections enter the sinusoids of the bone marrow vasculature to be taken up into the blood stream. Over a period of 5 days, a single megakaryocyte produces thousands of platelets, 30% of which are sequestered in the spleen to be

released upon a vascular crisis. On average, a human produces 100 x 10^9 platelets per day to maintain normal levels in the bloodstream (Daly, 2011).

Platelet characteristics

Physical characteristics

Figure 1-4 illustrates the unique features of a quiescent platelet. At rest, platelets are concave disk-like structures approximately 2-5 μm in diameter and 0.5 μm in thickness (Bizzozero, 1882). They contain large quantities of microtubules and microfilaments, the latter of which represents only 50% of all actin molecules within the platelet. Scanning electron microscopy reveals that the membrane surface of platelets is similar to the invaginations of the brain, giving a wrinkly appearance (White and Escolar, 1993). These invaginations are important in the hemostatic function of platelets, as their lipid bilayers are rigid (Behnke, 1970), and subsequent shape change of the platelet is dependent on the presence of additional membrane rather than its flexibility.

Platelets have a unique system of organelles that optimize their function within the bloodstream; they contain no Golgi complexes and have few (but efficient) mitochondria. The most prominent organelles present are alpha granules, which number between 50-80 per platelet and contain soluble and membrane-bound proteins (Frojmovic and Milton, 1982). The most abundant of these proteins are the αIIbβ3 integrins, fibrinogen, von Willebrand factor (vWF), and glycoprotein VI (GPVI). Many of the proteins in alpha granules promote platelet activation, but these granules also contain growth factors, including those that promote angiogenesis. Since the turn of the century, advances in proteomic technology has allowed the identification of over 300 unique proteins within alpha granules (Coppinger et al., 2004).

Dense granules are also specific to platelets. Although much smaller in number compared to alpha granules, these vesicles contain cations and small biomolecules at high concentrations. ADP and ATP have been measured in the sub-molar range, whereas [Ca^{2+}] was recorded above 2 M (Holmsen and Weiss, 1979).

Platelets also contain an open canalicular system (OCS) that is directly connected to the plasma membrane surface. As the OCS is in direct contact with circulating plasma, the total exposed surface area is approximately double that of the simple surface (Frojmovic et al., 1992). This massive tunneling of folded membranes allows the cell to expand its surface area over 400% when reorganizing from a disc-shape to a flattened fillipodia (Escolar et al., 1989). Interestingly, the OCS is not found in all platelets; it is present in humans, mice, and dogs yet absent in bovines, horses and camels (Choi et al., 2010).

One final organelle unique to platelets is the dense tubular system (DTS). This separate set of membranes is completely enclosed within the platelet and is formed from remnants of the endoplasmic reticulum (ER) of the parent megakaryocyte. The primary purpose of the DTS is to release Ca^{2+} into the cytoplasm upon receiving a signal in a similar manner that the ER of other cells release calcium.

Life span

The life span of platelets was first described by William Duke in 1910, who provided blood transfusions to patients with low platelet counts and remarked that the patients' ability to coagulate was only temporary (Duke, 1910). It wasn't for another 45 years that scientists were able to definitively measure a platelet's life span using quantifiable measures, which was calculated at approximately 10 days (Harker and Finch, 1969; Leeksma and Cohen, 1955).

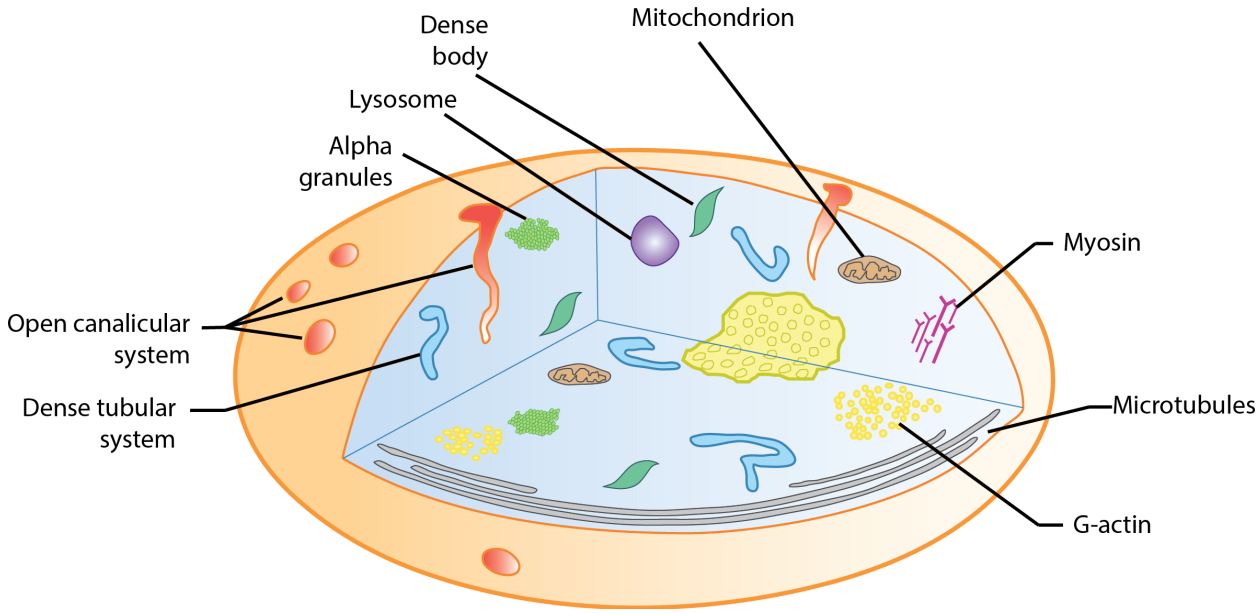

Figure 1-4. Organelle and structural schematic of a quiescent platelet.

Interestingly, platelets undergo apoptosis via the intrinsic pathway. B-cell lymphoma-extra large (Bcl-xL) has been shown to be critical for a normal platelet life span in mice (5 days) (Mason et al., 2007); conversely, mice that do not express Bcl-2 homologous antagonist/killer (Bak) have platelets with a doubled life span compared to wild type mice (Mason et al., 2007). This suggests tight regulation of the number of circulating platelets in the bloodstream.

Platelet count

A normal platelet count in humans ranges from 150-400 $\times 10^9$ platelets per liter; this has been shown in healthy Caucasians (Brecher and Cronkite, 1950), the elderly population (Ruocco et al., 2001) and the Spanish population (Lozano et al., 1998). However, the platelet count for the African or West Indies populations is slightly lower (Bain and Seed, 1986), but this does not reflect any increased incidence of platelet-related dysfunction in this group.

Platelet function

Primary platelet activation

A platelet's primary role in maintaining hemostasis is its ability to react at a vessel injury. The multitude of proteins expressed on the platelet membrane allow for rapid recruitment and activation through various pathways. Here, we will discuss the most common proteins involved in the critical stages of platelet activation.

Binding to injury site

When the sub-endothelium beneath the vessel wall is exposed to the blood plasma, several molecules are released into the blood stream. One such molecule is von Willebrand factor (vWF), which also is expressed in endothelial cells and can be found circulating within the plasma. vWF binds to glycoprotein Ib (GPIb), a crucial member of the GPIb-IX-V complex. vWF exhibits rapid on-off binding with GPIb and acts as a brake to slow the platelet down at the

injury site within a high shear flow environment. This mechanism makes the platelet appear to "roll" along the vessel wall.

As the platelet slows towards the injury site, other receptors on the platelets are able to bind to the exposed collagen from the subendothelial matrix. These receptors include the αII/βI integrin and glycoprotein VI (GPVI), which propagate "outside-in" signaling to further activate the platelet (which is described below). It has also been shown that the GPIb-IX-V complex is also able to bind to collagen under high shear situations (Farndale et al., 2004). This secondary adhesion securely attaches the platelet to the injury site.

The initial formation of a platelet matrix involves the platelet endothelial cell adhesion molecule (PECAM-1), also known as CD31. These proteins contribute to platelet-platelet adhesions at the injury site but are known to be secondary to integrin and glycoprotein-based adhesions (Burk et al., 1991; Duncan et al., 1999). PECAM-1 has been shown to interact with the platelet cytoskeleton upon activation (Newman et al., 1992), which is a critical component of platelet shape change and is described below.

Shape change

Gα12/13 mediated events

Shape change through the Gα12/13 pathway has been shown to be critical in thrombin- and thromboxane-driven platelet activation (Moers et al., 2003). Gα13 couples to the protease-activated receptors 1 (PAR1) and 4 (PAR4) in humans. These receptors are activated by thrombin, which cleaves their N-termini at an arginine residue and exposes a cryptic agonist(Jacques and Kuliopulos, 2003; Jacques et al., 2000). Because this newly revealed agonist is tethered to the receptor, it results in irreversible activation of the receptors. Upon dissociation of the heterotrimeric G-protein complex, Gα13 activates p115RhoGEF, which then activates Rho

(Fukuhara et al., 2001). Rho then acts upon the Rho-activated kinase p160ROCK (Klages et al., 1999) and LIM domain kinase (Pandey et al., 2006); these kinases phosphorylate myosin light chain kinase and cofilin, respectively, to influence actin filament polymerization.

Gαq mediated events

Gαq-directed shape change in platelets is mediated solely by ADP activation of the $P2Y_1$ receptor (Offermanns et al., 1997). GTP-bound Gαq activates PLCβ, which hydrolyzes PIP_2 to produce diacylglycerol and inositol triphosphate (IP_3), the latter of which binds to the IP_3 receptor expressed on the DTS. This releases Ca^{2+} into the cytoplasm, which binds to and activates the protein gelsolin (Hartwig et al., 1989; Yin and Stossel, 1979). Normally, gelsolin is autoinhibited in the absence of Ca^{2+}; however, Ca^{2+}-bound gelsolin is capable of binding to existing actin filaments and depolymerize them into globular actin. It was determined that gelsolin is required for platelet shape change (Witke et al., 1995).

Cytoskeletal reorganization

The combination of the mass gelsolin-mediated depolymerization of actin combined with the activation of cofilin and myosin light chain kinase produces a perfect storm for cytoskeletal reorganization. Along with the events described above, the barbed ends of existing filaments are removed, and Arp2/3 complexes are activated to provide branch points in actin polymerization. Phosphoinositides, which are produced from phosphatidylinositide 3-kinases (PI3K), have also been shown to be required for filament assembly (Hartwig et al., 1995). This massive reorganization and polymerization event is sufficient to force the cell from its resting discoid shape into what looks like a fried egg.

Integrin activation

Integrin activation is the key event that defines whether a platelet is activated or not. Upon conformational change of the cytoplasmic tails of the integrin αIIbβ3, an "inside-out" signal is propagated across the membrane to the extracellular domains that promotes the binding of the integrin to an Arg-Gly-Asp (RGD) peptide sequence. Three molecules that contain this sequence are fibrinogen, fibronectin, and vWF (Plow et al., 2000), with fibrinogen having the most repeats. Here, we describe the multiple pathways that converge to result in activation of αIIbβ3.

Gαq mediated events

Gαq couples to the $P2Y_1$, $5HT_{2A}$, PAR1, and PAR4 receptors in human platelets. Binding of GTP to the Gαq subunit from the heterotrimeric G protein complex results in the activation of PLCβ, which hydrolyzes PIP_2 into IP_3 and DAG. As described earlier, IP_3 induces the release of Ca^{2+} from the DTS stores. Along with activating gelsolin, cytoplasmic Ca^{2+} activates calcium and DAG-regulated GEF1 (CalDAG-GEF1) (Dupuy et al., 2001).

Gαi family mediated events

Two Gαi proteins are expressed in platelets: $Gαi_2$ and Gαz. Gi is primarily coupled with the $P2Y_{12}$ receptor (although the PAR1 receptor is capable of coupling with lower affinity), whereas the $α_{2A}$ adrenergic receptor couples to Gz. Activation of these alpha subunits results in a decrease in cAMP levels due to inhibition of adenylyl cyclase. More critical to platelet biology is the activation of PI3K (Trumel et al., 1999), which (in the case of the $P2Y_{12}$ receptor) results in sustained activation. Recent research has shown that PI3K inhibits Ras GTPase-activating protein 3 (RASA3), which functions as a GAP for Rap1B (Stefanini et al., 2015) and activates Akt (Woulfe, 2010).

Collagen-mediated events

As previously mentioned, collagen binding to GPVI and integrin α2/β1 aids the platelet in adhering to the exposed subendothelial matrix at the injury site. However, this binding also serves as an "outside-in" mechanism to activate the platelet as well. Src family kinases (SFKs) are recruited to the intracellular regions of these proteins upon collagen binding. This results in the recruitment and eventual phosphorylation of spleen tyrosine kinase (Syk), which then phosphorylates PLCγ (Poole et al., 1997). Similar to PLCβ, this enzyme is capable of hydrolyzing PIP2 into DAG and IP_3, the latter of which releases Ca^{2+} from the DTS.

Rap1B: the focal point

Activation of CalDAG-GEF1 leads the activation of Rap1B (Crittenden et al., 2004), a small GTPase. This activation is intensified by inhibition of the GAP RASA3. While little is known regarding the proteins involved between Rap1B activation and integrin αIIbβ3 activation, one study discovered that Rap1B activation induces increased activity of the Rap1-GTP-interacting adaptor molecule (RIAM), which is capable of binding talin (Lee et al., 2009). Talin is known to bind to the cytoplasmic tail of β3 (Wegener et al., 2007), which is a key step in activating integrin αIIbβ3. However, a RIAM knockout mouse was shown to have no loss of integrin activation using multiple agonists (Stritt et al., 2015), indicating that there are redundant molecules downstream of Rap1B that lead to αIIbβ3 activation. A schematic summarizing the multiple signaling cascades involved in platelet activation is shown in Figure 1-5.

Granule release

The release of alpha and dense granules is critical for effective platelet clot formation because it releases proaggregatory molecules into the immediate microenvironment, which can recruit nearby platelets and intensify the initial plug at the injury site. As described above,

activation of Gα13 leads to p160ROCK activation, which enhances myosin light chain phosphorylation by inhibiting the corresponding phosphatases. This increased phosphorylation of the myosin light chain promotes actin contraction. The corresponding shape change leads to the exocytosis of granules to the OCS (Stenberg et al., 1984). This is executed using vSNAREs and tSNAREs (Polgár et al., 2002; Polgár et al., 2003) in the presence of increased filamentous actin (F-actin) (Flaumenhaft et al., 2005). The release of the granule contents into the OCS promotes sustained activation of nearby platelets, thus providing a positive feedback loop at the injury site.

Inhibition of platelet activation

With platelets exhibiting such strong responses upon activation, maintaining a quiescent state under normal blood flow conditions is imperative. Endothelial cells both express and secrete proteins and molecules that keep platelets in an inactive state to prevent excessive thrombosis formation and maintain homeostasis.

Prostacyclin

Prostacyclin (PGI_2) is a product of the arachidonic acid pathway in endothelial cells. It is produced by prostacyclin synthase (PGIS) from prostaglandin H2 (PGH_2) (Oates et al., 1988; Weksler et al., 1977); interestingly, PGH_2 is the same substrate that is used to produce thromboxane in platelets. Upon release from the endothelial cells, PGI_2 binds to the prostacyclin (IP) receptor on platelets. The IP receptor is a Gs-coupled GPCR, and thus its activation results in increased cAMP levels within the platelet due to increased adenylyl cyclase activity (Tateson et al., 1977). Prostacyclin, though a potent inhibitor, has a brief half-life of 42 seconds (Cawello et al., 1994), thus providing quick inhibition in undamaged vessels yet allowing for rapid activation in its absence.

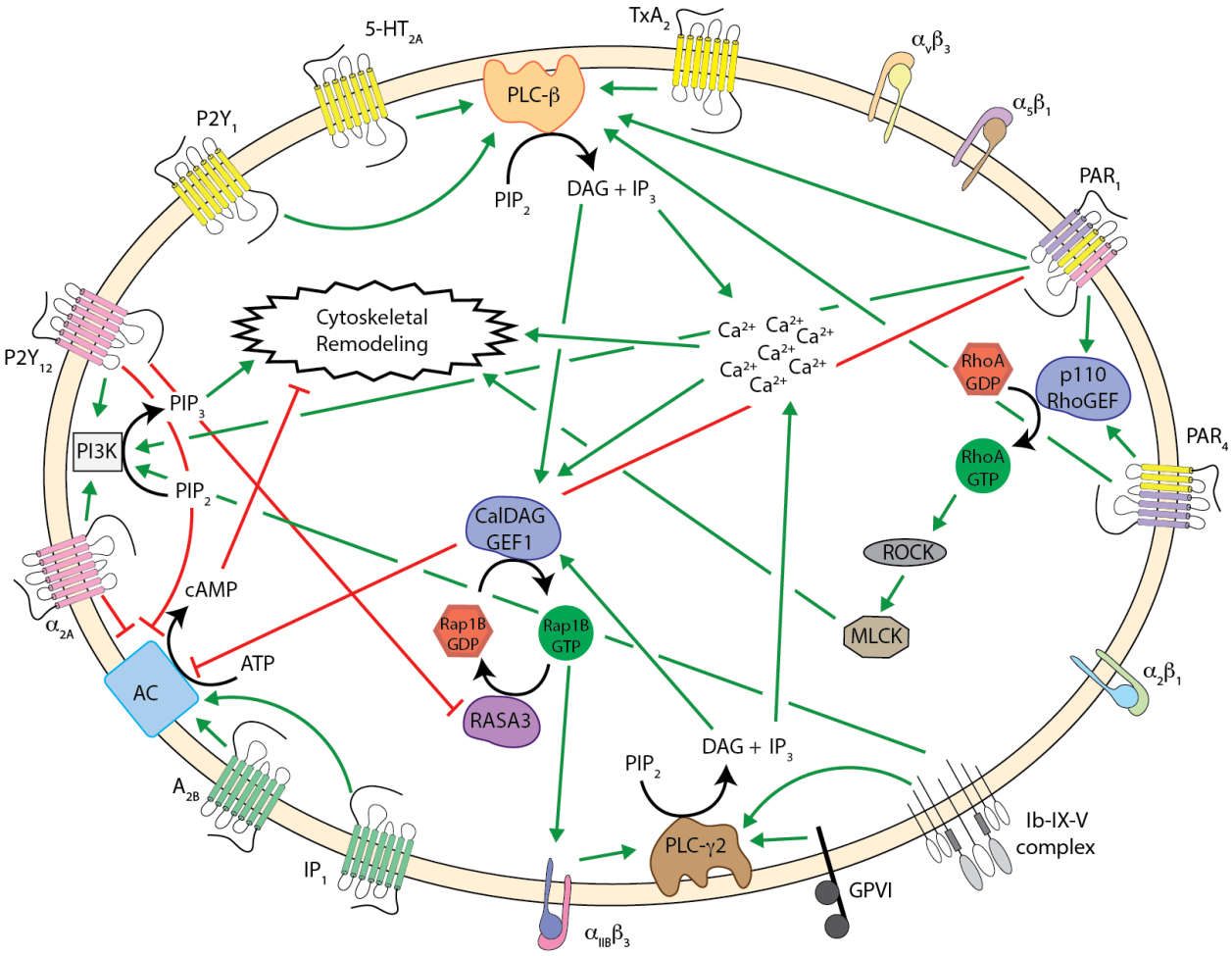

Figure 1-5. Overview of signaling involved in platelet activation.

Green GPCRs couple to Gs, pink GPCRs couple to Gi, yellow GPCRs couple to Gq, and purple GPCRs couple to G12/13.

Nitric Oxide

Nitric oxide is a highly stable free radical produced in endothelial cells by endothelial nitric oxide synthase (eNOS) and can freely cross the plasma membrane. NO binds to soluble guanylyl cyclase (sGC) within cells to increase cGMP levels; in platelets, this is similar to increasing cAMP levels and serves to lower its excitability state. sGC activation also reduces cytosolic Ca^{2+} levels (Moro et al., 1996).

Nucleotidase activity

The nucleotidases CD39 and CD73 are membrane proteins expressed on endothelial cells that break down ATP and ADP in the plasma to prevent purinergic-mediated platelet activation (Marcus et al., 1991). CD39 is an ecto-nucleotidase that metabolizes ATP to ADP and then to AMP; CD73 is a 5'-nucleotidase that converts AMP into adenosine (Dwyer et al., 2004). This is important because platelets express the A_{2A} adenosine receptor, which couples to Gs. Activation of the A_{2A} receptor results in increased cAMP levels, decreased intracellular Ca^{2+} concentrations, and platelet inhibition (Yang et al., 2010).

Secondary activation

The recruitment and activation of platelets serves as the first phase of thrombus formation. After the accumulation of activated platelets at the injury site, the secondary phase of clot stabilization transforms the loose platelet mesh into a gel-like plug to firmly block the site of injury and halt blood loss from the damaged vessel. This well characterized "waterfall cascade" has two initiating points that converge to retract the platelets and form a tight clot: the intrinsic pathway and the extrinsic pathway.

The intrinsic pathway is initiated primarily by the formation of a collagen-based complex with proteins within the subendothelial matrix, which derives the alternative moniker "contact

pathway". This complex converts Factor XII (FXII), a serine protease in its zymogen form, into FXIIa (its activated form) (Renné et al., 2012), although any negatively charged molecule can facilitate this activation. FXIIa then activates FXI to FXIa, which in turn converts FIX into FIXa, hence the descriptor "waterfall cascade". The extrinsic pathway is controlled by the exposure of tissue factor (TF) to the bloodstream at the injury site. Under normal conditions, FVII freely circulates in the blood plasma; however, when fibroblasts and leukocytes (which express TF) are present at an injury site, FVII binds with TF to form an activated TF-FVIIa complex.

Whether by FIXa (in conjunction with its cofactor FVIIIa) or the TF-FVIIa complex, FX is activated to FXa, which is the key node in secondary activation. FXa, with FV, Ca^{2+} and phospholipids as cofactors, cleaves prothrombin into thrombin, which not only serves as a potent platelet activator as described above, but also cleaves fibrinogen, activates FVIII and FV, and converts FXIII to FXIIIa. Fibrinogen cleavage produces insoluble fibrin, which serves as the core of a mature clot. These fibrin strands are cross-linked by FXIIIa to further stabilize the clot.

Platelets provide critical components of secondary activation. Phospholipids within these membranes are used for intrinsic FX activation as well as the prothrombinase activity of FXa. Both FV and FVIII are expressed on platelet membranes, and alpha granules contain high levels of fibrinogen (Harrison et al., 1989), FV (Camire et al., 1998) and FXIII (McDonagh et al., 1969).

Non-aggregatory functions

Inflammation

Platelet contribution to inflammation is a callback to its evolution from amebocytes, which are nucleated cells that serve as a "catch-all" for hemostasis in invertebrates. Along with its aggregatory nature, platelets serve to maintain the vessel barrier by interacting and

communicating with endothelial cells and leukocytes to determine the possibility of infection or foreign agents at the vessel injury site. Platelets aid in the inflammatory response by increasing vessel permeability to cause edema (Nachman and Polley, 1979) and release chemokines and cytokines (e.g., CXCL1, IL-1β, TGF-β) to recruit leukocytes at locations of activated endothelial cells.

Angiogenesis

Platelets have been shown to promote endothelial cell growth and proliferation (Gimbrone et al., 1969; Pintucci et al., 2002; Saba and Mason, 1975) and release the growth factors VEGF, bFGF, and PDGF (Brill et al., 2004). The combination of these growth factors plus a thrombus can induce tube formation (Pipili-Synetos et al., 1998) as well as direct the maturation of endothelial progenitors into mature cells (Langer et al., 2006).

There are data suggesting that platelets contain both pro- and anti-angiogenic factors within sequestered populations of granules (Italiano et al., 2008), which could serve as a node to determine what activators either enhance or inhibit vessel formation. This is of particular interest to cancer biologists, in which angiogenesis is a target for anti-cancer therapies. The first study reporting the involvement of platelets in cancer described a reduction in lung tumors in mice with low platelet counts that were injected with tumor cells compared to mice with normal platelet counts (Gasic et al., 1968). Further evidence has shown that the release of lysophosphatidic acid (LPA) from platelets can increase tumor growth, whereas pharmacologically blocking LPA activity results in reduced metastasis (Boucharaba et al., 2004).

Pathogenesis

Dysregulated platelet activity has two causes: irregular function and irregular numbers. Here, we describe both types of dysregulation as well as the therapeutic options for correcting

these conditions. We also discuss the role of platelets in cardiovascular disease as well as current platelet-related treatments.

Platelet count

The most common platelet dysfunction is a loss of platelets, defined as thrombocytopenia, due to decreased production or increased destruction of platelets. Decreased production can be due either to hereditary diseases (e.g., Bernard-Soulier syndrome, gray platelet syndrome, Wiskott-Aldrich syndrome) or secondary reactions to other medical maladies (e.g., folic acid deficiency, liver failure). Increased platelet destruction can be caused by autoimmune disorders (e.g., immune thrombocytopenic purpura), hypersplenism, von Willebrand disease, or heparin treatments. Patients undergoing myelosuppressive treatment regimens are also at risk for developing thrombocytopenia. The primary treatment options for patients with low platelet counts are corticosteroids, platelet transfusions, plasmapheresis, and bone marrow transplants.

At the other end of the spectrum, thrombocytosis is the excessive production of platelets. Primary thrombocytosis is congenital and usually the result of myeloproliferative disorders, including chronic myelogenous leukemia (CML) and essential thrombocythemia. Much more common is secondary thrombocytosis, which can be due to an elevated inflammatory state (e.g., bacterial diseases, rheumatoid arthritis, surgery), loss of spleen or spleen function, or Kawasaki disease, among other causes. Although many cases of secondary thrombocytosis do not require intervention, patients suffering from extreme thrombocytosis (i.e., count >1000 x 10^9/liter of blood) may benefit from a low dose aspirin regimen.

Platelet function

As there are a plethora of proteins and processes involved in aggregation, dysfunction of even one of these can have deleterious effects on hemostasis, resulting in prolonged bleeding

times and poor thrombus formation. The inability to properly transduce signals within the platelet due to either missing components or inactive enzymes can disrupt hemostasis and lead to severe illness.

A common loss-of-function node occurs with the inability of platelets to adhere to their target proteins. There are two characterized diseases from the absence of the GPIb-vWF interaction: Bernard-Soulier syndrome and von Willebrand disease. The former is caused by the loss of expression of GPIb and is also associated with low platelet counts (described above) and abnormally large platelets; the latter is caused by the loss of either vWF expression or function. Platelets from patients who suffer from Glanzmann thromasthenia no longer bind fibrinogen due to either inefficient expression or defective activity αIIbβ3, thus resulting in increased bleeding times.

Granule dysfunction is less common but still just as detrimental to platelet function. These diseases are often classified as platelet pool storage defects regardless of whether the alpha or dense granules are affected. The majority of these disorders is congenital and thus has minimal treatment options. Patients with either Hermansky-Pudlak syndrome or Chediak-Higashi syndrome have defects in their dense granules, whereas gray platelet syndrome is characterized by a lack of alpha granules. A recent study discovered a new autosomal dominant alpha granule-related disorder, termed "White platelet syndrome" named after the ancestor of the commonly affected people, in which platelets had more megakaryocytic morphology and incomplete alpha granule formation (White et al., 2004).

Cardiovascular disease

Cardiovascular disease remains the leading killer of Americans as of 2013, responsible for nearly 30% of all deaths. Normally, platelets recruit leukocytes to sites of vessel injury to

promote endothelial healing and prevent infection. However, increased inflammatory conditions within the vessels can cause platelets to bind to undamaged endothelial cells (Bombeli et al., 1998; Gawaz et al., 1997). The primary causes of this inflammation are the accumulation of oxidized low-density lipoproteins (LDLs) and the release of chemokines. Once congregated, the P-selectin expressed on platelets binds to the inflamed endothelium (Huo et al., 2003). As leukocytes are recruited, they extravasate into the subendothelial space and accumulate. Over time, these fatty deposits develop into plaques and constrict the vessel diameter, which is a hallmark of coronary artery disease (CAD). Though the increased pressure due to the decreased vessel width can cause discomfort, the greater risk to patients with CAD is vessel occlusion. As platelets accumulate at plaque sites, smaller thrombi can break off of instable clots and block vessels – a dangerous combination.

Brain ischemia

Extended occlusion of arteries and arterioles can cause hypoxia and tissue death in the affected areas, more commonly referred to as ischemia. The most deadly of these occlusions occurs in the brain and causes a stroke, as minutes without oxygen can cause irreparable harm. Most of the thrombi that cause stroke are formed in the heart, aortic arch and carotid arteries. Several studies have found that stroke patients have higher levels of activated platelets due to increased beta-thromboglobulin, a protein released after platelet activation, compared to healthy individuals (Hoogendijk et al., 1979; Landi et al., 1990; Taomoto et al., 1983). Conversely, increased bleeding due to loss of platelet function can result in brain hemorrhage and increased intracranial pressure.

Anti-platelet therapies

Fortunately, there have been great advances in treating patients suffering from cardiovascular disease. The most well known anti-platelet medication is low dose aspirin. Aspirin, a non-steroidal anti-inflammatory drug (NSAID), irreversibly inhibits the cyclooxygenases that produce thromboxane, with a slightly higher affinity for the COX-1 isozyme. Because of the rapid turnover of platelets, low dose aspirin can prevent excessive thrombus formation without permanently disabling hemostasis. Another established class of anti-platelet drugs is thienopyridines, which are antagonists of the $P2Y_{12}$ receptor. The majority of these compounds, including clopidogrel and ticlopidine, irreversibly block the $P2Y_{12}$ receptor by binding covalently to the extracellular loops. This only partially blocks platelet function but reduces the size of the thrombi that are formed. Finally, integrin αIIbβ3 antagonists prevent fibrinogen from forming the thrombus matrix that is critical for aggregation. The three most commonly administered drugs (abciximab, eptifibatide, and tirofiban) all have relatively short half-lives in the blood stream and are used primarily in hospitalized patients to temporarily prevent aberrant blood clots. All of these anti-platelet therapies aid in preventing excessive thrombus formation from causing myocardial infarction, stroke, and ischemia. Despite the available therapies, there still remains a significant need for more targeted anti-platelet therapies in patients who do not respond to thienopyridines as well as more reversible treatments in patients who require surgery or other procedures with increased bleeding risks.

With the majority of the pharmaceutical focus on the $P2Y_{12}$ receptor and other major players in integrin activation, other studies have begun to unravel the role of the $P2Y_1$ receptor in platelet function. There is evidence that the $P2Y_1$ receptor desensitizes in human platelets, which may be physiologically relevant to homeostatic platelet function. The experiments in this

dissertation are aimed at testing the hypothesis that $P2Y_1$ receptor desensitization limits excessive thrombosis and maintains hemostatic homeostasis.

CHAPTER 2: DESENSITIZATION OF THE $P2Y_1$ RECEPTOR IN MULTIPLE HUMAN AND MOUSE MODELS OF PLATELET ACTIVATION

Introduction

Understanding thrombosis is the first step in determining how to control it. The tight regulation of clot formation and dissolution appears benign in the realm of scraped knees and bruises, but there are a myriad of points within platelet function and coagulation that can tip the scale towards either bleeding or thrombosis. Thrombosis plays a vital role in cardiovascular disease (CVD), which is the leading cause of death in the United States. CVD affects more than 85 million Americans as of 2013, with approximately 50% under the age of 60 (Mozaffarian et al., 2016). The primary end result of the process of hemostasis is a thrombus, which is comprised of platelets within a fibrous network that serves to plug the endothelium of a damaged blood vessel to prevent blood loss. Excessive thrombus formation can result in vessel occlusion, , causing ischemia of the surrounding tissues. When this occurs in the brain, it causes stroke. In adults with CAD, the vessels are at a higher propensity to form clots in the constricted space; complete blockage here results in a myocardial infarction (MI). Thus, the ability to prevent thrombus formation in patients at risk for stroke or MI is critical for long-term prognosis and overall health.

Thrombi generally form upon exposure to the extracellular matrix, which simultaneously disrupts inhibitory influences from the endothelial vessel walls on platelets and promotes pro-aggregatory pathways within platelets. Initial activation causes the release of other pro-

aggregatory factors from alpha and dense granules; release of these secondary mediators results in positive feedback on other nearby platelets to amplify the aggregation response and prevent excessive blood loss. The critical aspect of thrombus formation is localization, that is, containment of the positive feedback and aggregation to the injury site. If positive feedback is permitted to balloon out of control, the thrombus can grow to sizes that occlude the entire vessel and cause ischemia to tissues supplied by the affected artery. Furthermore, smaller thrombi from an oversized aggregate can embolize and flow downstream into smaller arterioles and capillaries, which can also result in tissue ischemia. Vessel occlusion is more prominent in patients suffering from atherosclerosis, whereby vessel diameters are greatly reduced because of fatty buildup and inflammation under the endothelial cell layer and increasing the likelihood of blockage.

There has already been significant scientific and clinical progress made in controlling thrombus formation. Aspirin inhibits cyclooxygenase that functions in the arachidonic acid pathway; in platelets this prevents the formation of thromboxane A2 (TxA_2), a potent platelet agonist. Many CVD patients are currently on a low-dose aspirin regimen, as this is an inexpensive yet effective anti-platelet therapy. The current gold standard of anti-platelet therapies, particularly those at higher risk of strokes and myocardial infarctions, is thienopyridines, which inhibit the $P2Y_{12}$ receptor and prevent ADP-induced platelet activation. This class of molecules includes clopidogrel, prasugrel and ticagrelor and functions by reducing the secondary amplification of platelet activation, thereby producing smaller thrombi, but activation via thrombin is unaffected – the platelets are still responsive to their primary activator and do not lose all function.

The current model of platelet activation focuses on Rap1B activity in platelets, which is described in more detail in Chapter 1. An emerging theory regarding the synergy between Gq

and Gi signaling hinges on the regulation of Rap1B activity – Gq signaling activates CalDAG-GEF1 and results in Rap1B activation, but this activation is transient and insufficient to result in integrin activation because of the inhibitory GAP activity of RASA3. Activation of the Gi signaling pathway results in the inhibition of the RASA3, thereby allowing for prolonged Rap1B activity. Only when the two pathways are activated simultaneously does sufficient Rap1B-GTP accumulate and promote downstream platelet activation.

Much of the work in developing antiplatelet therapies has focused primarily on direct integrin inhibition and optimizing the thienopyridines, but each of these therapies has limitations. For example, approximately 4-30% of the population is resistant to clopidogrel treatment (Nguyen et al., 2005). Therefore, recent interest has shifted to other potential targets that influence platelet activation; one such target is the $P2Y_1$ receptor. The role of the $P2Y_1$ receptor in platelet aggregation was first reported by Gachet and colleagues by assessing the pharmacological selectivity of $P2Y_1$ receptor inhibitors in blocking ADP-promoted platelet aggregation (Hechler et al., 1998). A subsequent study analyzing mice lacking CD39, an ATP diphosphohydrolase expressed on endothelial cells, reported an unusual result: mice without CD39, instead of displaying an expected hyperaggregatory phenotype due to the lack of ATP/ADP metabolism, actually showed a loss of platelet aggregation in response to ADP (Enjyoji et al., 1999). This loss of platelet aggregation was subsequently shown to be due to loss of $P2Y_1$ receptor activity, suggesting that the higher levels of ADP in the blood promoted desensitization of the receptor and a loss of ADP-promoted aggregation. These results suggest that $P2Y_1$ receptor-selective antagonists could be developed as an alternative anti-platelet therapy. Also, targeting secondary activators of aggregation are preferable because they are less potent compared to thrombin-based and collagen-based activation, which can reduce excessive

bleeding and increase reversibility of platelet reactivity under extenuating circumstances (e.g., surgery).

Bourdon et al. showed that the $P2Y_1$ receptor on human platelets desensitized rapidly in the presence of the $P2Y_1$ receptor-selective agonist, MRS2365, with a half-life of the loss of receptor activity of ~18 sec, which is extremely rapid for a GPCR (Bourdon et al., 2006). Based on the observations that the platelet $P2Y_1$ receptor undergoes rapid desensitization, we hypothesize that this property is both physiologically relevant and important in normal homeostasis. We further hypothesize that rapid desensitization of the $P2Y_1$ receptor in platelets provides both an early reversible brake that prevents unwanted aggregation, thereby saving the platelet after a shape change event that does not ultimately result in a clot, and by limiting excessive thrombus formation by preventing aggregation in the loose outer sphere of a platelet clot at sites of vascular injury. Because the $P2Y_1$ receptor is the initiator of aggregation, its desensitization is expected to limit excess thrombosis and thus maintains proper hemostasis. The experiments in this dissertation are aimed at testing this hypothesis and elucidating the physiological importance of $P2Y_1$ receptor desensitization.

Materials and Methods

Mouse studies

$P2Y_1^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred to C57/BL6J mice (Jackson Laboratories) to maintain genomic heterogeneity. Mice were housed in hot-washed cages under a 12-h light/dark cycle and provided food and water *ad libitum*. All protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill.

Reagents

The $P2Y_1$ receptor agonist MRS2365 was purchased from Tocris Biosciences (Bristol, UK). The TxA_2 receptor agonist U46619 was acquired from Cayman Chemicals (Ann Arbor, MI, USA). ADP, serotonin, and epinephrine-HCl were purchased from Sigma Aldrich (St. Louis, MO, USA). The phycoerythrin (PE)-labeled JON/A antibody was kindly provided by Dr. Wolfgang Bergmeier in the Biochemistry Department at the University of North Carolina at Chapel Hill. Monoclonal antibody against the hemagglutinin tag (Covance, Princeton, NJ, USA) was labeled with AlexaFluor 647 dye as previously described (Bergmeier et al., 2002).

Human whole blood aggregation

Adult human subjects (>18 years of age) weighing more than 110 pounds who had not donated blood within the previous 4 weeks were eligible for this study. Exclusion criteria were either pregnancy or ingestion of one of the following drugs within 72 hours of donation: aspirin, acetaminophen, ibuprofen, naproxen, or any other antiplatelet medications (e.g., clopidogrel, etc.). Enrolled subjects provided written informed consent. After sterilizing the arm and applying the tourniquet, an 18G needle was inserted into either the basilic or the cephalic vein depending on the anatomy of the subject. Blood was collected by gravity into a sterile 50 mL conical tube containing 3 mL heparin sulfate (100 U/mL) to a total volume of 30 mL. The blood-heparin mixture was gently inverted and placed at 37°C until further testing.

For each sample, 500 μL of whole blood was combined with sterile 0.9% saline and incubated in a cuvette (Chrono-Log, Havertown, PA) containing a magnetic stir bar (Chrono-Log) at 37°C for approximately 5 minutes. Prior to data acquisition, the samples were placed in the 592A aggregometer (Chrono-Log) and recorded for 1 minute to record baseline; any sample that did not pass the baseline was repeated until achieved. Drugs were added at either 19× or 20×

depending on the time of drug addition and number of adds for the sample. The data were recorded for 4 minutes after the final drug addition to allow for slower activation, and the final volume after all additions was 1 mL.

Mouse whole blood aggregation

Male and female mice were anesthetized under 3.5% isoflurane for 3-4 minutes with 1.5% O_2 and bled via retro-orbital capillary insertion into a microcentrifuge tube containing 30 U/mL of heparin. Approximately 600 µL of blood per animal was collected, and blood from mice of the same genotype was pooled into a 15 mL conical tube, which was stored at 37°C.

For each sample, 300 µL of whole blood was combined with sterile 0.9% saline and incubated in a cuvette containing a stir bar at 37°C for approximately 5 minutes. Prior to data acquisition, the samples were placed in the 592A aggregometer and the baseline recorded for 1 minute; samples that yielded unstable baselines were repeated until achieved. The data were recorded for 4 minutes after the final drug addition to allow for slower activation, and the final volume after all additions was 1 mL.

Washed mouse platelets

Mice were anesthetized as previously described, and either 350 µL or 525 µL was collected via retro-orbital capillary insertion into a microcentrifuge tube containing 150 µL or 225 µL, respectively, of 30 U/mL of heparin solution. After the mouse recovered, the blood was centrifuged at 130 g for 5 minutes with the brake off to maximize separation of the red blood cells and plasma layers. After transfer of the plasma layer and top of the RBC layer into a new microcentrifuge tube, the mixture was centrifuged at 100 g for 5 minutes with the brake off. The plasma layer was then collected and transferred into a fresh microcentrifuge tube, after which it was centrifuged at 700 g for 5 minutes. The supernatant was carefully discarded, and the platelet

pellet was resuspended in 1 mL of Tyrode's buffer containing 1 mM Ca^{2+}, 0.2 U/mL of apyrase (Sigma) and 2 μg/mL of prostaglandin (Cayman Chemicals). The platelets were incubated at 37°C for 5 minutes and centrifuged at 700 g for 5 minutes. The supernatant was discarded, and the platelet pellet was resuspended in approximately 50-70 μL of Tyrode's buffer containing 0.2 U/mL of apyrase and 2 μg/mL of prostaglandin I2. To determine the platelet count, 1 μL of the platelet solution was diluted into 999 μL of sterile-filtered PBS, and 25 μL of this solution was run through the BD Accuri C6 flow cytometer (Becton Dickinson, San Jose, CA, USA).

Mouse integrin activation

Washed platelets were resuspended to a concentration of 5 x 10^8 per mL in Tyrodes buffer containing 1 mM Ca^{2+} and 0.2 U/mL apyrase as a concentrated platelet suspension. For each sample, 2.5 uL of platelets were added to Tyrode's buffer containing 1 mM Ca^{2+}. Each of the drugs added to the samples were made at 4 to 5× depending on the time of the drug addition and number of additions per sample. After the final drug addition (to bring the reaction volume to 25 μL), the samples were incubated at room temperature for 1 minute, after which 5 μL of phycoerythrin (PE)-labeled JON/A antibody was added to each sample to a final concentration of 0.75 μg/mL. The samples were incubated for an additional 9 minutes at room temperature and diluted with 1 mL of sterile-filtered PBS to halt the reaction. The samples were read on the Accuri C6 until 10,000 events within the platelet-defined gate were collected.

Statistical analysis

Data are presented as the mean ± standard deviation of the respective measurements. Two-way analysis of variance (ANOVA) was used to compare multiple groups. A p-value less than 0.05 was defined as statistically significant. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, LaJolla, CA, USA).

Results

$P2Y_1$ vs. $5HT_{2A}$ receptor desensitization in human platelets

To determine if previous desensitization of the $P2Y_1$ receptor in washed human platelets could be recapitulated in whole blood, samples were pretreated with 3 μM MRS2365 for various amounts of time before adding 10 μM ADP. The results show that there is a similar loss of signaling capabilities in response to ADP following longer pretreatments of MRS2365 (Figure 2-1) as was observed with washed platelets by Bourdon *et al*. The average half-life for desensitization of $P2Y_1$ receptor signaling was 13.12 seconds. To determine if another Gq-coupled receptor could desensitize on this time scale, platelets were pretreated with serotonin to activate the $5\text{-}HT_{2A}$ receptor, followed at various time intervals by addition of epinephrine, which activates the Gz-coupled α2-adrenergic receptor. Unlike the $P2Y_1$ receptor, the $5\text{-}HT_{2A}$ receptor showed little to no desensitization upon pre-treatment with serotonin (Figure 2-1). Significant differences between the serotonin-pretreated platelets and MRS2365-pretreated platelets were observed at 15, 30, 60, and 120 seconds ($p<0.05$, $p<0.01$, $p<0.0001$, and $p<0.01$, respectively). After 2 minutes of serotonin pretreatment before addition of epinephrine, the platelets retained approximately 81% of the maximum serotonin-epinephrine response. This is significantly higher than the responsiveness of $P2Y_1$-pretreated platelets (37% of maximum ADP response; $p<0.01$). These data suggest that desensitization of the $P2Y_1$ receptor in platelets is specific to the $P2Y_1$ receptor and does not occur with other Gq-coupled receptors.

$P2Y_1$ receptor desensitization in mouse platelets

Mouse platelet aggregometry

Because of the difficulty in biochemically manipulating human platelets as well as the lack of a strong platelet-like cell culture system, the use of mice was considered necessary for

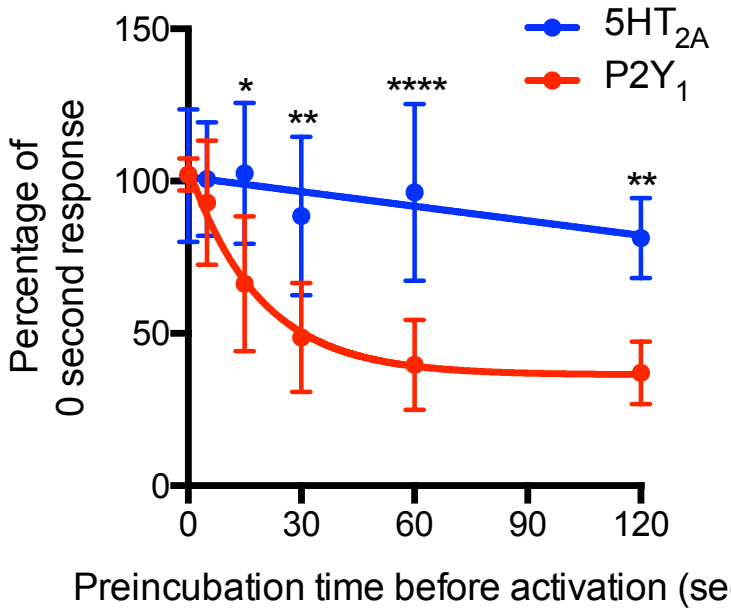

Figure 2-1. Gq-based desensitization in human whole blood aggregation.

Platelets were pretreated with either 3 μM MRS2365 ($P2Y_1$) or 10 μM serotonin ($5HT_{2A}$) prior to the addition of 10 μM ADP and 5 μM epinephrine, respectively. The data are presented as the mean ± standard deviation of the percentage of the amplitude at 0 seconds of pretreatment. *p<0.05; **p<0.01; ****p<0.0001. N = 6.

future experiments. To test the validity of a mouse model, washed mouse and human platelets were pretreated with MRS2365 for various amounts of time prior to the addition of ADP. The data show that mouse platelets desensitize to ADP activation in a similar rapid manner as human platelets (Figure 2-2), indicating that $P2Y_1$ receptor desensitization also occurs in mouse platelets. To validate that the loss of aggregation was due to desensitization of the $P2Y_1$ receptor in mouse platelets and not some other mechanism, a rescue experiment was performed using serotonin as an alternate activator of the Gq signaling pathway. As shown in Figure 2-3, the aggregation response to ADP was significantly decreased when the blood was pretreated with 3 μM MRS2365 for 90 seconds ($p<0.0001$). Furthermore, when serotonin was co-administered with ADP, the loss of aggregation observed with the MRS2365 pretreatment was abolished. These same experiments were performed in $P2Y_1^{-/-}$ mice, which showed that the combination of ADP + serotonin was capable of promoting aggregation, whereas ADP alone was without effect. These results bolster the credibility of using mouse blood in place of human blood in studying the desensitization of the $P2Y_1$ receptor in platelets.

Flow cytometry-based integrin activation of mouse platelets

With the disadvantage of a low blood volume, studying hemostatic properties using mice can result in requiring large broods of animals with few data points. To bypass this limitation, an assay using flow cytometry and the JON/A monoclonal antibody, which binds specifically to the activated conformation of mouse integrin αIIbβ3, requires <300 μL of blood but can provide dozens of data points in triplicate. Thus, we assessed whether $P2Y_1$ receptor desensitization could also be observed in washed platelets using this flow cytometry assay. Figure 2-4 shows that the mouse $P2Y_1$ receptor in platelets desensitizes upon pretreatment with MRS2365 prior to the addition ADP in the flow cytometry assay. Although the loss of ADP-promoted activation of

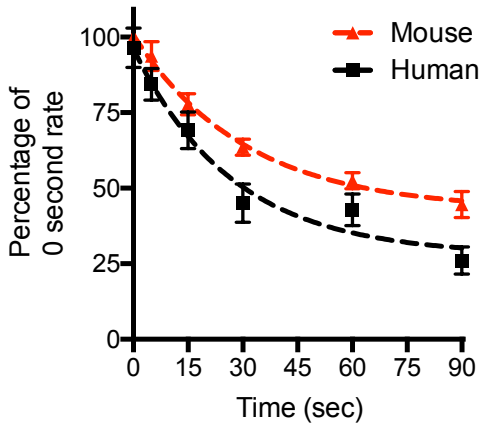

Figure 2-2. Comparison of $P2Y_1$ receptor desensitization in mouse and human platelets.

Washed platelets from either mouse or human platelets were pretreated with MRS2365 prior to the addition of ADP. The data are presented as the mean ± standard deviation of the percentage of the rate of aggregation at 0 seconds of pretreatment. $t_{1/2}$(mouse) = 21.25 sec; $t_{1/2}$(human) = 18.72 sec. N = 12 for human; N = 9 for mouse.

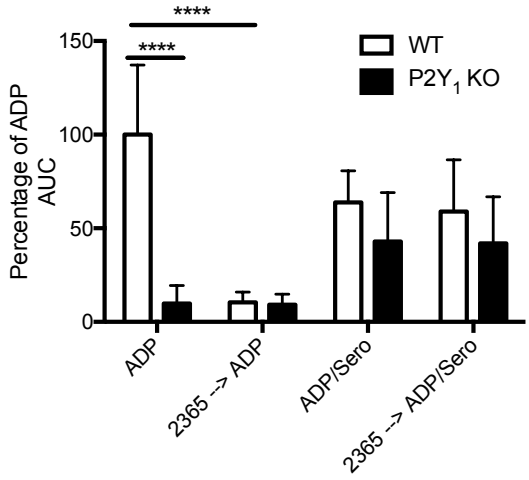

Figure 2-3. Whole blood aggregation response of mouse platelets.

ADP, 10 μM ADP. 2365 → ADP, 90 second pretreatment of 3 μM MRS2365 followed by 10 μM ADP. ADP/Sero, 10 μM ADP and 10 μM serotonin. 2365 → ADP/Sero, 90 second pretreatment of 3 μM MRS2365 followed by 10 μM ADP and 10 μM serotonin. The data are presented as the mean ± standard deviation of the percentage of the amplitude at 0 seconds of pretreatment. ****$p<0.0001$. N = 6 for WT; N = 7 for $P2Y_1^{-/-}$.

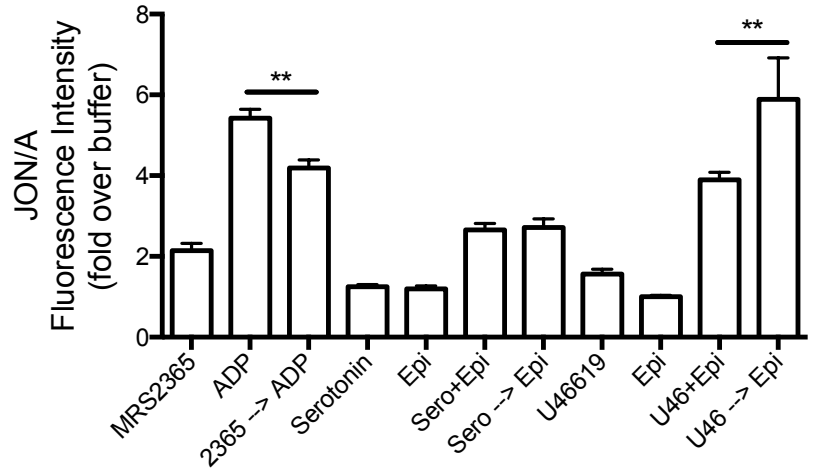

Figure 2-4. Measurement of mouse integrin activation using several secondary activators of platelet aggregation.

MRS2365, 3 μM; ADP, 10 μM; 2365 → ADP, 90 second pretreatment with MRS2365 before ADP addition; Serotonin, 10 μM serotonin; Epi, 5 μM epinephrine; Sero+Epi, 10 μM serotonin and 5 μM epinephrine; Sero → Epi, 90 second pretreatment with serotonin before epinephrine addition; U46619, 1 uM U46619; U46+Epi, 1 μM U46619 and 5 μM epinephrine; U46 → Epi, 90 second pretreatment with U46619 before epinephrine addition. **$p<0.01$. N = 5.

αIIbβ3 is decreased upon MRS2365 pretreatment and is significant ($p<0.01$), it is not as pronounced as the decrease observed in the whole blood and washed platelet aggregometry. Additionally, 90-sec pretreatment with either serotonin or U46619 (a stable thromboxane analog) did not show any loss of integrin activation after addition of epinephrine; interestingly, the U46619 pretreatment alone resulted in a significant increase in integrin activation ($p<0.01$) compared to the simultaneous addition of U46619 and epinephrine. Based on these results, we concluded that the JON/A flow cytometry assay was sufficient for measuring $P2Y_1$ receptor desensitization.

Discussion

Understanding the mechanism of receptor regulation has been an evolving and active area within the GPCR field. A large percentage of commercially available drugs currently target GPCRs, but elucidating how these proteins are regulated within different tissues could result in the development of more effective treatments. An example of this tissue-specific regulation is the $P2Y_1$ receptor, which does not desensitize in cultured cell lines [e.g., HEK293, Madin-Darby canine kidney (MDCKs), and C6 glioblastoma cells] but desensitizes in platelets on the order of seconds. Furthermore, we show that this desensitization occurs in physiological blood conditions and is not observed with the $5HT_{2A}$ receptor.

It was important to show that the loss of ADP-promoted platelet aggregation following pre-activation of the $P2Y_1$ receptor occurs in the most physiologically relevant system available. The original study reporting this phenomenon used washed platelets (Bourdon et al., 2006), which are devoid of many plasma proteins and molecules. Here we quantify desensitization of the platelet $P2Y_1$ receptor by measuring aggregation of washed platelets and in whole blood, as well as measuring platelet activation by flow cytometry with an antibody detecting the active

conformation of the αIIbβ3 integrin complex. Furthermore, we showed that $P2Y_1$ receptor desensitization is observed in both mouse and human platelets, which opens the door for biochemical and genetic manipulation of mouse platelets. Interestingly, the degree of desensitization was dependent on the method used to quantify this phenomenon; the most striking difference was observed in the JON/A flow cytometry assay. Although we cannot say for certain why this is so, one possibility for the smaller levels of desensitization determined by flow cytometry is that the extremely high affinity of the JON/A antibody to the active conformation of the integrin αIIbβ3 complex may mask a graded aggegratory state of platelets. That is, the activation state of the αIIbβ3 integrins may be in conformational equilibrium, with the integrin shifting in and out of its active state. Following MRS2365 treatment, the αIIbβ3 integrins could be rapidly fluxing in and out of the active state, but the high affinity of the antibody for the active conformation could irreversibly lock the integrin into its active conformation. Thus, the flow cytometry assay may not accurately reflect the actual number of integrin complexes that are stably activated and can bind fibrinogen in a temporal aggregation assay. Nonetheless, only pretreatment of mouse platelets with ADP showed any loss of maximal integrin activation and aggregation compared to pretreatment of platelets with $5HT_{2A}$- and TxA_2-selective agonists (followed by epinephrine to activate Gz-coupled α2-adrenergic receptors), suggesting that the desensitization of the $P2Y_1$ receptor in platelets is unique in both mice and humans.

Conclusions

The data presented here strongly support the claim that $P2Y_1$ receptor desensitization is not only specific to this receptor compared to other Gq-coupled receptors expressed in platelets, but that this desensitization occurs in mouse platelets as well as human platelets. Furthermore,

the murine $P2Y_1$ receptor shows similar specificity regarding desensitization compared to the other Gq-coupled receptors. Because of the observed desensitization activity using the labeled JON/A antibody, the flow cytometry assay can be used to measure this phenomenon using small populations of mouse platelets – this is critical for measuring integrin activity in platelets from a potential mouse model that introduces mutant $P2Y_1$ receptors, as described in Chapter 3.

CHAPTER 3: OPTIMIZATION OF A VIRAL TRANSDUCTION MODEL TO EXPRESS MUTANT $P2Y_1$ RECEPTORS IN BONE MARROW CELLS

Introduction

Model Systems

One of the overarching themes of scientific discovery is the understanding of the human body – development, function, and pathogenesis. Because of the ethics and complications involved to study diseases in humans, researchers rely on model systems to translate various biological processes to human analogs. Model animals can vary widely depending on the process studied, from *Saccharomyces cerevisiae* to zebrafish to primates. As the species being studied becomes more evolved, increasing ethical standards are applied, with all research institutions housing an internal committee known as the Institutional Animal Care and Usage Committee (IACUC) to oversee all animal research and work closely with government agencies to ensure animal safety and wellbeing.

Regarding disease states and pathogenesis, the most prominent animal model is the common house mouse, as they breed rapidly and share approximately 90% of their genome with humans (Consortium et al., 2002). Although mice were initially used to determine cause and effect, genetic manipulation has allowed mouse models to become a powerful tool in a researcher's repertoire. The earliest mouse studies involved the administration of a drug or

treatment followed by monitoring of vital signs and behaviors. However, the advent of genetic engineering of mice has allowed scientists to perform biochemical experiments *in vivo* and to study the effects of knocking out a specific protein in an animal versus *in vitro* cell culture, the previous standard method for understanding cell function upon loss of a gene product. First established in 1981, genetically modified mice were the perfect tool to translate test tube and bench science into possible therapeutic targets for clinical applications (Gordon and Ruddle, 1981). Further developments by Oliver Smithies, Mario Capecchi and Martin Evans resulted in the ability to target specific genes within the genome to create "knock-out mice" (Thomas and Capecchi, 1987), a discovery that was awarded with the Nobel Prize in Physiology & Medicine in 2007. With the mouse genome fully decoded in 2002 (Consortium et al., 2002), the technology of genetically engineered mice has evolved from simple global gene knockout into sophisticated targeting that can control gene expression in specific tissues at predetermined times.

The first instance of DNA modification in a mouse came from Jaenisch and colleagues in 1974, who reported that when mouse embryos were injected with a DNA-based virus, the entire animal globally expressed the foreign DNA (Jaenisch and Mintz, 1974). However, these mice were incapable of passing the foreign DNA to their offspring, as the germ line was unaffected. Some years later, other researchers were able to inject purified DNA directly into the blastocyst of mouse embryos. This method not only proved successful in the incorporation of non-native DNA into the genome but also allowed for the passage of the DNA into the offspring of these transgenic animals (Brinster et al., 1981; Costantini and Lacy, 1981). DNA could be randomly inserted into the mouse genome to create modified mice that either expressed foreign proteins or overexpressed native proteins.

However, scientists were also interested in the physiological processes in animals lacking certain proteins. In 1987, two groups of researchers utilized homologous recombination to recombine large fragments of DNA into the native chromosome at the locus site. The design of the construct allowed for the selection of positively modified cells, but more importantly could be used to alter or eliminate a gene at its natural locus, thus creating "knock-out" mice. When the mouse genome was fully sequenced, scientists could knock out any gene they wished.

One significant downside of global gene knockout is the limit to the types of genes that can be studied using this system. For example, genes essential for development and survival such as actin and β-arrestin are embryonic lethal if knocked out in a homozygous manner. This phenotype both excites and frustrates researchers, as reaching an understanding as to why these types of proteins are critical would be illuminating, but the optimal tool for studying their importance is unavailable. This changed when the Cre-recombinase mouse was created in 1992 (Orban et al., 1992). Cre recombinase is an enzyme that targets a 10 base pair sequence (known as a loxP site) in the genome and cleaves it; however, the recombinase only functions when there are two loxP sites in relative proximity. When this is met, the enzyme cuts at the two loxP sites and "stiches" them together, thereby removing the intervening sequence. The creation of mice that express Cre recombinase in selective tissues provided a method to study a gene in a specific tissue type without affecting the other organ systems of the mouse and thus allowed researchers in many cases to leave the gene untouched in tissues that lead to the block in development.

Another method of controlling gene expression in mice is the introduction of tetracycline-controlled transcriptional activation. By introducing a tetracycline response element (TRE) directly upstream of the open reading frame of the gene, often the Cre recombinase, researchers can treat the mice with either doxycycline or tetracycline to either promote or inhibit gene

expression, depending on the design of the transactivator (tTA) protein. The "Tet-On" system only expresses the gene in the presence of the antibiotic, whereas the "Tet-Off" system prevents gene expression under the same conditions. The implementation of this system was first described in 1992 (Gossen and Bujard, 1992) and has been used to study developmentally required genes that are embryonic lethal.

More recently, scientists have taken advantage of a bacterial defense mechanism known as CRISPR/Cas to rapidly create gene knock-outs, knock-ins, and other genetic manipulations in mice. This is a prokaryotic immune system that eliminates any inserted foreign DNA (e.g., bacteriophages, plasmids) and protects the bacterium from future attacks. CRISPR is a series of short repeating sequences separated by spacers that are generated by previous encounters with foreign DNA. When a CRISPR region is transcribed, the resulting RNA is used in conjunction with Cas9 (an endonuclease) to cleave the invading DNA or RNA. The precision of this system has allowed researchers to target specific regions in the mouse genome for excision to produce highly targeted deletions, insertions, or mutations at the native locus.

Platelet study

In studying different tissue types, most scientists use cell lines derived from the tissue in question and perform experiments *in vitro* to acquire a basic understanding of their particular interest before investing into animal models because cell culture is less expensive and can be more easily manipulated. However, there are several tissue types that do not lend themselves to cell culture, a prime example of which are platelets. Platelets are small anuclear protein sacs that arise from the maturation and breakdown of megakaryocytes in the bone marrow, a process described in more detail in Chapter 1. They have a life span of 7-10 days in the bloodstream and are incapable of mitosis. The closest cell culture model to platelets is the use of megakaryocyte

cell lines, which are primarily derived from cancer cells and have different protein expression patterns compared to mature platelets. Thus, platelet studies require the use of whole blood from either human subjects (which cannot be molecularly manipulated in the same manner as cell culture and are generally lacking in any sort of mutations) or animal subjects. Despite the fact that platelet-specific knockout mice can be generated using Pf4-Cre mice (Tiedt et al., 2007), there still remains an inability to study the effect of mutant proteins in platelet biology without creating a new mouse, which can be expensive both in cost and time.

The study of bone marrow transplantation has helped to provide treatment options in patients suffering from blood diseases such as leukemia. Implementing total body irradiation (TBI) at specific doses and time intervals can successfully destroy the existing bone marrow without having a significant effect on other vital organs (e.g., heart, lung, brain). This procedure, combined with the introduction of healthy bone marrow cells, is the gold standard of treatment for leukemia and other blood disorders. This technique can also be performed in mice, allowing for the replacement of the original bone marrow with a different source of hematopoietic stem cells to either correct or introduce a novel population of blood cells. Because of advances made in culturing primary cells, it is now possible to remove bone marrow cells from a mouse and culture the cells *in vitro* to promote sustained (or even enhanced) pluripotent hematopoietic stem cell growth. Thus, the application of cell culture techniques with these primary cells may allow for the genetic manipulation of platelets that are usually afforded only to cell culture.

Studies in our lab have focused on agonist-promoted internalization and the role of phosphorylation in this process in a variety of cultured cell lines (Qi et al., 2011). However, receptor desensitization is not observed in any of these cell lines with the rapidity observed in platelets, thus leading to the question of why $P2Y_1$ signaling is drastically reduced seconds after

activation in platelets but not cell culture. Based on our data that phosphorylation of Ser residues in the C terminus of the $P2Y_1$ receptor are critical for receptor internalization in cultures cell lines, we hypothesized that phosphorylation of Ser/Thr amino acids on the C-terminus also may play a crucial role in $P2Y_1$ receptor desensitization observed in platelets. To test this hypothesis, a mouse model was developed to introduce mutant constructs of the $P2Y_1$ receptor into platelets to study the effects of these mutations in a mouse system. Here, we highlight viral production efficacy, bone marrow culturing and transplantation procedures to maximize the uptake of virally transduced bone marrow into recipient animals as well as the functionality of platelets from successfully transduced chimeric mice. To best study the effects of the introduction of this protein, we are using mice that have a global $P2Y_1$ receptor knockout to ensure that all measured effects are due to the introduction of this protein into the platelets.

Materials and Methods

Reagents

Dulbecco's Modified Eagle medium containing 4.5 g/L glucose (DMEM-H), Iscove's Modified Dulbecco medium (IMDM), fetal bovine serum (FBS), newborn calf serum (NCS) penicillin/streptomycin (P/S) and phosphate buffered saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cytokines interleukin 3 (IL-3), interleukin 6 (IL-6) and murine stem cell factor (mSCF) were purchased from Peprotech (Rocky Hill, NJ, USA).

Plasmids

The pMigRI (Addgene plasmid # 27490) and pCL-Eco (Addgene plasmid # 12371) plasmids were kindly provided by Dr. Wolfgang Bergmeier from the University of North Carolina at Chapel Hill. The multiple cloning site (MCS) of pMigR1 between 1411-1447 bp was

reconfigured to include the restriction sites (5' to 3') EcoRI, XhoI, BamH1 and NotI. The plasmids were verified by restriction enzyme digestion.

Animals

C57BL/6 wild type mice and mice globally lacking the $P2Y_1$ receptor ($P2Y_1^{-/-}$) were acquired from Jackson Laboratories (Sacramento, CA, USA) and bred in house to maintain sufficient populations. $P2Y_1^{-/-}$ mice were backcrossed to wild type mice once a year to prevent excessive inbreeding and to create similar genetic backgrounds for comparison. The mice were housed in micro-washed cages under a 12-h light/dark cycle and provided food and water *ad libitum*. All protocols and procedures were approved by the International Animal Care and Usage Committee at the University of North Carolina at Chapel Hill.

PCR and cloning of the $mP2Y_1$ receptor

Genomic DNA from a wild type C57BL/6 mouse (Jackson Laboratory) was extracting using the PureLink Genomic DNA Mini kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. PCR was carried out using Pfu polymerase and the following primers: forward, 5'- GACTACGCGTTGAGTCTCTCGCCGCTGCT-3'; reverse, 5'- GACTCTCGAGTGCCTTCACAAACTCGTGTC-3'. The resulting fragment was purified and digested with MluI and XhoI (sites underlined in the aforementioned primers) prior to ligation into a pLXSN vector containing a hemagglutinin (HA) tag immediately upstream of the MluI site within the vector; this allowed for the addition of the HA tag onto the 5' end of the $P2Y_1$ receptor. Once cloned into the pLXSN vector, it was subsequently digested with EcoRI and XhoI to allow for ligation into the pMigRI vector. After transformation into chemically competent *E. coli*, the bacteria were grown on LB agar plates containing carbenicillin to select for cells harboring the recombinant plasmids. Success of the insertion was verified by sequencing.

To create a mutant form of the mP2Y$_1$ receptor that lacked phosphorylatible residues on the C-terminus, we created two primers that mutated all Ser and Thr residues to Ala downstream of Thr339 (P2Y$_1$ 340-0P), as previous studies in cell culture systems had shown that phosphorylation of Ser and Thr on the C-terminus downstream of Thr339 is critical for agonist-dependent internalization of the P2Y$_1$ receptor (Qi et al., 2011). These primers incorporated the necessary point mutations, and the differences between the two receptors are illustrated in Figure 3-1.

Virus production

Virus production in cell lines requires that they express gag-pol to convert transfected plasmid into functioning virus. Because the gag-pol elements are located trans to the target plasmid, the virus is considered to be "replication incompetent"; that is, once the virus infects a cell, it is unable to complete the replication cycle after incorporating its DNA into the host cell genome. Here, we describe the procedure used to form virus and the optimal conditions that produce the greatest viral titer. We used the pMigRI retroviral plasmid as the target plasmid because it has been previously shown to transduce bone marrow cells.

Cells

HEK293T cells that stably overexpress integrins α_v and β_3 (TAB22 cells) were kindly provided by Dr. John Olson from the University of North Carolina at Chapel Hill. The expression of these integrins allow for greater adherence of the cells to the culture dish compared to regular HEK293T cells, as viral production requires several liquid removal and application steps. TAB22 cells were grown in DMEM-H containing 10% FBS and 1× penicillin/ streptomycin (1× P/S) in a humidified incubator at 37°C with 5% CO_2.

wt: FRRRLSRATRKASRRSEANLQSKSEEMTLNILSEFKQNGDTSL
340-0P: FRRRLSRATRKAARRAEANLQAKAEEMALNILAEFKQNGDAAL

Figure 3-1. Differences in the C-termini of the wild type $P2Y_1$ receptor and mutant 340-0P $P2Y_1$ receptor.

All Ser/Thr residues are indicated in red; all mutations created are shown in green. The PDZ domain is indicated in blue.

Mouse 3T3 cells were kindly provided by Dr. Klaus Hahn at the University of North Carolina at Chapel Hill. These cells were grown in DMEM-H containing 10% FBS and 1× P/S in a humidified incubator at 37°C with 5% CO_2.

Transfection

On day 1, 6.5-7 x 10^6 TAB22 cells were plated in a 10 cm tissue culture-treated dish (BD Falcon) and incubated overnight. One dish produces enough virus to infect 5 million bone marrow cells and ideally should be approximately 80-90% confluent the following day. On day 2, a total of 30 μg of target and helper plasmid DNA was combined with 9 μg of VSVG in a solution containing 250 mM $CaCl_2$ at a volume of 300 μL per 10 cm plate. The ratio of target to helper plasmid DNA was adjusted to determine the optimal conditions that would produce the best viral titer. The following ratios of pMigRI:pCL-Eco were tested: 1:1, 1.5:1, 2:1 and 2.5:1. To the $CaCl_2$-DNA mixture, 300 μL of 2× HEPES-buffered saline (HBS) was added drop-wise and gently agitated to promote the formation of a Ca^{2+}/DNA precipitate. After incubating at room temperature for 30 minutes, 600 μL of the DNA solution was added drop-wise to cells in a 10 cm plate in which the media had been replaced with 6 mL of DMEM-H containing 6% FBS and 1× P/S. The plates were incubated overnight at 37°C in 5% CO_2. On day 3, the media on the plates was replaced with 8 mL DMEM-H containing 2% FBS, 1× P/S and 10 mM sodium butyrate to promote virus production.

Viral harvest

On day 4, the medium from the plate was collected and filtered through a 0.45 μm PES filter and spun at 5000 g for 22 hours at 4°C in a swinging bucket rotor. This slower speed allowed the virus to be concentrated without damaging the pCL-Eco vector-based protein coating, which is less stable than other protein coats. The resulting viral pellet was resuspended

in 1 mL of IMDM and either frozen at -80°C until further use or applied immediately to bone marrow cells.

Viral titer testing

Mouse 3T3 cells were seeded at a density of 1.5×10^5 cells in a T-25 flask (Corning, source) in DMEM-H containing 10% FBS and 1× P/S and incubated overnight at 37°C with 5% CO_2. The following day, the media was removed and replaced with 1.5 mL of DMEM-H containing 8 μg/mL of Polybrene, which neutralizes the repulsion charge on the cell surface and improves the infection rate, and one of the following volumes of concentrated virus: 500 μL, 250 μL, 125 μL, or 50 μL. The cells were incubated at 37°C for 4 hours followed by removal of the virus and the addition of fresh DMEM-H containing 10% FBS and 1× P/S. The cells were then incubated for an additional 72 hours.

Prior to removal from the plates, the cells were imaged to qualitatively assess the infection efficiency of each treatment. After imaging, the cells were washed with PBS and treated with 5 mM EDTA in PBS to gently lift the cells from the plate. The cells were then centrifuged at 1200 rpm for 5 min and resuspended in 2 mL of PBS. These cells were then analyzed for GFP expression via the UNC flow cytometry core facility.

Collection and enrichment of hematopoietic stem cells from bone marrow for viral infection

The two primary sources of murine hematopoietic stem cells are fetal livers (usually taken between E14-E18) and bone marrow cells (BMCs) of young adult mice (6-8 weeks). Here, we discuss the procedure used to collect and enrich BMCs to promote the pluripotency of HSCs. A full diagram of the process to collect, transduce and transplant these BMCs is shown in Figure 3-2.

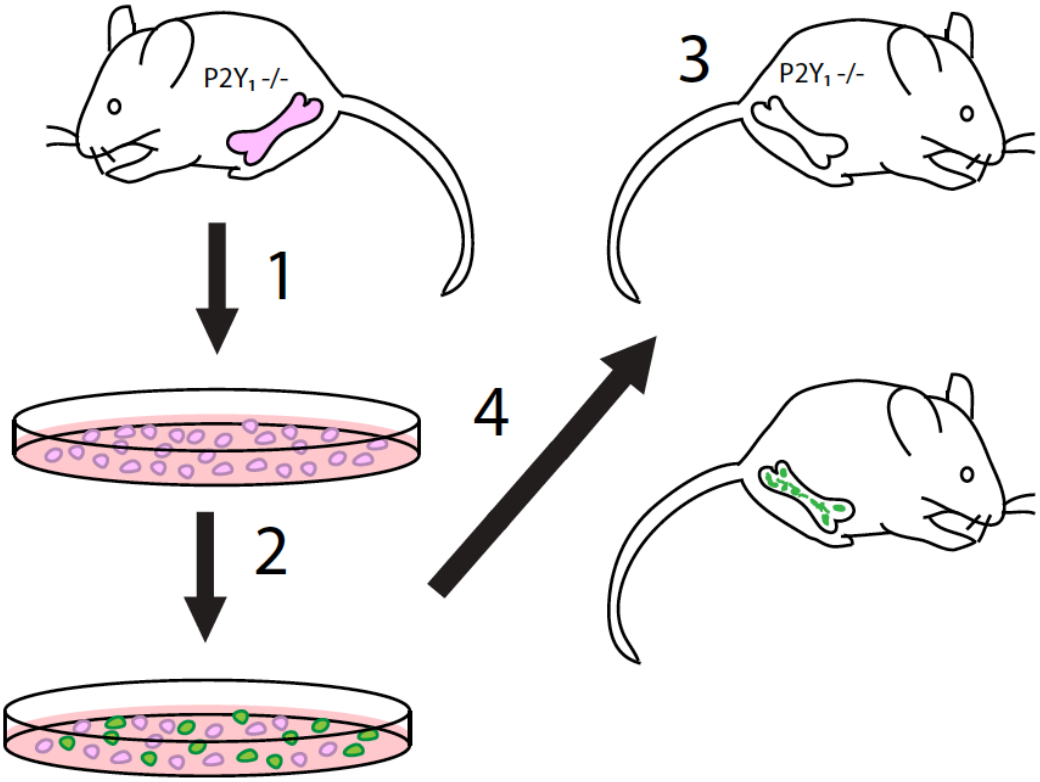

Figure 3-2. Schematic of mouse model system used to create chimeric mice expressing various types of $P2Y_1$ constructs in platelets.

The bone marrow from a $P2Y_1^{-/-}$ mouse is harvested (1) and plated in BMC medium containing IL-3, IL-6, and mSCF to promote pluripotency of hematopoietic stem cells. After incubation overnight, the cells are transduced with virus containing a plasmid that expresses both GFP and the $P2Y_1$ construct of interest (2). Concurrently, recipient $P2Y_1^{-/-}$ mice are irradiated to deplete existing bone marrow (3) and administered antibiotics to assist in fighting infection. The majority of the transduced bone marrow cells are injected into the irradiated mouse (4), and the mice are allowed to recover for at least 4 weeks prior to blood cell measurement to determine the effectiveness of the engraftment. At 48 hours post injection, remaining BMCs are run on flow cytometry to determine the percentage of BMCs transduced (72 hours post-infection).

Bone marrow harvest

A C57BL/6 $P2Y_1^{-/-}$ mouse (aged 6-8 weeks) was anesthetized with 3.5% aerosolized isoflurane (source) until there was a lack of response from multiple toe pinches. The mouse was then cervically dislocated and sprayed with 70% ethanol prior to dissection. The skin and fur were stripped from the hind legs of the animal to expose the muscles, and the femur was dislocated from the hip socket. The legs were then cut away from the main body, and the feet were removed with scissors. The muscle tissue was gently stripped away from the bones using surgical scissors and a scalpel to avoid any damage to the bone. Once completely stripped, the patella was removed and discarded to produce a clean femur and clean tibia. The hip socket was then cut off to expose an opening on the lateral end of the femur, and a 25G needle on a 10 mL syringe containing wash buffer (PBS containing 2% NCS, sterile filtered) was inserted into one of the exposed openings on the ends of the femur or tibia. Approximately 2-3 mL of wash buffer was used to flush the bone marrow cells for each bone (maximum of 10 mL per animal) into a clean 10 cm plate. Prior to flushing, the bones were kept in BMC media (IMDM containing 10% FBS and 1× P/S, sterile filtered) on ice.

After flushing, the needle was replaced with an 18G needle, and the bone marrow suspension was siphoned several times to break up any large tissue clumps and to form a single cell suspension, which was then filtered through a 40 μm filter (BD Falcon). The plate was then washed 3 times with wash buffer and subsequently filtered. The suspension was centrifuged at 420 g for 5 min, and the supernatant is discarded. The pellet was resuspended in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.4) and incubated at room temperature for 5 minutes to lyse the red blood cells (RBCs). The reaction was halted with the addition of 5 mL of wash buffer, and the suspension was spun again at 420 g for 5 min. After the

supernatant was discarded, the cells were resuspended in BMC media containing the cytokines IL-3 (20 ng/mL), IL-6 (10 ng/mL) and mSCF (100 ng/mL) to promote the pluripotency of existing hematopoietic stem cells. The cells were then seeded in a volume of 2.5 mL at a density of 5×10^6 cells/mL (12.5×10^6 cells total) in a 12-well cell culture plate (BD Falcon). The cells were incubated overnight at 37°C with 5% CO_2.

Infection of bone marrow cells

Following overnight incubation, non-adherent BMCs were removed from the plates (including a single wash with PBS) and centrifuged at 700 g for 5 minutes. After removal of the supernatant, each well of recovered cells was resuspended either in concentrated virus or in BMC media alone (mock) containing IL-3, IL-6 and mSCF as well as 4 μg/mL of polybrene. The cells were plated into a new 12-well plate and centrifuged at 1500 g for 50 min (referred to as a "spinfection"). This centrifugation step compacts the cells into a single layer to promote more equivalent viral infection of all cell sizes. After centrifugation, the cells were incubated for 2.5 hours at 37°C with 5% CO_2, at which point 2 mL of BMC media containing the 3 cytokines were added to each well. The cells were then incubated overnight at 37°C with 5% CO_2.

The following day, the non-adherent cells were collected and centrifuged at 700 g for 5 min. After removal of the supernatant, the cells were then washed with 1 mL of PBS, from which 200 μL of cells from each condition were set aside for flow cytometry analysis. The remaining 800 μL was centrifuged at 700 g for 5 min, and the cells were resuspended in approximately 320 μL of PBS for injections into irradiated recipient mice. The 200 μL fractions were replated into 2 mL of BMC media containing the 3 cytokines in a 12-well plate and incubated at 37°C with 5% CO_2 for an additional 48 hours.

Irradiation and transplantation of recipient mice with virally transduced bone marrow cells

The technique of total body irradiation (TBI) and bone marrow engraftment has been hailed as an effective treatment of patients with leukemia and other blood diseases. Although many animal studies that utilize this technique focus on graft-versus-host disease (GvHD) and relapse rates related to donor-recipient relationships, here we describe the application of this technique as it pertains to the ability of virally transduced bone marrow cells to successfully engraft irradiated mice.

Preparation of animals

Approximately 2-4 days prior to bone marrow harvest, C57BL/6 $P2Y_1^{-/-}$ mice (aged 12-13 weeks) were administered water containing 2 g/L of neomycin sulfate (source) as a prophylaxis for any potential infection that the animals might incur following irradiation. On the same day that the harvested BMCs were spinfected (day 0), the animals receiving the treated water were irradiated in an XRAD 400 X-ray irradiator to sufficiently kill bone marrow but allow for survival of animals receiving a bone marrow transplant. After irradiation, the animals were transferred to sterile caging and given irradiated food and autoclaved water treated with 2 g/L of neomycin sulfate.

The following day, the irradiated mice were anesthetized with 3.5% aerosolized isoflurane for 3 min and maintained under anesthesia with 2% isoflurane. For each mouse, approximately 90-100 μL of resuspended transduced BMCs (as described above) were injected retro-orbitally into the right eye with a 32G insulin syringe (Becton Dickinson, Franklin Lakes, NJ, USA). The mice were allowed to recover until they regained their righting reflex and were monitored for 5 min after regaining consciousness. The mice were monitored daily for signs of

lethargy and weight loss, and the treated water was changed weekly. After 4 weeks of recovery, the animals were subjected to a retro-orbital bleed (50 μL) to determine platelet counts and estimate success of the engraftment.

Fluorescence imaging

Mouse 3T3 cells were subjected to epifluorescence imaging 72 hours after initial infection. The plated cells were imaged on an IX70 inverted microscope (Olympus, Waltham, MA, USA) under bright field and excitation at 488 nm at 40× to detect GFP.

Flow cytometry

To measure GFP expression in the 3T3 cells, samples were run on an Accuri C6 flow cytometer (BD Falcon). Untransduced cells served as a negative control to determine the fluorescence cutoff for positive GFP expression. The values are expressed as the average GFP fluorescence intensity and the percentage of GFP positive cells per group. A minimum of 10,000 gated events were run per sample.

Statistical Analysis

The fluorescence data collected from the flow cytometer is expressed as the mean intensity (confidence interval). The number of GFP+ cells is expressed as the mean percentage ± standard error of the mean (SEM) of the corresponding number of replicate experiments. Differences between the groups were analyzed using two-way analysis of variance (ANOVA), with a p-value < 0.05 set for statistical significance. All data were analyzed using GraphPad Prism 6.0 (GraphPad Software, LaJolla, CA, USA).

Results

Insertion of a second gene alters the optimal ratio of target:helper plasmid

To determine the optimal ratio of target:helper plasmid in TAB22 cell transfections that produce the highest viral titer, we tested 4 different ratios using pMigRI plasmids that contained no $P2Y_1$ insert (empty vector, EV) or the wild type $P2Y_1$ receptor ($P2Y_1$ wt). We then graphed the overall percentage of GFP+ cells (Figure 3-3A) and the average GFP fluorescence intensity of these positive cells (Figure 3-3B). Transfections with the 1:1 ratio of target:helper plasmid with the EV had the highest titers (determined indirectly by quantifying the percentage of transduced 3T3 cells) and also produced the highest average GFP fluorescence compared to the other ratios, In contrast, transfections at a 1.5:1 ratio of target:helper plasmids were the most effective with plasmids expressing the $P2Y_1$ receptor. Moreover, we observed a decreased average GFP fluorescence as well as a decreased percentage of transduced 3T3 cells. The drop in average GFP fluorescence for the $P2Y_1$ virus compared to the EV virus is likely due to the bicistronic transcription of the plasmid containing the $P2Y_1$ receptor gene construct, as more resources can be dedicated to the transcription of GFP in the EV virus. Thus, for the remainder of the experiments, the ratio of 1.5:1 pMigRI:pCL-Eco was used for all transfections for virus production.

Mutating the C-terminal region of the $P2Y_1$ receptor has no impact on viral transduction in 3T3 cells

Next we sought to determine if mutations on the C-terminus of the $P2Y_1$ receptor would have an impact on viral transduction in 3T3 cells. We produced concentrated viral stocks containing either the wild type or 340-0P mutant forms of the $P2Y_1$ receptor parallel and transduced 3T3 cells with varying amounts of the virus. Figure 3-4A shows the microscope

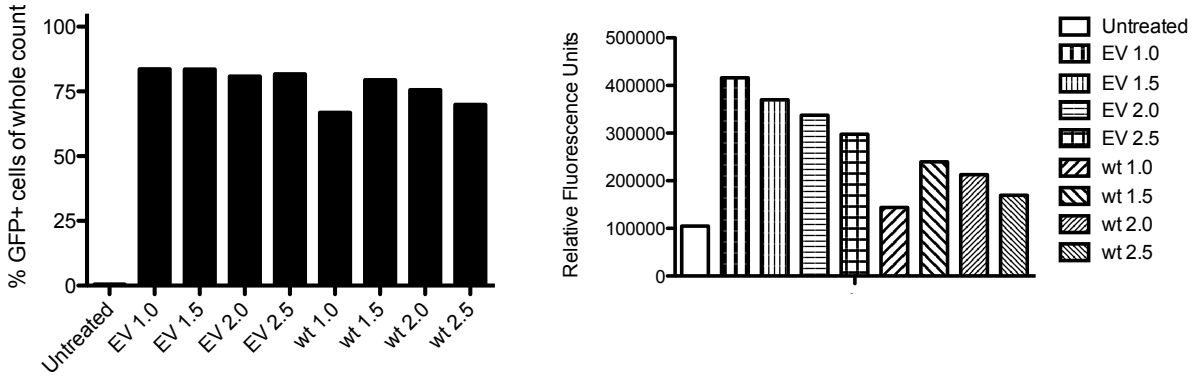

Figure 3-3. Analysis of GFP+ mouse 3T3 cells 72 hours after viral transduction.

A, Percentage of GFP+ cells. The cells (total number) were analyzed by flow cytometry and gated based on the population of untreated cells. The numbers in each column represent the ratio of pMigRI:pCL-ECO plasmid upon transfection of the TAB22 cells. **B**, Average fluorescence intensity of GFP+ cells. The numbers in each variable represent the ratio of pMigRI:pCL-ECO plasmid upon transfection of the TAB22 cells. EV, empty pMigRI vector; wt, pMigRI containing the $P2Y_1$ wild type receptor. EV, empty pMigRI vector; wt, pMigRI containing the $P2Y_1$ wild type receptor.

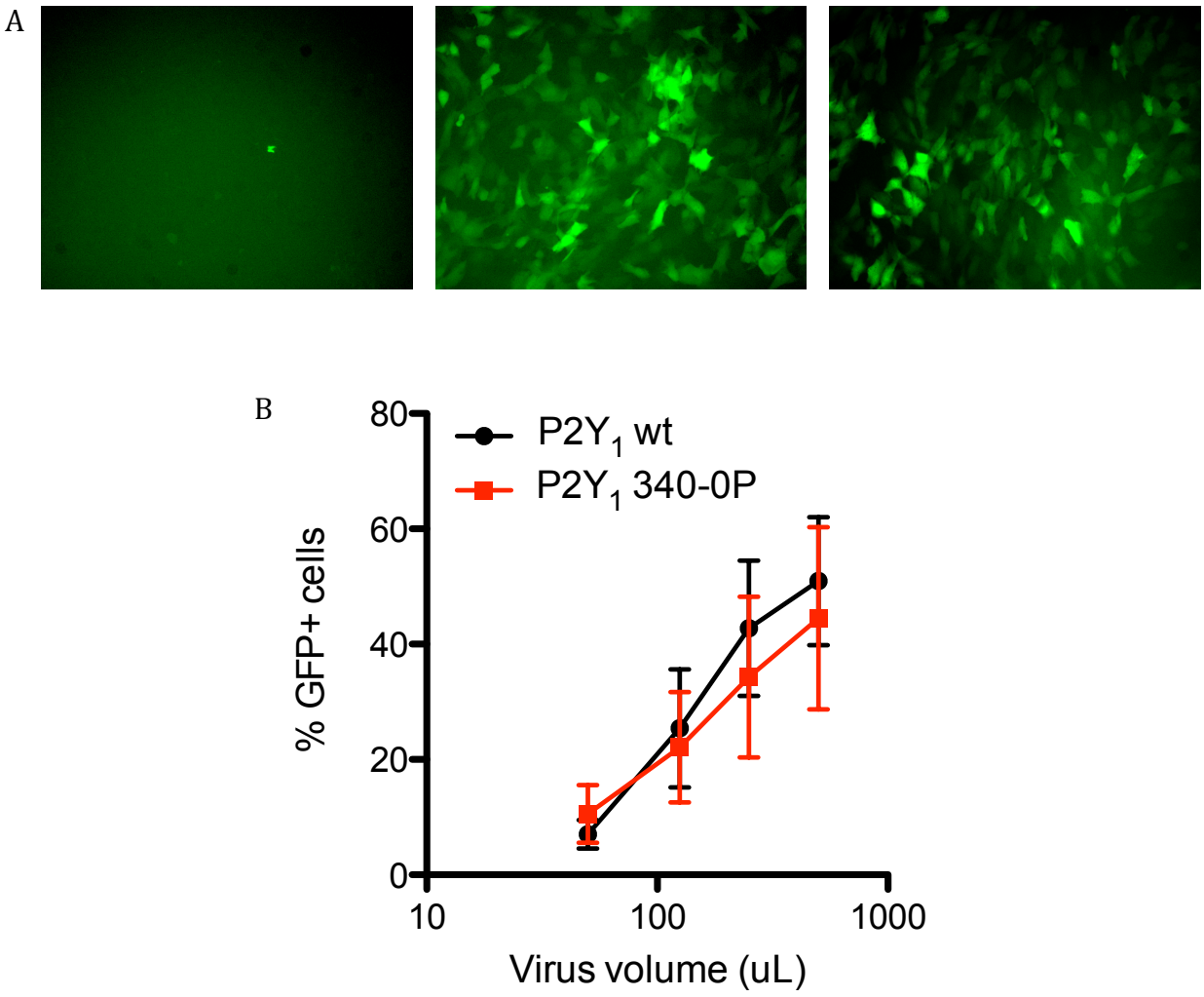

Figure 3-4. Qualitative and quantitative data of viral infection of mouse 3T3 cells.

A, Fluorescence microscopy of transduced mouse 3T3 cells. (From left) Mock-transduced, $P2Y_1$ wild type-transduced, $P2Y_1$ 340-0P-transduced. **B**, Assessment of viral titer in mouse 3T3 cells. Varying amounts of concentrated virus were added to mouse 3T3 cells; after wash off, the cells were grown for 72 hours prior to GFP measurement. The values represent the percentage of cells that were GFP^+. N = 4.

images under bright field and excitation at 488 nm of 3T3 cells infected with 500 μL of virus containing either of the two $P2Y_1$ constructs. Qualitatively, both viruses show significant transduction compared to mock-treated cells. The quantitative measurements of the transduction percentages (as determined by flow cytometry) for the wild type and 340-0P $P2Y_1$ viruses at the tested volumes are as follows: 500 μL, 50.94 ± 11.09 and 44.50 ± 15.83; 250 μL, 42.77 ± 11.76 and 34.30 ± 13.95; 125 μL, 25.41 ± 10.24 and 22.13 ± 9.60; and 50 μL, 7.04 ± 2.44 and 10.58 ± 5.00, respectively. The data show that the transduction efficiencies of both viruses are similar ($p > 0.05$ for all volumes), suggesting that differences in viral production or expression between the wild type and 340-0P $P2Y_1$ constructs are likely to be negligible (Figure 3-4B). This is crucial, as differences in protein expression could alter any signaling data acquired downstream of receptor activation.

Transduction of wild type and mutant $P2Y_1$ receptor in primary bone marrow cells shows similar efficiencies

To assess the transduction efficiency of the virus into primary bone marrow cells, approximately 20% of the transduced BMCs were left in culture until 72 hours post-infection to measure the percentage of GFP+ cells. When analyzed by flow cytometry, there were 3 distinct cell populations within the bone marrow culture based on forward and side scattering. The population of the largest cells contains approximately 90-95% of the GFP+ cells; thus, this population was the only gate assessed. Cells transduced with virus containing either the wild type or 340-0P $P2Y_1$ receptor showed similar percentages of successful transduction (11.37 ± 1.35; 95% CI 8.25-14.50 and 11.40 ± 1.11; 95% CI 8.84-13.96, respectively), with only 0.60 ± 0.14 of the mock-treated cells expressing GFP (95% CI 0.22-0.85, Figure 3-5A). Furthermore, the difference between the two treated groups and the mock group were statistically significant

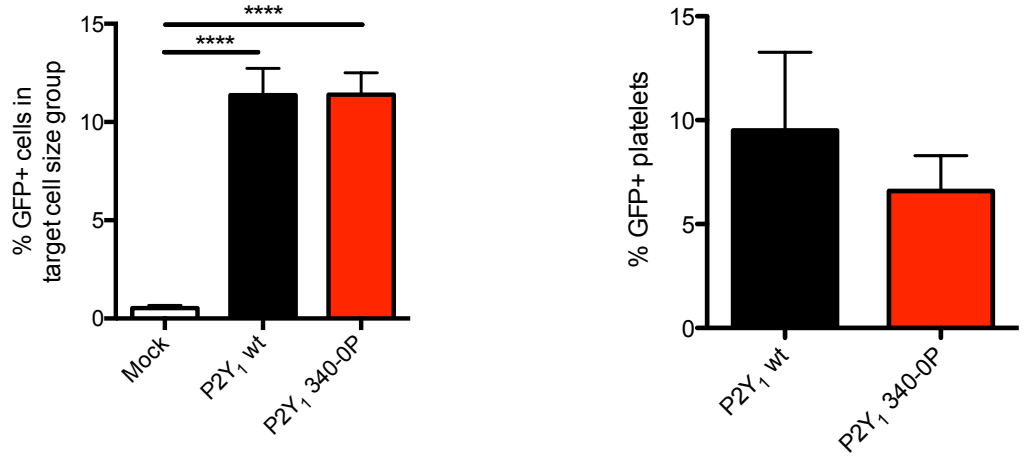

Figure 3-5. Assessment of viral transduction in cultured bone marrow cells and recovered platelets.

A, Percentage of GFP+ BMCs 72 hours after infection. Harvested cells were mock treated (white) or treated with virus containing pMigRI that expressed either $P2Y_1$ wt (black) or $P2Y_1$ 340-0P (red). The values represent the percentage of cells in the target gate that were GFP^+. ****$p<0.0001$. N = 9. **B**, Percentage of GFP+ platelets collected from mice 4 weeks after injection with bone marrow cells virally transduced with pMigRI vectors containing either $P2Y_1$ wt (black) or $P2Y_1$ 340-0P (red). The values represent the percentage of platelets in the target gate that were GFP^+. N = 4.

($p < 0.0001$ for both comparisons). These data are indicative that the virus is able to successfully transduce the bone marrow cells in culture and that 10% of the target BMCs that were injected into the irradiated mice contain the plasmid.

GFP expression in platelets from mice transplanted with virally transduced bone marrow

Blood samples from mice receiving either mock-treated or transduced BMCs were collected 4 weeks after the procedure to measure the platelet count and determine the percentage of GFP+ platelets. Figure 3-5B shows that the mean percentages of the platelets positive for GFP were similar between the wild type and 340-0P-transduced bone marrow transplanted into irradiated recipients (9.51 ± 3.76 and 6.60 ± 1.70, respectively; $p > 0.05$). This indicates that, although lower than the observed percentage of transduced bone marrow cells, HSCs were successfully infected with the plasmid DNA and produced platelets that expressed GFP.

Integrin activity of virally transduced platelets

Validation of model system

With a standardized assay in place, we moved forward in implementing an optimized chimeric mouse model introducing the $P2Y_1$ receptor into platelets via viral transduction of bone marrow cells from $P2Y_1^{-/-}$ mice. We determined if viral transduction of bone marrow cells of the pMigRI sequence (and expression of the GFP reporter protein) would impact purinergic signaling in platelets. Following the viral transduction procedure, we obtained one chimeric mouse with transplanted bone marrow cells transduced with the empty vector (EV mouse) and another chimeric mouse transplanted with bone marrow transduced with the vector expressing the HA-tagged $P2Y_1$ receptor ($P2Y_1$ wt mouse). The limited number of transduced mice made solid conclusions difficult, but preliminary data with the few mice that were transduced showed that the platelets from the EV mouse did not activate $\alpha IIb\beta 3$ in response to ADP regardless of

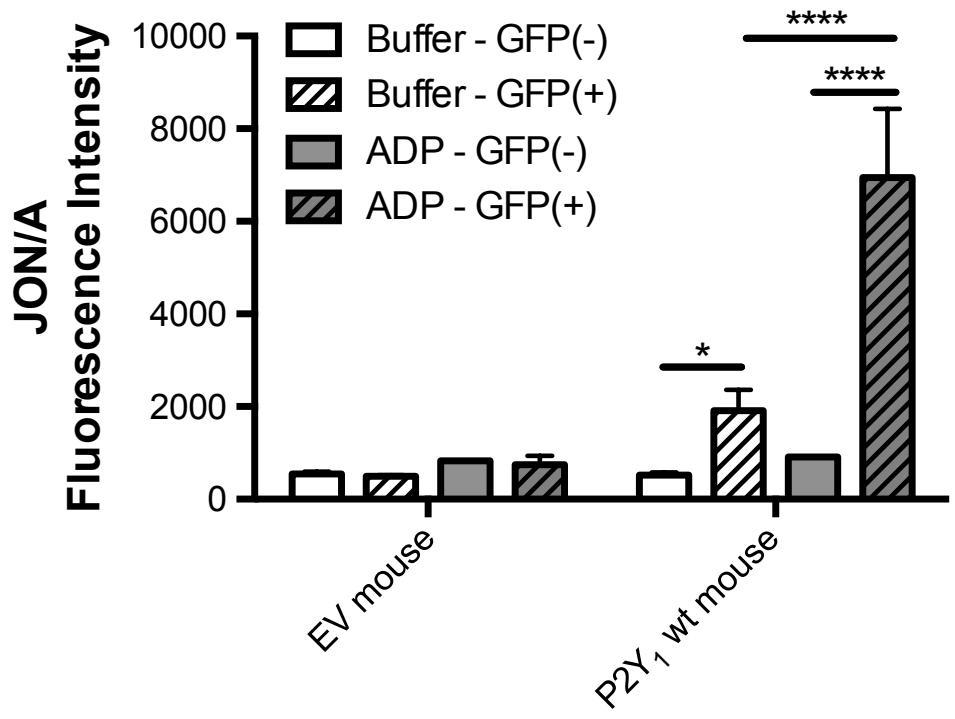

Figure 3-6. Comparison of ADP responses in virally transduced platelets.

Preliminary data compares ADP-based integrin activation of $P2Y_1^{-/-}$ mice grafted with bone marrow cells transduced with virus containing the pMigRI vector expressing either empty vector (EV mouse) or the wt $P2Y_1$ receptor ($P2Y_1$ wt mouse). *$p<0.05$; ****$p<0.0001$.

GFP expression (Figure 3-6). Likewise, GFP^- platelets from the mouse transduced with the $P2Y_1$ wt virus also did not activate αIIbβ3 in response to ADP. In contrast, GFP^+ platelets from these same mice showed significant integrin activation in response to ADP compared to both GFP^+ buffer-treated platelets and GFP^- ADP-treated platelets ($p<0.0001$ for both comparisons). Based on these preliminary data, we proceeded with the introduction of mutant $P2Y_1$ receptors.

Activity of transduced $P2Y_1$ receptors

Mouse BMCs were transduced with either HA-WT $P2Y_1$ receptor or HA-340-0P receptor and transplanted back into irradiated mice. From this procedure, there were three successful chimeras obtained: one that expressed the HA-WT-$P2Y_1$ receptor and two that expressed the HA-$P2Y_1$-340-0P receptor. Figure 3-7 shows the response of platelets from the different chimeric mice to MRS2365 and ADP. None of the GFP^- platelets in any of the chimeric mice activated αIIbβ3 integrin in response to any of the treatments; in contrast, the GFP^+ platelets in the HA-WT chimera were capable of activating integrin αIIbβ3 in response to ADP but not MRS2365 as expected. Unexpectedly, the GFP^+ platelets from both HA-$P2Y_1$-340-0P mice showed significant integrin activation in response to MRS2365 alone compared to the HA-WT-$P2Y_1$ GFP^+ platelets treated with MRS2365 ($p<0.0001$). ADP-promoted αIIbβ3 integrin activation in the HA-$P2Y_1$-340-0P GFP^+ platelets was 60% higher than that of the HA-WT GFP^+ platelets ($p<0.0001$). These preliminary data strongly suggest that the $P2Y_1$-340-0P mutant receptor stimulates the Gq signaling pathway better (or longer) compared to the wild type receptor.

An additional caveat of these experiments is that the highest ranges of receptor expression within the GFP^+ population could account for the increased responsiveness to ADP and MRS2365. To account for this possibility, we repeated these experiments in the presence of PE-labeled JON/A and Alexa467-labeled anti-HA antibodies and performed flow cytometry

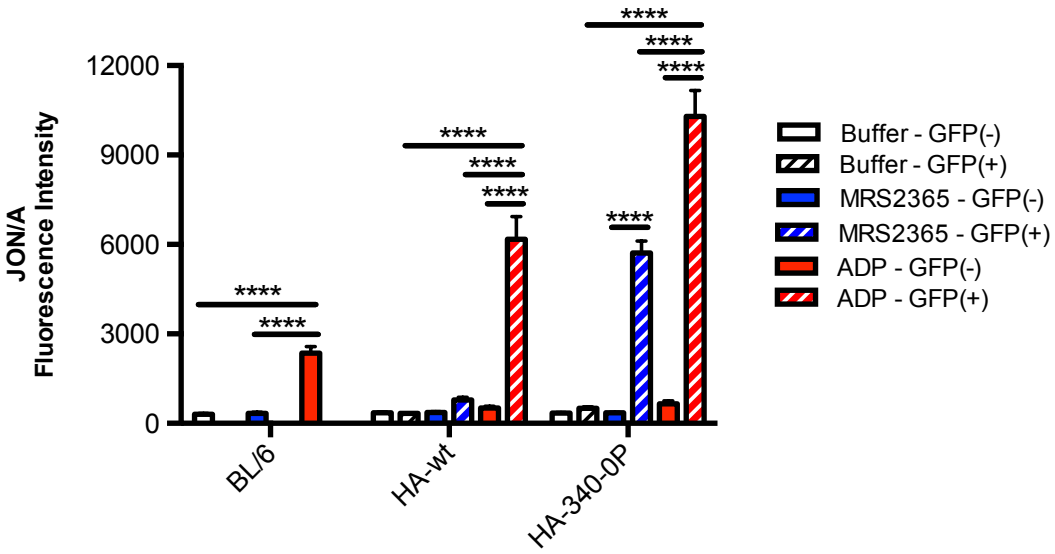

Figure 3-7. Raw preliminary results of purinergic activation of platelets with mutant $P2Y_1$ receptors.

Data show responses from a BL/6 mouse (N = 1) and $P2Y_1^{-/-}$ mice transplanted with bone marrow transduced with virus expressing either the wt HA-$P2Y_1$ receptor (N = 1) or the HA-340-0P mutant receptor (N = 2). The data are presented as the mean ± standard deviation of the raw fluorescence values. ****$p<0.0001$.

simultaneously measuring the two fluorophores; we then binned platelets based on the levels of HA antibody binding. We first selected platelets expressing WT $P2Y_1$ receptors that activated αIIbβ3 integrins to the same level as native platelets, and used the range of HA antibody binding from the selected set to bin $P2Y_1$-340-0P-expressing platelets that expressed the same levels of receptor. Using these sets of selected platelets, we observed that integrin activation in response to MRS2365 in the HA-$P2Y_1$-340-0P receptor-expressing mice was approximately the same as the response to ADP in both a BL/6 wild type mouse and the HA-WT-$P2Y_1$ receptor mouse (Figure 3-8). Furthermore, the ADP response in HA-$P2Y_1$ 340-0P receptor-expressing mice was twice that observed in platelets from BL/6 wild type or HA-WT-$P2Y_1$ receptor-expressing mice. These data provide a basis for the possibility that, at native levels, mutating several Ser and Thr residues on the C-terminus of the $P2Y_1$ receptor could result in platelets that are more sensitive to nucleotide-based integrin activation.

Due to the capacity of MRS2365 alone to activate αIIbβ3 integrins in platelets expressing the HA-$P2Y_1$-340-0P receptor, we ascertained the concentration-response relationship for MRS2365 to determine the potency of the agonist and to determine if any differences could be observed between the two mutant receptor chimeric mice. Both mice showed similar EC_{50} values (13.3 nM for 3386 and 10.8 nM for 3387), and the difference between the efficacies was insignificant (Figure 3-9). Additionally, the HA-WT mouse showed no response to 3 μM MRS2365, which was shown to be a maximal concentration for both HA-$P2Y_1$-340-0P receptor-expressing mice.

To determine whether the increased activity of ADP and MRS2365 observed in GFP^+ chimeric platelets as detailed above was due solely to activation of the $P2Y_1$ receptor or a

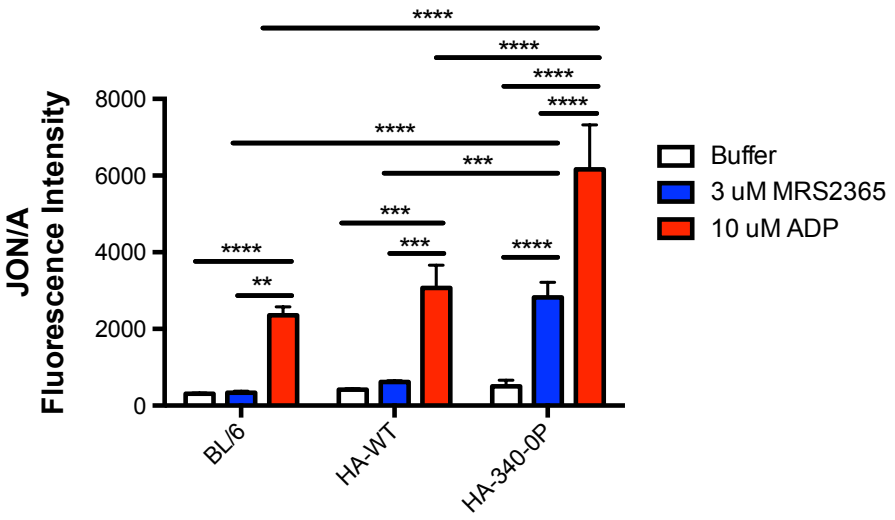

Figure 3-8. Gated preliminary results of purinergic activation of platelets with mutant $P2Y_1$ receptors.

Data from a BL/6 mouse (N = 1) and $P2Y_1^{-/-}$ mice transplanted with bone marrow transduced with virus expressing either the wt HA-$P2Y_1$ receptor (N = 1) or the HA-340-0P mutant receptor (N = 2) based on GFP and HA-antibody correlation between the BL/6 response and HA-WT response. The data are presented as the mean ± standard deviation of the raw fluorescence values. **p<0.01; ***p<0.001; ****p<0.0001.

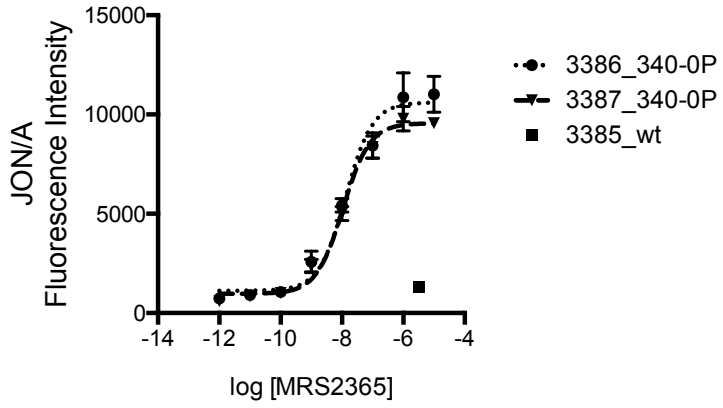

Figure 3-9. MRS2365 dose response of GFP+ chimeric $P2Y_1^{-/-}$ platelets.

Platelets expressing either HA-WT $P2Y_1$ (3385_wt) or HA-340-0P $P2Y_1$ (3386_340-0P, 3387_340-0P) were treated with multiple doses of MRS2365. The data are presented as the mean ± standard deviation of the raw fluorescence values. The calculated EC_{50} values for the two HA-340-0P chimeras were 13.3 nM (3386) and 10.8 nM (3387).

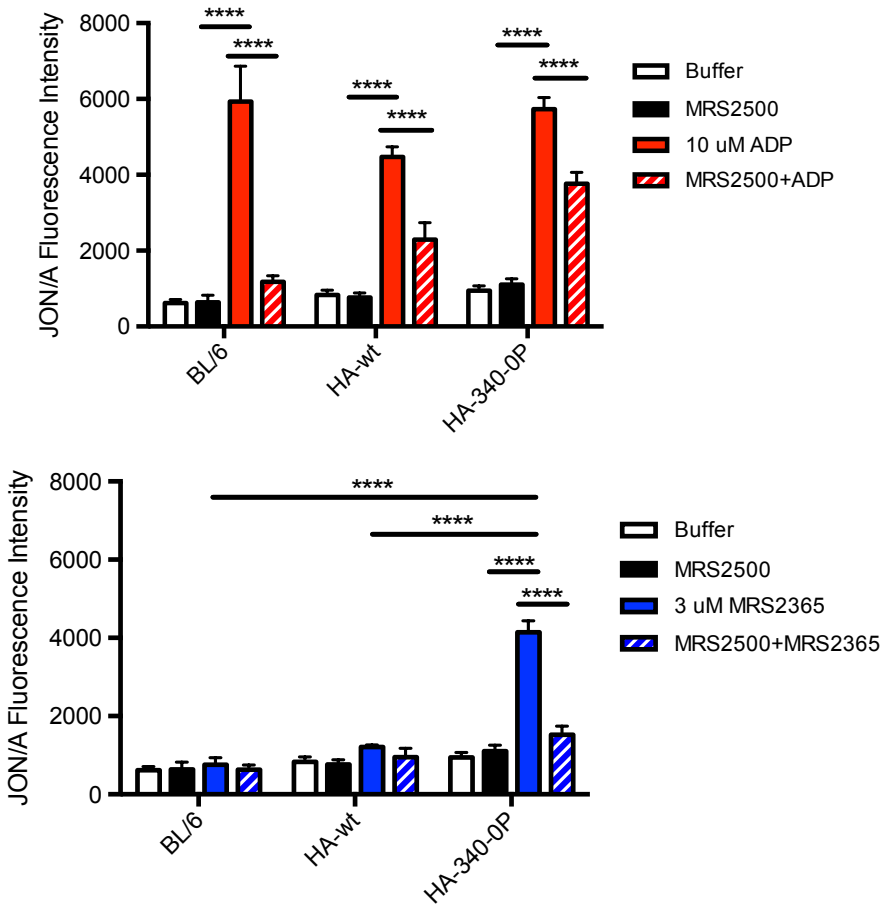

Figure 3-10. Purinergic platelet activation in the presence of MRS2500.

A, Preliminary data showing inhibition of ADP-induced integrin activation in BL/6 wt mice (N = 3) and binned GFP^+ chimeric $P2Y_1^{-/-}$ platelets expressing either HA-WT $P2Y_1$ (N = 1) or HA-340-0P $P2Y_1$ (N = 1) using the $P2Y_1$-selective antagonist MRS2500 (30 uM). The data are presented as the mean ± standard deviation of the raw fluorescence values. **B**, Preliminary data showing inhibition of MRS2365-induced integrin activation in BL/6 wt mice (N = 3) and binned GFP^+ chimeric $P2Y_1^{-/-}$ platelets expressing either HA-WT $P2Y_1$ (N = 1) or HA-340-0P $P2Y_1$ (N = 1) using the $P2Y_1$-selective antagonist MRS2500 (30 uM). The data are presented as the mean ± standard deviation of the raw fluorescence values. ****$p<0.0001$.

combination of other signaling pathways, platelets were pretreated with 30 μM MRS2500, a $P2Y_1$-selective antagonist, prior to addition of ADP or MRS2365. For the ADP-treated platelets, 30 μM MRS2500 blocked approximately 90% of the signal in the BL/6 wild type mice but only 59% and 42% of the signal in the HA-WT and HA-340-0P mice, respectively (Figure 3-10A). For the MRS2365-treated platelets, only the HA-340-0P mouse showed integrin activation, and this response was antagonized by approximately 87% in the presence of MRS2500 (Figure 3-10B). These data suggest the increased integrin activation upon MRS2365 treatment occurs through the mutant $P2Y_1$ receptor alone.

Variability of transplantation in mouse groups

The complexity of transducing BMCs is further confounded by differences among the responsiveness of mice to the irradiation procedure. Initial tests used two doses of 450 cGy, but control mice not receiving a transplant survived, indicating that the dose was insufficient for full bone marrow death (data not shown). A dose of 1200 cGy was determined empirically for the $P2Y_1^{-/-}$ mice. Table 3-1 summarizes the survival and GFP expression in platelets of mice injected with transduced BMCs. The first several rounds of irradiation and transplantation treatments through 2013 showed good survival but highly variable GFP transduction into platelets, with a 0-100% success rate in surviving transduced animals. However, under the same conditions with $P2Y_1^{-/-}$ mice having the same lineage as their predecessors, none of the 33 mice transplanted after February 2014 survived past 19 DPT, suggesting that the radiation dosage was too strong for the transplanted cells to overcome. Another empirical test was performed to reestablish the appropriate dosage, which was determined to be 950 cGy (data not shown). Unfortunately, the two sets of transplanted mice irradiated at this lower dose still showed low survival (37.5% and 14.3%), with none of the surviving mice expressing GFP^+ platelets. The repetitive failures with

Date	Irradiation dose	Transduced mice	4-wk survival	>2% GFP^+ platelets	6-wk survival	>2% GFP^+ platelets
10/30/12	1200 cGy	6	6	2	6	2
2/5/13	1200 cGy	12	11	2	11	2
3/20/13	1200 cGy	5	5	5	N/A	N/A
7/16/13	1200 cGy	12	12	6	N/A	N/A
9/16/13	1200 cGy	6	6	4	5	4
12/21/13	1200 cGy	4	4	0	4	0
2/6/14	1200 cGy	8	8	1	7	0
3/11/14	1200 cGy	6	0	N/A	N/A	N/A
3/18/14	1200 cGy	7	0	N/A	N/A	N/A
3/28/14	1200 cGy	8	0	N/A	N/A	N/A
5/5/14	1200 cGy	5	0	N/A	N/A	N/A
6/9/14	1200 cGy	7	0	N/A	N/A	N/A
1/21/16	950 cGy	8	3	0	3	0
2/26/16	950 cGy	7	1	0	1	0

Table 3-1. Summary of irradiation treatments and survival of mice transplanted with transduced BMCs.

the multiple groups of mice that were irradiated and transplanted highlight the difficulty in using this model testing the different transduced $P2Y_1$ receptors.

Discussion

The ability to study mutant proteins in an environment conducive to platelet function is challenging and cumbersome. Between the lack of culturing techniques and the monetary and temporal expense of mouse engineering, there are limited options in using biochemistry to determine the effects of a mutant protein within a native platelet system. Here, we optimized several established techniques to produce a viable mouse model that stably expresses one of two types of $P2Y_1$ receptor in live platelets to be assayed for activity and expression. The entire process of viral transduction of HSCs prior to transplantation has been developed by Mark Kahn at the University of Pennsylvania; however, maximizing virus production and infection is critical in targeting the 0.01% of HSCs within the bone marrow population to consistently produce platelets that express the protein of interest.

The ratio between the targeted plasmid and enveloping plasmid is critical to ensure that there is neither an excess of envelope nor plasmid within the production cells. As the transfection of large quantities of DNA could damage the production cells, 39 μg was set as the maximum amount, with 9 μg as a fixed volume of VSVG. Thus, the remaining 30 μg was separated into 4 ratios, with the amount of target plasmid gradually increasing. For the 3T3 cells infected with empty vector, the percentage of infected cells as well as the average fluorescence intensity decreased inversely with the target:helper ratio. This effect was also observed with the cells infected with virus containing the $P2Y_1$ wild type receptor, but the 1:1 ratio showed the lowest percentage of infected cells as well as the lowest average fluorescence intensity. Based on these

results, we decided to proceed as using the 1.5:1 target:helper ratio (i.e., 18 μg pMigRI:12 μg pCL-Eco) for all subsequent experiments.

With the virus production in place, we then sought to determine if the 340-0P construct of the $P2Y_1$ receptor would have any effect on either the virus transduction or the expression levels in mouse 3T3 cells. We transduced cells with multiple volumes of virus containing either the wild type or 340-0P construct of the $P2Y_1$ receptor and observed that the transduction efficiencies were similar as measured by the percentage of GFP+ cells. However, the average fluorescence intensities of the positive cells were highly variable within each transduction group (data not shown); this could be attributed to the health of the targeted cells, the location of the inserted DNA (insertions into active parts of the genome would express higher levels than those going into silent parts of the genome), and the number of copies integrated per infection. Despite the high variability, both the wild type and 340-0P $P2Y_1$ constructs exhibited a minimum of a 5-fold increase in average fluorescence compared to either the mock-transduced or untreated cells (data not shown). This fluorescence increase supported the notion that transduced cells could be easily separated from non-transduced cells by measuring GFP levels.

The critical phase of this optimization was the transduction of harvested BMCs from mice. Retroviral transduction only affects actively dividing cells, which can skew the transduction efficiencies due to the mitotic rates of different hematopoietic subpopulations. As the target population of HSCs is approximately 0.01% of all bone marrow cells, collecting a sufficient population and treating with a substantial amount of virus increases the likelihood of transduction into these pluripotent cells. In measuring GFP expression in a subset of BMCs that were not transplanted, we observed distinct subpopulations based on cell size and complexity. The gate denoting larger, more complex cells contained the majority of the GFP-expressing cells

and was thus used as the primary marker for detecting GFP measurement. With the cell counts nearly identical in both treatment groups as well as the mock control group, our data showed that approximately 11% of the selected gated cells were successfully transduced as measured by GFP expression. Both the wild type and 340-0P $P2Y_1$ receptor constructs had highly similar transduction rates and 95% CIs. Furthermore, the percentage of GFP expression was over 18-fold higher than that of the control BMCs. These data indicate that retroviral transduction of BMCs using the transfection parameters and viral concentration is effective.

Our data show that approximately 7-9% of platelets in irradiated mice that received a bone marrow transplant containing experimentally transduced cells were GFP+. A non-transplanted mouse will succumb to immunodeficiency between 10 and 20 days after a lethal irradiation dose. During that time, the injected BMCs will uptake to bone and proliferate to replace the dying cells. Platelet production and release from mature megakaryocytes takes approximately 4-10 hours (Patel et al., 2005); therefore any platelets observed 4 weeks after irradiation and implantation are derived from the transplanted bone marrow. Although the BMC transduction rate was approximately 11% for both $P2Y_1$ constructs, the lower GFP+ rate in platelets may be attributed to the lineage of transduced cells at the time of infection, i.e., lymphoid progenitors, myeloblasts, and other BMCs downstream of the pluripotent hematopoietic stem cells may account for a large percentage of the observed GFP+ cells in the BMC measurements. However, the existence of GFP+ platelets indicates that a small number of hematopoietic stem cells were successfully transduced and produced GFP+ megakaryocytes, which would give rise to the positive platelets.

Our preliminary data with the chimeric mouse model showed that introduction of the $P2Y_1$ receptor into bone marrow cells via viral transduction restores the capacity of ADP to

activate integrin αIIbβ3 in receptor-expressing platelets. These data also showed that expression of GFP alone had no influence on platelet activation by ADP. Despite having data from only 2 test mice regarding the effectiveness of the mouse model, the data were compelling enough that we moved forward with creation of chimeric mice to measure the integrin activation of platelets expressing mutant $P2Y_1$ receptors. There were a total of three successfully transduced mice: one mouse expressed the HA-$P2Y_1$ wild type receptor, and two mice expressed the HA-$P2Y_1$-340-0P receptor. The analyses of platelets from these mice were surprising: whereas integrin activation in platelets expressing the WT HA-$P2Y_1$ receptor was essentially identical to those from native C57BL/6 mice, we unexpectedly observed integrin activation in platelets expressing HA-$P2Y_1$-340-0P receptors platelets in response to MRS2365 alone.

One explanation for these results was that these platelets express very high amounts of mutant receptors, as overexpression of even WT $P2Y_1$ receptors is known to make platelets hyperaggretory (Hechler et al., 2002). Therefore, we sorted the flow data to reflect platelets with the lowest expression of WT and mutant receptors, and the MRS2365-promoted integrin activation was still observed in the HA-$P2Y_1$-340-0P transduced platelets. These results suggest that specific Ser and/or Thr residues in the C-terminus of the $P2Y_1$ receptor are involved in receptor desensitization, and that mutations in these residues are capable of bypassing the otherwise required activation of Gi-coupled signaling to induce integrin activation, a feat that has not been observed previously in platelets. Because we believe that this mutant receptor is incapable of desensitizing, the continued signaling of the $P2Y_1$ receptor results in increased activation of CalDAG-GEF1 that can overcome the baseline RASA3 GAP activity without a Gi signaling pathway, as evidenced by activated integrin αIIbβ3 in the presence of MRS2365 alone.

To clarify that the observed activation of integrins using the $P2Y_1$-selective agonist was not due to off-target effects, platelets were treated with the $P2Y_1$-selective antagonist MRS2500. In the presence of 30 μM MRS2500, over 90% of the integrin activation observed with 10 μM of ADP in BL/6 wild type platelets was inhibited; however, only 59% and 42% of integrin activation was inhibited in the HA-WT and HA-340-0P platelets, respectively. The inability of MRS2500 to block integrin activation in the chimeric platelets likely reflects the increased expression of $P2Y_1$ receptors in these platelets following viral transduction; that is, there was not enough MRS2500 to fully inhibit a 10 μM dose of ADP in the chimeric platelets. Based on Gaddum's equation, approximately 8% of receptors in platelets from C57BL/6 mice would not be inhibited at the concentrations of ADP and MRS2500 used. As the chimeric platelets likely have much higher variability in $P2Y_1$ receptor expression, it appears likely that the receptor number on some of the platelets is significantly higher than the reported native expression levels (Hechler et al., 2002). Regarding the partial inhibition of ADP-treated HA-340-0P platelets, there are three possibilities: 1) increased sensitivity of the $P2Y_1$ receptor may result in increased integrin activation with fewer receptors, 2) the receptor is expressed at higher numbers on the membrane, or 3) the remaining activity acts through other signaling pathways independent from $P2Y_1$. However, inhibition of MRS2365-induced integrin activation in the HA-340-0P platelets with the same concentration of MRS2500 indicates that the observed activity operates primarily through the $P2Y_1$ receptor.

A considerable limitation to this procedure is the extreme variability in the penetrance of successfully transplanted and transduced BMCs that convey GFP expression to platelets. Though the data from the available chimeric mice are compelling, no definitive conclusions can be drawn from these studies as only a single HA-$P2Y_1$-WT and two HA-$P2Y_1$-340-0P mice were

successfully created, which are insufficient numbers to make a strong statistical conclusion regarding the differences in activity. Furthermore, these chimeric mice deteriorated before any other experiments could be performed, namely treatments in the presence of a $P2Y_{12}$ receptor-selective antagonist to eliminate any influence of the $P2Y_{12}$ receptor on the signaling of the transduced $P2Y_1$ receptors. Power calculations based on the preliminary data revealed that a total of 6 mice for each chimeric genotype were necessary for the data to hold any statistical significance. Unfortunately, multiple attempts to create additional chimeric mice were unsuccessful.

Of the 14 groups of mice subjected to the transplantation procedure, only three groups had more than 50% of the animals producing GFP^+ platelets. Of these groups, two of them were further subjected to an additional harvest and transplantation into a second set of irradiated mice, a procedure termed second-generation transplantation. The original goal was to sort the GFP^+ BMCs from the successful mice to produce mice that produced >90% of GFP^+ platelets; this would allow for more uniform activation responses. However, all mice receiving sorted GFP^+ BMCs died within 7 days after receiving the transplant. This procedure has been successful with introducing a mutant CalDAG-GEF1 protein into platelets (Stolla et al., 2011), but only 4 mice were produced in this publication. Based on these exciting data, we attempted to repeat the generation of chimeric mice using the same protocol, but unfortunately all subsequent attempts were unsuccessful. While the number of chimeric mice tested did not have enough statistical power to make meaningful conclusions, these preliminary data were provocative and provided the impetus to create knock-in mice expressing the HA-$P2Y_1$-340-0P receptor using CRISPR/Cas technology. These experiments are described in Chapter 4.

Conclusions

With the success of these optimization experiments, the protocol tested the functionality and activity of platelets derived from transduced BMCs expressing either wild-type or 340-0P $P2Y_1$ receptors. Introduction of the wild type receptors should elicit a restoration of function and serve as a control for the unknown properties of the 340-0P receptors. Because of the low GFP+ rate in platelets, large numbers of mice are necessary to best elucidate any possible changes in signaling and/or activation of the mutant receptor.

The three chimeric mice produced provide a small glimpse into the importance of Ser and/or Thr residues on the C-terminus of the $P2Y_1$ receptor in platelet activation and regulation. Mutation of these residues results in integrin activation following activation of only the $P2Y_1$ receptor, which is not observed in wild type platelets. Unfortunately, the extremely high failure rate of the multiple injection groups hinders the use of this mouse model to study $P2Y_1$ receptor desensitization. However, the preliminary data give credence to generating knock-in mice to better understand the physiological consequences of $P2Y_1$ receptor desensitization.

CHAPTER 4: VIABILITY AND PLATELET CHARACTERISTICS OF $P2Y_1$ RECEPTOR KNOCK-IN MICE CONTAINING MUTATIONS ON THE C-TERMINUS

Introduction

Data from Chapter 3 strongly suggest that mutation of Ser and Thr residues downstream of Thr339 in the $P2Y_1$ receptor ("HA-$P2Y_1$-340-0P") has a significant impact on platelet aggregation. Unfortunately, multiple attempts to repeat the generation of chimeric mice were unsuccessful, thereby limiting the statistical power of the experiment due to too few mice available for testing. Moreover, it was apparent from our analyses that viral transduction of WT and mutant $P2Y_1$ receptors into bone marrow cells resulted in a high variability of receptor expression in platelets, which confounded the interpretation of the experiments. Therefore, we proceeded with generation of a knock-in mouse expressing the HA-$P2Y_1$-340-0P receptor. Although time consuming initially, replacing the coding region of the native allele with the coding sequence containing the mutations would allow for physiological expression levels of the mutant receptor and provide more accurate data on how this mutation affects platelet activity. Because the mutant receptor also contains an HA tag on the N-terminus of the receptor, a second knock-in mouse with an HA tag on the N-terminus of the wild type $P2Y_1$ receptor was also created to determine if the HA tag alone has any effect on ADP-induced platelet activation, as well as providing the means to assess receptor expression using an anti-HA antibody.

Materials and Methods

Cloning of mP2Y$_1$ locus for genomic translocation

To integrate the hemagglutinin (HA) tag into the locus of the P2Y$_1$ receptor, the previously cloned mP2Y$_1$ receptors (the wild type receptor and receptor containing 12 point mutations to create the 340-0P mutant as described in Chapter 3) were used as templates for overlap extension PCR to combine the HA-P2Y$_1$ receptor coding sequence (either WT or mutant) from the pMigRI vector with the 1560-bp section immediately upstream of the start codon of the P2Y$_1$ receptor in the chromosomal locus. The primers used are listed in Table 4-1. The upstream genomic sequence was amplified from C57BL/6 genomic DNA and the coding sequences of the mutant and wild type P2Y$_1$ receptors were amplified from the respective pMigR1 plasmids, the fragments were purified, and then the two coding sequences were combined with the upstream genomic sequence in separate reactions and amplified with the outside primers to produce single bands of 2727 bp. As the outside primers contained a SacI (fwd) or BamHI (rev) at their 3' ends, the fragments were digested with SacI and BamHI and cloned into similarly digested pUC18. To clone the region downstream of the P2Y$_1$ receptor coding sequence, the appropriate primers from Table 4-1 were used to amplify the sequence from C57BL/6 genomic DNA; these primers contained on their 5'ends a BamHI site (fwd primer) and an SphI site (rev primer). The fragment was digested with these enzymes and cloned into similarly digested wild type and mutant plasmids generated above to yield the final contructs. Colonies were identified by digestion with MluI and XbaI; the correct digestion produces bands of 3328 bp, 2447 bp, and 1174 bp. Selected colonies were confirmed by sequencing with the primers listed in Table 4-1.

Primer	Fwd/ Rev	Sequence
Primers for generating $P2Y_1$ receptor targeting construct		
To amplify genomic DNA upstream of $P2Y_1$ receptor coding sequence	Fwd	5'-ACTG**GAGCTC**CCCTCAACAAATGAACCCTCT-3' (SacI site bolded/underlined)
	Rev	5'-TGG**ACGCGT**AGTCGGGCACGTCGTAGGGGTACATCCTC TTCTTTCCAACTCAG-3' (MluI site bolded/underlined)
To amplify the HA-tagged $P2Y_1$ receptor coding sequence	Fwd	5'-GAGTTGGAAAGAAGAGGATGTACCCCTACGACGTGC-3'
	Rev	5'-AGTC**GGATCC**TTCACAAACTCGTGTCTC-3' (BamHI site bolded/underlined)
To amplify the HA-tagged $P2Y_1$ -340-0P receptor coding sequence	Fwd	5'-GAGTTGGAAAGAAGAGGATGTACCCCTACGACGTGC-3'
	Rev	5'-AGTC**GGATCC**TTCACAAAGCCGCGTCTCC-3' (BamHI site bolded/underlined)
To amplify genomic DNA downstream of $P2Y_1$ receptor coding sequence	Fwd	5'-ACGA**GGATCC**TAGCTCCTGAGTTTTG -3' (BamHI site bolded/underlined)
	Rev	5'-ACTG**GCATGC**CCTACTGGGCACAAAGGTTG-3'(SphI site bolded/underlined)
Primers for sequencing $P2Y_1$ receptor targeting construct		
pUC rev primer	Fwd	5'-GAGCGGATAACAATTTCACACAGG-3'
Seq751	Fwd	5'-CTCTGCTTCCAGAGGCCA-3'
Seq1542	Fwd	5'-AGCTGCCTGAGTTGGAAAGA-3'
Seq2341	Fwd	5'-CTGGACAACTCTCCGCTCC-3'
Seq3140	Fwd	5'-TCTATCCTTTAAACAATTTGGCA-3'
pUC -40 primer	Rev	5'- GCCAGGGTTTTCCCAGTCACGA-3'
Primers for genotyping recombinant mice		
HA-tag genotyping	Fwd	5'-AGC TGC CTG AGT TGG AAA GA-3'
HA-tag genotyping	Rev	5'-TTG GGG ACA ACC GAC CAA-3'
$P2Y_1$ Wild-type genotyping	Fwd	5'-TCAAGCAGAATGGAGACACG-3'
$P2Y_1$-340-0P genotyping	Fwd	5'-GgcTGAGGCCAATTTACAAg-3'
Common Rev primer for WT/340-0P genotyping	Rev	5'-AAAAGGAGGAAGGGGAAGTG-3'

Table 4-1. Primers used to generate genomic targeting construct.

The vectors containing the HA-tagged wild type and mutant $P2Y_1$ receptors were given to Dr. Dale Cowley at the UNC Mouse Core, who designed guide RNAs and subjected the vectors to the CRISPR/Cas9 system to integrate the HA-tagged receptors into the locus on chromosome 3. There were 3 successfully integrated HA-tagged wild type mice and 1 successfully integrated HA-tagged 340-0P mutant mouse. These mice were bred with C57BL/6J mice to eventually produce homozygous HA-tagged mice.

Genotyping of $P2Y_1$ knock-in mice

To detect the difference in the wild type and inserted coding regions, PCR primers surrounding the start site of the coding sequence of the $P2Y_1$ receptor mRNA were used (primer sequences listed in Table 4-1). The wild type locus produces a PCR product of 56 bp, whereas both HA-tagged receptors produce a PCR product of 89 bp. To differentiate between mice expressing wild type $P2Y_1$ and $P2Y_1$-340-0P receptors, two forward primers were used that targeted the wild type or the mutant,sequences paired with an identical reverse primer. The wild type allele produced a PCR product of 264 bp, whereas the 340-0P mutant PCR product was 326 bp.

Measurement of intracellular Ca^{2+}

Washed platelets (protocol described in Chapter 2) were resuspended at a concentration of 1 x 10^9 per mL in Tyrode's buffer containing 0.2 U/mL of apyrase as a stock solution. For each sample, 1 µL of platelets was added to 109 µL of Tyrode's buffer containing 0.1 µL of either 500 mM Fluo-4 AM dye (Thermo Fisher Scientific, Waltham, MA, USA) or 500 mM Fura-Red AM dye (Thermo Fisher Scientific). The platelets were incubated in black microcentrifuge tubes (Argos Technologies, Elgin, IL, USA) at 37°C for 30 minutes. The platelets were then briefly vortexed and run on an Accuri C6 flow cytometer. After 10 µL of

solution was run (approximately 10 seconds), 100 µL of 2× drug was added (total volume 200 µL) and the sample continued to record. For samples with two additions, 100 µL of 2× drug was added at 1 minute 40 seconds after sample read initiation (total volume 200 µL), and the sample continued to record. The sample was halted either 3 minutes after the final addition or when the number of counts reached 1,000,000, whichever came first.

Statistical analysis

Data are presented as the mean ± standard deviation of the respective measurements. Two-way analysis of variance (ANOVA) was used to compare multiple groups. A p-value less than 0.05 was defined as statistically significant. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, LaJolla, CA, USA).

Results

Breeding of HA-P2Y$_1$ wild type mice

Upon receipt, the founder animals were bred with C57BL/6J mice to produce larger numbers of heterozygous HA-WT/WT mice. Once sufficient populations of heterozygous animals were available, they were crossbred together to produce homozygous HA-WT/HA-WT mice. Pups from these litters were of similar sizes to litters born to C57BL/6J wild type mice (6-12 mice), suggesting that reproduction and/or development was not affected by the insertion of the HA tag at the N-terminus of the receptor. Thus, HA-WT/WT mice were used to continue breeding of homozygous HA-WT mice and to prevent excessive inbreeding of the animals. Of the 137 live births from heterozygous HA-WT/WT animals, there were 29 wild type, 70 heterozygous, and 38 HA-WT homozygous progeny, which is within the bounds of the predicted Mendelian distribution (Table 4-2).

Breeding of HA-P2Y$_1$ 340-0P mutant mice

Upon receipt, the founder animal was bred with C57BL/6J mice to produce larger numbers of heterozygous HA-WT/WT mice. As there was only a single founder, the descendent HA-340-0P/WT heterozygous mice were bred together to produce HA-340-0P/HA-340-0P mice. However, of the 68 live pups born among the breeding pairs, there were 45 heterozygous mice (HA-340-0P/WT) and 23 WT mice (WT/WT), with no live births of HA-340-0P homozygous mice. This outcome deviates markedly from the predicted distribution of progeny (Table 4-2). Although this is a small sample size, the 66/34 distribution of the heterozygous and wild type mice (respectively) is highly suggestive of lethality of embryos homozygous for the HA-340-0P allele.

Genotyping and sequencing of HA-P2Y$_1$ wild type and HA-P2Y$_1$ 340-0P mice

Figure 4-1 shows the expected PCR products of mice containing two wild type alleles, one HA-WT allele and one wild type allele, and two HA-WT alleles. The difference in size of the amplified products allowed for quick identification of homozygous HA-WT mice for further experiments. An agarose gel showing the results of PCR genotyping for detecting the 340-0P mutant allele is also shown in Figure 4-1. The alleles of C57BL/6J wild type and HA-WT/HA-WT homozygous mice were amplified and sequenced to determine any significant changes. The wild type allele was consistent with the genome sequence present in the NCBI database. However, the HA-WT allele showed an 81 base pair deletion downstream of the stop codon of the genome within the intron region of the chromosome. It is uncertain if this deletion exerts a significant effect on either the transcription or translation of the HA-WT protein, although the observation that platelets from HA-P2Y$_1^{+/+}$ mice showed normal activation properties in response to ADP suggest that this deletion had little effect on P2Y$_1$ receptor levels (see below).

	Wild type homozygous	Heterozygous	Knock-in homozygous
Predicted HA-WT (%)	34.25 (25%)	68.5 (50%)	34.25 (25%)
Actual HA-WT (%)	29 (21.2%)	70 (51.1%)	38 (27.7%)
Predicted HA-340-0P (%)	17 (25%)	34 (50%)	17 (25%)
Actual HA-340-0P (%)	23 (33.8%)	45 (66.2%)	0 (0%)

Table 4-2. Predicted and observed genotyping of offspring from HA-WT/WT and HA-340-0P/WT heterozygous parents.

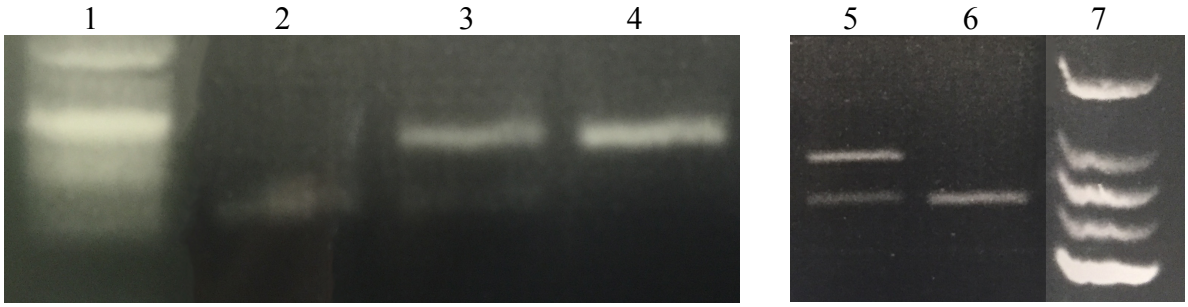

Figure 4-1. Images of genotyping PCR of wild type, HA-WT, and HA-340-0P alleles of the $P2Y_1$ receptor.

Lane 1, low molecular weight ladder (bright band, 100 bp). Lane 2, homozygous wild type. Lane 3, HA-WT/WT heterozygote. Lane 4, homozygous HA-WT. Lane 5, HA-340-0P/WT heterozygote. Lane 6, homozygous wild type. Lane 7, low molecular weight ladder (bright band, 200 bp).

$P2Y_1$ receptor activity in platelets

To determine if the $P2Y_1$ proteins expressed in the knock-in mice had similar activity to the wild type mice, integrin αIIbβ3 activation using the JON/A antibody was performed as described in Chapter 2. A concentration-response curve to ADP was generated using platelets from wild type, HA-$P2Y_1^{+/+}$, and HA-$P2Y_1$-340-0P/WT mice to detect any changes in ADP-promoted integrin activation. Figure 4-2 shows that platelets from all three mice have similar EC_{50} values to ADP (2.6 ± 0.2 μM, 3.4 ± 0.3 μM, and 2.9± 0.2 μM for wild type, HA-WT, and HA-340-0P/WT, respectively). Because ADP-promoted activation of αIIbβ3 integrins involves both the $P2Y_1$ and $P2Y_{12}$ receptor, it is not a direct measure of $P2Y_1$ receptor activation; therefore, we also measured $P2Y_1$ receptor-dependent calcium release to get a more direct representation of $P2Y_1$ receptor activation. Figure 4-2 shows the concentration-response relationship of MRS2365 for Ca^{2+} mobilization in platelets from HA-$P2Y_1^{+/+}$ and HA-$P2Y_1$-340-0P/WT mice, which indicate that platelets from both of these mice have similar EC_{50} values (180 pM for HA-WT and 360 pM for HA-340-0P/WT). Interestingly, platelets from the HA-340-0P/WT heterozygous mice have a higher maximal AUC in response to MRS2365, though this difference is not significant. Taken together, these data support three notions: 1) introducing the HA tag on the N-terminus of the $P2Y_1$ receptor does not impact (positively or negatively) the integrin-activating capabilities of the receptor, 2) the 81 bp deletion in the HA-$P2Y_1^{-/-}$ mice has no effect on the EC_{50} of ADP, and 3) the heterozygous HA-340-0P/WT mice have similar calcium and integrin responses to MRS2365 and ADP, respectively, compared to wild type and HA-WT mice.

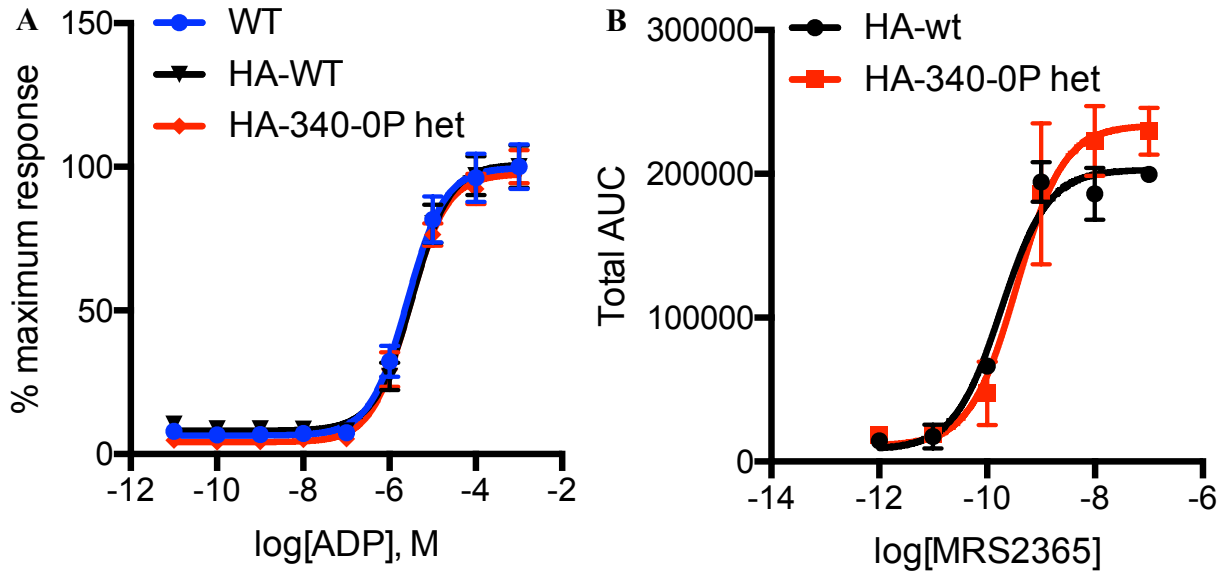

Figure 4-2. Dose response of integrin activation on platelets from $P2Y_1$ knock-in mice.

A, Dose response curve of ADP on integrin activation in platelets from wild type (WT), HA-WT homozygous, and HA-340-0P heterozygous mice. The EC_{50} values were 2.58 ± 0.20 μM, 3.38 ± 0.25 μM, and 2.85 μM ± 0.18 for wild type, HA-WT, and HA-340-0P/WT heterozygous mice, respectively. N = 6 for wild type mice; N = 4 for the HA-WT and HA-340-0P heterozygous mice. **B**, Dose response curve of MRS2365 on calcium release from platelets from HA-WT homozygous and HA-340-0P/WT heterozygous mice. The EC_{50} values were 181.9 ± 47.0 pM for the HA-WT platelets and 357.7 ± 129.5 pM for the HA-340-0P/WT heterozygous platelets. The data are represented as the mean ± SEM. N = 3 for both mouse lines.

Changes in extended signaling of the HA-340-0P/WT mouse

To determine if platelets from HA-340-0P/WT heterozygous mutant mice showed any differences in $P2Y_1$ receptor signaling kinetics, the levels of integrin activation using the JON/A antibody were assessed as described in Chapter 2. Platelets from HA-$P2Y_1^{+/+}$ or the HA-340-0P/WT mice treated with either buffer or 3 μM of MRS2365 showed no increase in integrin activation. The heterozygous mutant platelets treated with ADP alone displayed a significant increase in integrin responsiveness compared to platelets from HA-$P2Y_1^{+/+}$ mice ($p<0.0001$). ADP treatment of platelets preincubated with either MRS2500 or PSB0739 showed similar levels of inhibition of integrin activation in both HA-$P2Y_1^{+/+}$ and HA-340-0P heterozygous mice. Platelets from HA-$P2Y_1^{+/+}$ mice pretreated with MRS2365 for 90 seconds prior to the addition of ADP exhibited a similar decrease in integrin activation to a subsequent challenge with ADP to that observed with wild type mice (see Chapters 2, 3); in contrast, platelets from the heterozygous mutant mice showed a highly significant increase in integrin activation compared to platelets from HA-$P2Y_1^{+/+}$ mice in the MRS2365 pretreatment paradigm ($p<0.0001$). The MRS2365-pretreated platelets from heterozygous mutant mice had a slight increase in integrin integrin activation in the MRS2365 pretreatment paradigm compared to the response of the same platelets challenged with ADP directly, but this increase was not significant.

Because of the increased ADP-induced integrin activation observed in platelets from HA-340-0P heterozygous mice, we sought to determine if this increase could be attributed to increased $P2Y_1$ receptor signaling in platelets from the heterozygous mice. Platelets from HA-$P2Y_1^{+/+}$ and heterozygous HA-340-0P mice were treated with thrombin to measure the maximum achievable JON/A signal. Figure 4-4 shows the normalized integrin activation response to both thrombin and ADP to determine the influence of thrombin-induced granule release on platelet

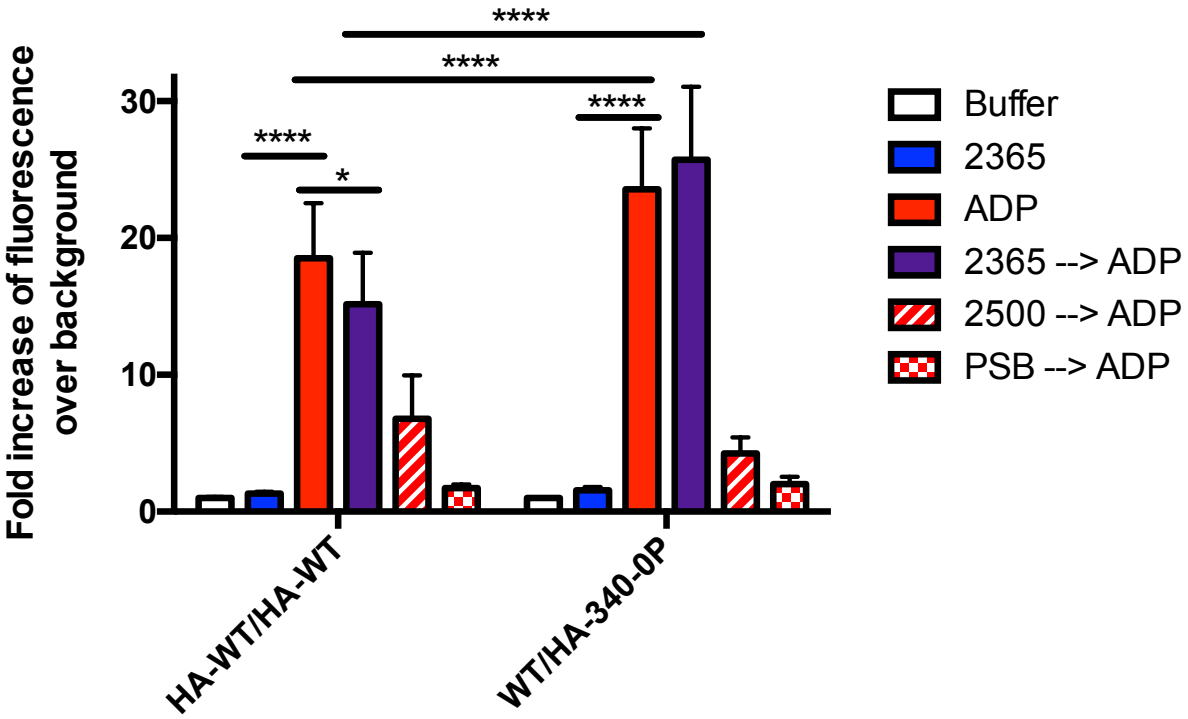

Figure 4-3. αIIbβ3 activation response to agonist stimulation of homozygous HA-WT mice and heterozygous HA-340-0P mice.

Buffer, buffer only; 2365, 3 μM of MRS2365; ADP, 10 μM of ADP; 2365 → ADP, 90 second pretreatment with 3 μM of MRS2365 followed by 10 μM of ADP; 2500 → ADP, platelets pretreated with 100 μM of MRS2500 before addition of 10 μM of ADP; PSB → ADP, platelets pretreated with 10 μM of PSB0739 before addition of 10 μM of ADP. The data are represented as the mean ± SEM. N = 6 for both genotypes.

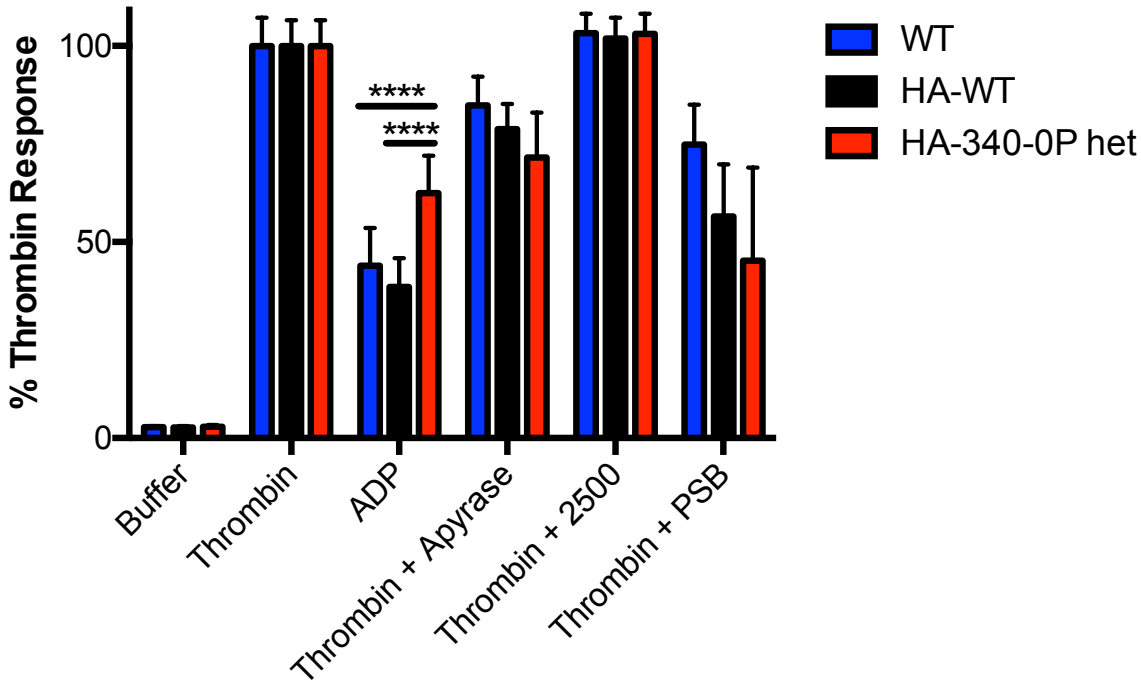

Figure 4-4. αIIbβ3 activation response to agonist stimulation of wild type, homozygous HA-WT, and heterozygous HA-340-0P mice.

Buffer, buffer only; Thrombin, 0.5 U/mL of thrombin; ADP, 10 μM of ADP; Thrombin + apyrase, 0.5 U/mL of thrombin in platelets treated with 1 U/mL of apyrase; Thrombin + 2500, platelets pretreated with 100 μM of MRS2500 before addition of 0.5 U/mL of thrombin; Thrombin + PSB, platelets pretreated with 10 μM of PSB0739 before addition of 0.5 U/mL of thrombin. The data are represented as the mean ± SEM. N = 5 for all three genotypes.

activation. The raw fluorescence values from both sets of platelets in response to thrombin treatment were similar (data not shown), thus all of the values were normalized to the thrombin response. The levels of integrin activation in response to ADP in the heterozygous HA-340-0P/WT platelets were significantly higher than those in both the wild type and HA-WT platelets ($p<0.0001$ for both), suggesting that the platelets have similar maximum responses but the HA-340-0P/WT platelets have stronger ADP-induced integrin activation. The thrombin responses in the presence of apyrase and MRS2500 were similar for all three genotypes, but preincubation with PSB0739 was more effective on both knock-in mice compared to the wild type platelets ($p<0.0001$ for both vs. wild type).

Discussion

The use of the CRISPR/Cas9 system at the UNC Mouse Core Facility generated three mice with successfully integrated HA-$P2Y_1$ alleles and one mouse with a successfully integrated HA-340-0P allele. The mice with the HA-WT alleles were able to breed normally, with full homozygous HA-WT mice produced within 5 months of receiving the founder mice. These animals presented no obvious changes in phenotype or behavior and had a Mendelian distribution of progeny, suggesting that the insertion of the HA tag at the N-terminus had no negative effects on mouse development. Surprisingly, although the HA-340-0P allele was able to transmit from parent to offspring, there were no live births of mice homozygous for two mutant alleles. The number of heterozygous mice and wild type mice progeny follows a 67/33 distribution of genotypes, indicative of embryonic lethality of HA-340-0P homozygous fetuses. This is potentially the first known instance of a mutant GPCR at the native locus conferring embryonic lethality; other published instances of embryonic lethality relating to GPCRs involve homozygous knockout mice, including the orphan receptor GPR126, with full lethality by E13.5

(Waller-Evans et al., 2010), and the sphingosine 1-phosphate receptor 1 ($S1P_1$) (Liu et al., 2000) and calcitonin receptor-like receptor (CLR) (Dackor et al., 2006), both of which have full lethality by E14.5. Further studies using timed pregnancies are necessary to determine the time point of lethality. Currently, breeding is underway with the HA-340-0P/WT heterozygous mice with $P2Y_1$ knockout mice to determine if an HA-340-0P hemizygous mouse is viable, which would allow for further study of the expression and activity of the HA-$P2Y_1$-340-0P mutant receptor in the absence of a wild-type allele.

The integrin activation responses in platelets from wild type mice and both knock-in mice were measured to determine if the incorporation of the HA tag on the N-terminus would exert any effect (positive or negative) on the ability of the $P2Y_1$ receptor to activate platelets. Because of the observed embryonic lethality, all experiments comparing the changes in integrin activation in HA-340-0P mice were performed using the heterozygous mice. At face value, the concentration-response relationships for ADP in the integrin activation assay were virtually identical among the wild type, HA-WT homozygous, and HA-340-0P heterozygous platelets. These data suggest that 1) the N-terminal HA tag does not influence ADP signaling in platelets, and 2) the heterozygous mutant exerts a similar integrin signal in response to ADP. However, these data do not account for the influence of the $P2Y_{12}$ receptor in integrin activation, which is more responsible for maintaining the integrin response (Stefanini et al., 2015). To measure $P2Y_1$ receptor signaling more directly, calcium release from the DTS was measured using platelets loaded with a calcium-sensitive dye. The calcium dose response to MRS2365 showed that the homozygous HA-WT and heterozygous HA-340-0P mice had similar EC_{50} values, but the Hill slopes were vastly different (6.08 for HA-WT; 1.40 for HA-340-0P heterozygote).

Although the concentration-response curves were similar in response to both ADP and MRS2365, these data do not reflect any potential changes in the kinetics of $P2Y_1$ receptor activation and inactivation in platelets. Thus, homozygous HA-WT and heterozygous HA-340-0P platelets were preincubated with MRS2365 for 90 seconds prior to the addition of ADP to determine if either the HA tag or a single copy of the mutant allele had an effect on the loss of ADP-induced integrin activation. Although not as pronounced as wild type platelets (Chapter 2), the HA-WT platelets showed a significant reduction in ADP-induced integrin activation when the $P2Y_1$ receptor was stimulated for 90 seconds before activation of the $P2Y_{12}$ receptor by adding ADP, indicating that the HA tag does not affect normal receptor activity. The heterozygous mutant platelets, however, had increased integrin activation upon ADP stimulation alone and increased (but not significant) activity upon preincubation with MRS2365. These data suggest that there is some penetrance of mutant receptor expression on platelets. Assuming that the receptors are transcribed and translated equally from both copies of the chromosome, approximately 75 of the receptors on the platelet surface would have the mutations. Although this is insufficient to reproduce the data observed in the transplanted chimeric mice, it does support the notion that low expression levels of C-terminal mutant $P2Y_1$ receptor can influence ADP-induced activation of integrin $\alpha IIb\beta 3$.

Of course, it is possible that the introduction of these HA tags onto the $P2Y_1$ receptor could increase the overall excitability of platelets. This was addressed by treating homozygous HA-WT and heterozygous HA-340-0P platelets with a high dose of thrombin to determine the maximum integrin response measurable using the JON/A antibody. The raw thrombin responses were similar between the two genotypes, thus any differences in the purinergic-based responses were solely due to the mutations on the C-terminus of the HA-340-0P allele. Although not direct,

taken together these data support the hypothesis that the Ser and Thr residues on the C-terminus of the $P2Y_1$ receptor are involved in the inactivation of the receptor upon initial stimulation.

One significant limitation in the interpretation of the data is proving the expression of the HA tag on the N-terminus of the knock-in $P2Y_1$ receptor proteins. This tag was originally incorporated into the coding region of the receptor because current antibodies against the $P2Y_1$ receptor are insufficient for either pulldown or detection. The HA tag begins immediately after the start codon, after which there are two amino acids followed by a second Met codon. Thus, there exists the possibility that the ribosomal machinery does not recognize the first Met as the proper start site for translation. Thus, tissues that express high levels of the $P2Y_1$ receptor (brain, heart, intestine) will be processed to perform Western blots and pull-down assays to determine if the HA tag is expressed on the protein. There are several transgenic designer receptor exclusively activated by designer drug (DREADD) mouse lines that successfully express the HA tag on the N-terminus (Rogan and Roth, 2011), thus detecting and identifying the HA-tagged $P2Y_1$ receptors should be straightforward.

Conclusions

Introduction of the HA-tagged $P2Y_1$ receptor into the locus of BL/6J wild type mice showed no phenotypic differences regarding development, procreation and ADP-induced platelet activity compared to BL/6J wild type mice. However, the HA-340-0P $P2Y_1$ receptor mutants were more intriguing, as the homozygous mutants were embryonic lethal and the heterozygous HA-340-0P/WT mice showed increased integrin activation upon ADP treatment as well as increased instead of decreased activity when pretreated with the $P2Y_1$-selective agonist MRS2365. Further studies regarding the expression of the HA tag as well as the time point of embryonic lethality will provide more solid evidence regarding the importance of the C-terminal

Ser and Thr residues in $P2Y_1$ receptor desensitization in platelets, as well as during embryonic development.

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