G Protein-coupled Receptor Signaling: Role of PAR2 in Breast Carcinoma Metastasis and Regulation of Agonist-Promoted Internalization of P2Y1 Receptors

Dionne Renee Glast

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Approved by

Advisor: Robert Nicholas, Ph.D

Reader: Gary Johnson, Ph.D.

Reader: Channing Der, Ph.D.

Reader: T. Kendall Harden, Ph.D.

Reader: Ellen Weiss, Ph.D.

Abstract

Dionne Renee Glast
G Protein-coupled Receptors: A Function in Breast Carcinoma Metastasis and Insight Into the Regulation of Agonist-promoted Internalization
(Under the direction of Dr. Robert A. Nicholas)

This dissertation is comprised of two projects investigating the function of GPCR signaling and regulation in cancer and/or cardiovascular physiology. The first study delineated the functional importance of PAR2 in breast cancer migration and invasion by using small interfering RNAs (siRNAs) to deplete highly invasive breast cancer cells of endogenous PAR2 protein. Our findings strongly suggest that PAR2 is critical for MDA-MB-231 and BT549 breast cancer cell migration and invasion towards NIH-3T3 fibroblast conditioned medium. We also examined the importance of PAR2 in mediating factors VIIa and Xa responses. We showed that MDA-MB-231 cells depleted of PAR2 exhibit a marked inhibition of VIIa and Xa signaling to phosphoinositide hydrolysis and ERK1/2 activation, whereas signaling by VIIa and Xa remained intact in PAR1 deficient cells. Factors VIIa and Xa-induced cellular migration was also impaired in MDA-MB-231 cells deficient in PAR2 but not in cells lacking PAR1. The results from these studies reveal the novel findings that PAR2 has a critical role in breast cancer cell migration and invasion and functions as the endogenous receptor for coagulant proteases VIIa and Xa in these cells.

For the second study, we investigated the regulation of agonist-promoted $P2Y_1$ receptor internalization in Madin-Darby Canine Kidney cells. Our studies revealed that Ser336 within a highly conserved $S^{336}RAT^{339}$ sequence regulates agonist-promoted $P2Y_1$

internalization. We showed that mutation of Ser336 to Ala resulted in internalization rates faster than wild type receptors in response to agonist. Agonist-promoted [^{32}P]phosphate incorporation studies indicated that increased phosphorylation was not the cause of enhanced internalization. Arrestin-GFP mobilization and internalization studies in arrestin2- and arrestin3-null mouse embryonic fibroblasts revealed that both wild type and mutant receptors required arrestins to undergo agonist-promoted internalization. We propose a model in which Ser336 regulates arrestin binding to an active conformation of the receptor. This model predicts that mutation of Ser336 to Ala increases the rate of internalization as a result of increased arrestin binding. These studies increase our understanding of the internalization and regulation of the $P2Y_1$ receptor, a GPCR critical in regulation of various processes including ion and water transport across epithelia, smooth muscle relaxation, and platelet activation.

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2MeSADP	2-Methylthiol ADP
2MeSAMP	2-Methylthiol AMP
5-HT_{2A}	Serotonin receptor
7TM	Seven transmembrane
AAK1	AP2-associated kinase
ABC	ATP-binding cassette
ACR	Apyrase conserved regions
ADP	Adenosine diphosphate
Akt/PKB	Protein kinase B
ANTH	AP180 N-terminal homology
AP	Alkaline phsphatases
AP2	Adaptor protein complex-2
APC	Activated protein C
ARH	Autosomal recessive hypercholesterolemia
AT1aR	Angiotensin receptor subtype 1a
ATP	Adenosine triphosphate
B2R	Bradykinin B2 receptor
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
C5ar	Anaphylatoxin chemotactic receptor
Ca^{2+}	Calcium
CALM	Clathrin assembly lymphoid myeloid leukemia protein

cAMP	Cyclic adenosine monophosphate
CCR5	Chemokine receptor 5
CD73	Surface-associated ecto-5'-nucleotidase
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CK1	Casein kinase 1
CK2	Casein kinase 2
CNS	Central nervous system
CRD	Cystein-rich domain
CXCR	Chemokine receptor
D	Aspartic acid
Dab2	Disabled-2
DAG	Diacylclycerol
DRM	Detergent-resistant membrane
E	Glutamin acid
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EBP50	ERM-binding phosphoprotein 50
EC_{50}	Effective concentration at which half maximal response is achieved
ECM	Extracellular matrix
Ede1	EH domains and endocytosis
EGFR	Epidermal growth factor receptor

EH	Epsin homology
ELISA	Enzyme linked immunosorbent assay
E-NPP/NPP	Ecto-nucleoside pyrophosphatase
Ent	Yeast epsin homologues
ENTDPase/NTPDases	Ecto-nucleoside triphosphate diphosphohydrolases
ENTH	Epsin N-terminal homology
EP4R	Prostaglandin receptor 4
Eps15R	Eps15 related
Epsin	Eps15-interacting protein
ERK	Extracellular signal-regulated kinase
EST	Expressed sequence tag
ETAR	Endothelian A receptor
ETBR	Endothelian B receptor
F	Phenylalanine
FBS	Fetal bovine serum
G proteins	GTP-binding proteins
G418	Geneticin
$GABA_B$	γ-aminobutyric acid type B
GAPs	GTPase-activating proteins
GDP	Guanosine 5'-diphosphate
GEFs	Guanine nucleotide exhange factors
GFP	Green fluorescent protein
GPCRs	G protein-coupled receptors

GPI	Glyvosylphosphatidylinositol
GRKs	G protein receptor kinases
GTP	Guanine triphosphate
HDL	High density lipoprotein
HIP1	Huntington-interacting protein-1
HIP1R	HIP1 related protein
HMECs	Human mammary epithelial cells
HRP	Horseradish peroxidase
HSP90	Heat shock protein 90
I	Isoleucine
IP_3	Inositol-1,4,5-triphosphate
K	Lysine
kDa	Kilodaltons
L	Leucine
LDL	Low-density liproprotein
M2R	Muscarinic m2 receptor
MDCK	Madin-Darby canine kidney epithelial cells
Mdm2	Mouse double minute-2
MEFs	Mouse embryo fibroblasts
Mg	Magnesium
MMP	Matrix metalloprotease
mRNA	Messenger RNA
MRS2179	2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate

MRS2365	(N)-methanocarba-2-methylthioadenosine-diphosphate
MRS2500	(N)-methanocarba-2-deoxyadenosine-3-bisphosphate
N	Asparagine
N-	Amino
NANC	Noncholinergic nerves
NaOH	Sodium hydroxide
NDPK	Nucleoside diphosphate kinase
NMP	Nucleoside monophosphate kinase
NO	Nitric oxide
NPF	Asn-Pro-Phe
P1	Adenosine purinergic receptors
P2X	Ligand-gated P2 purinergic receptors
P2Y	G protein-coupled P2-purinergic receptors
PAR	Protease-activated receptor
PFA	Paraformaldehyde
pH	A measure of the acidity or alkalinity of a solution
PI	Phosphoinositide
PI-3K	Phosphatidylinositol-3' kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLCβ	Phospholipase Cβ
PTB	phosphotyrosine-binding domain

RGS	Regulators of G protein signaling
RIA	Radioimmunosassay
*R*luc	*Renilla* luciferase
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylimide gel electrophoresis
Ser	Serine residue
siRNA	Small interfering RNA
SNARE	Soluble N-ethyl malemide sensitive factor attachment protein receptor
Ste	Sterol receptor
TF	Tissue factor
TIRF	Total internal reflection fluorescence
TNAP	Tissue non-specific alkaline phosphatase
TPα	Thromboxane α receptor
TPβ	Thromboxane β receptor
TxA2	Thromboxane A2
UBDs	Ubiquitin-binding domains
UDP	Uracil diphosphate
UIM	Ubiquitin-interacting motifs
US28	Human viral chemokine receptor
UTP	Uracil triphosphate
V1aR	Vaspopressin V1a receptor
VFTM	Venus flytrap module
Vps26	Vacuolar protein sorting 26 protein

VTR	Vasotocin receptor
vWF	von Willedbrand factor
X	Any amino acid
Y	Tyrosine
YFP	Yellow fluroescent protein
Zn	Zinc
β2AR	Beta2-adrenergic receptor
Φ	Bulky, hydrophobic residue

Chapter 1

General Introduction

1.1. G protein-coupled receptors

Maintaining homeostasis in any living multicellular organism requires cells to sense their external environment, communicate with each other, and respond rapidly to extracellular signals. These extracellular signals that regulate many important physiological processes are regulated via multiple cell-surface signaling receptors, including G protein-coupled receptors.

G protein-coupled receptors (GPCRs) were initially discovered through studies examining the dose-dependent activity of neurotransmitters, peptide hormones, and natural and synthetic drugs in animal models, isolated organs, and tissues. The targets for most of these molecules turned out to be GPCRs and ion channels. GPCRs are expressed in a wide variety of tissues and represent the largest and most versatile group of cell surface receptors. This seven transmembrane (7TM) superfamily (~800 functional genes) responds to a diverse range of stimuli, such as light, odor, taste, pheromones, and neurotransmitters. More than thirty percent of all drugs currently used target GPCRs [1]. Theses drugs have therapeutic actions across a wide range of human diseases, including neurodegenerative, psychiatric and immune disorders, cardiovascular, gastrointestinal, renal, and pulmonary diseases, pain, and cancer [1-2] (TABLE 1.1).

GPCRs generally fall into one of three main receptor families: rhodopsin-like family A, secretin-like family B, or metabotropic glutamate/pheromone-like family C[3]. Sequences within each family generally share over twenty-five percent sequence homology in the seven transmembrane core regions. The rhodopsin-like family A is the largest subgroup and contains olfactory, small-molecule/peptide hormones, protease-activated, and purinergic GPCRs. Family A GPCRs are characterized by several highly conserved amino acids in the seven transmembrane bundle. In addition, there is usually a disulfide bridge linking extracellular loops E1 and E2. Most family A receptors have at least one cysteine residue in the intracellular carboxyl-terminal tail that is palmitoylated, thus promoting its interaction with the membrane.

Secretin-like family B GPCRs include approximately 50 receptors for peptides such as secretin, calcitonin, and parathyroid hormone. These receptors are characterized by a relatively long amino-terminus and contain a network of three conserved disulfide bridges defining a globular domain structure. The family B receptors show a number of conserved proline residues within the seven transmembrane segments that are thought to be essential for the conformational shape of the receptors. Secretin-like receptors preferentially couple to the $G\alpha_s$ protein (over $G\alpha_i$, $G\alpha_{12}$, and $G\alpha_q$) to activate adenylyl cyclase [4].

In addition to the metabotropic glutamate (mGluR) receptors, family C GPCRs also include receptors for γ-aminobutyric acid type B ($GABA_B$), Ca^{2+} and several amino acids. Of the 17 members of family C receptors, the majority are characterized by having both a large amino-terminus and carboxyl-terminal tail, a disulfide bridge connecting the first and second extracellular loops, and a very short, well-conserved third intracellular

loop. A number of the highly conserved residues of class A GPCRs are also conserved in class C GPCRs, consistent with class A and class C receptors sharing a common ancestor. The ligand-binding site, which is located in the amino-terminus, is composed of the venus flytrap module (VFTM) that shares sequence homology with bacterial periplasmic amino acid-binding proteins. In all class C GPCRs, except the $GABA_B$ receptor, a cysteine-rich domain (CRD) consisting of nine conserved cysteine residues links the VFTM to the seven transmembrane domain[5].

1.2. Classical G protein-coupled receptor signaling

1.2.1. Heterotrimeric GTP-binding proteins (G proteins)

GPCRs couple to heterotrimeric GTP-binding proteins (G proteins) to regulate a variety of cell responses. G proteins are composed of three protein subunits-α, β, and γ, with the latter two subunits forming an obligate dimer. Some G proteins are ubiquitously expressed while others are expressed in selective tissues. There are 16 mammalian Gα subunits that are categorized into four families based upon primary structure and signaling properties: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. Gβγ dimers are combinations of five isoforms of the Gβ subunit and thirteen isoforms of the Gγ subunit.

In the basal state, the inactive GDP-bound Gα subunit is complexed with a Gβγ dimer and a GPCR. Receptor activation leads to the exchange of GDP for GTP on the α subunit, which promotes dissociation of the G protein complex into the GTP-bound Gα subunit and the Gβγ dimer, both of which have the capacity to signal individually to downstream effectors. Thus, activated receptors function as guanine nucleotide-

exchange factors (GEFs) that stimulate the dissociation of GDP from Gα and the binding of GTP from the cytosol.

Hydrolysis of GTP to GDP by the intrinsic GTPase activity in Gα subunits leads to signal termination and re-association of the heterotrimeric complex. GTPase-activating proteins (GAPs) increase the rate of GTP hydrolysis. Regulators of G protein signaling (RGS) proteins are GAPs that enhance the intrinsic GTPase activity of the Gα subunit. There are ~25 RGS proteins encoded in the human genome and these proteins are critical in regulating G protein-mediated responses.

The dissociation of the heterotrimeric G protein activates its two components in different ways. GTP binding causes a conformational change that affects the association of the Gα subunit with the Gβγ complex and allows the Gα subunit to interact with downstream target proteins. The Gβγ complex does not change, but does interact with downstream effectors as well.

1.2.2. G protein-coupled receptor signal transduction

Signal transduction is achieved through the coupling of G proteins to various secondary pathways involving ion channels, adenylyl cyclases, and phospholipases to regulate a broad range of physiological processes, including gene transcription, chemotaxis, and ion channel opening. This coupling results in production of second messengers.

$G\alpha_s$ activates adenylyl cyclase to stimulate intracellular cyclic AMP (cAMP) levels. $G\alpha_{i/o}$ inhibits adenylyl cyclase and triggers other signaling cascades. $G\alpha_{q/11}$ activates phospholipase Cβ (PLCβ) resulting in the hydrolysis of phosphatidylinositol-

4,5-bisphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). DAG increases the activity of protein kinase C (PKC) and IP_3 triggers the release of Ca^{2+} ions from intracellular stores. $G\alpha_{12/13}$ activates Rho family proteins. $G\beta\gamma$ dimers signal to a large number of effectors including ion channels, phospholipases, phosphoinositide kinases, and the ras/raf/ERK (extracellular signal-regulated kinase) pathways.

1.3. Regulation of G protein-coupled receptor signaling

Following GPCR activation, the receptor must be desensitized in order to terminate receptor signaling. Receptor desensitization and signal termination usually occur as a consequence of three different mechanisms: 1) uncoupling and desensitization of the receptor from G proteins in response to receptor phosphorylation and arrestin binding, 2) receptor internalization to intracellular compartments, and 3) downregulation of the total amount of receptor due to lysosomal sorting and subsequent degradation of pre-existing receptors. The time frames over these mechanisms range from seconds (phosphorylation) to minutes (internalization) to hours (downregulation). A number of factors, including receptor structure and cellular environment, regulate the extent of receptor desensitization which varies from complete signal termination (visual and olfactory receptors)[6] to attenuation of agonist potency and maximal responsiveness (β_2AR)[7-8].

1.3.1. G protein-coupled receptor desensitization by uncoupling the G protein in response to phosphorylation

Ligand binding to GPCRs and activation of signaling pathways leads to covalent modification of the receptor through phosphorylation by intracellular kinases. In general,

second messenger-dependent protein kinases [i.e. cAMP-dependent protein kinase (PKA) and protein kinase C (PKC)] and G protein receptor kinases (GRKs) phosphorylate GPCRs on serine and theronine residues in both the intracellular loops and carboxyl-terminal tail[9-11]. As their name indicates, second messenger-dependent protein kinases are activated by second messengers (see below), which results in phosphorylation of both the activated receptor and other receptors that are not in the activated state. In contrast, GRKs specifically phosphorylate only activated receptors. Arrestin binds to phosphorylated receptors and promotes the uncoupling of G proteins from the receptor to terminate signaling[7,12].

1.3.1a. Multiple sites of G protein-coupled receptor phosphorylation

Mutagenesis[13-20] and antibody studies[21] on a wide range of receptors demonstrate that GPCRs are phosphorylated at multiple sites. Early studies on rhodopsin showed that phosphorylation is sequential or hierarchical, which is also believed to be true for nonvisual GPCRs[22]. For example, the chemokine N-formyl peptide C5a receptor is basally phosphorylated by an unknown kinase, which primes the receptor for agonist-induced phosphorylation[18,23-24]. The δ-opioid receptor is phosphorylated primarily at Ser363 in the carboxyl tail, and a S363A mutation inhibits phosphorylation at other sites on the carboxyl tail[24]. Additionally, *in vitro* phosphorylation experiments of the M_3-muscarinic receptor by protein kinase casein kinase 2 (CK2) revealed that mutating one consensus site resulted in the loss of a number of phosphoylation events on the third intracellular loop. These data suggest that the kinase phosphorylates the receptor at multiple sites in the region and that the phosphorylation is hierarchical[25]. Finally, basal

phosphorylation of the bradykinin B_2 receptor at Ser346 on the carboxyl tail is followed by multiple phosphorylation that was both time-dependent and ligand-concentration dependent. This phosphorylation pattern is due to multiple kinases that include GRKs, PKC, and at least one unidentified protein kinase[26].

1.3.1b. Second messenger-dependent kinases

The second messenger-dependent kinases, PKA and PKC, are activated in response to GPCR-stimulated increases in intracellular second messengers such as cyclic AMP (cAMP), Ca^{2+}, and diacylglycerol and participate in GPCR signaling by phosphorylating downstream effector proteins. Additionally, second messenger-dependent kinases also phosphorylate GPCRs at consensus sites within the intraceullar loop and carboxyl tail to facilitate desensitization in a feedback mechanism. For example, upon activation of the β_2AR, PKA is activated and subsequently phosphorylates the receptor in at least one of two PKA consensus sites[13,27-28].

1.3.1c. G protein receptor kinases

The GRK family consists of seven family members (GRK 1-7) that share significant sequence homology and structure. Each GRK contains a central catalytic domain, an amino-terminal domain that contains an RGS-like domain and is important for substrate recognition, and a carboxy-terminal domain that is important for targeting to the plasma membrane. Most GRKs are localized in the cytosol in the basal state, and following receptor activation they translocate to the plasma membrane to phosphorylate GPCRs at serine and threonine residues in the third intracellular loops or carboxy-

terminal tails. In addition to promoting desensitization, the functional role of GPCR phosphorylation by GRKs is to promote arrestin binding and signaling.

1.3.1d. Other receptor kinases

The studies mentioned above (1.3.1a) involving M_3-muscarinic receptor phosphorylation by CK2 and bradykinin B_2 receptor phosphorylation by an unidentified kinase indicate a role for the involvement of multiple protein kinases in GPCR phosphorylation. In addition to CK2, whose cellular effect is to regulate Jun-kinase signaling upon GPCR phosphorylation, casein kinase 1 (CK1) has been shown to phosphorylate the M_3-muscarinic receptor to regulate ERK1/2 signaling[29-30]. Evidence for a role of other protein kinases is also supported by the fact that some GPCRs, including the β_2-adrenergic and insulin receptors, are tyrosine phophorylated[31-34]. Phosphorylation of the β_2-adrenergic receptor by the Akt/PKB (protein kinase B) protein regulates agonist-induced internalization and cellular migration. Interestingly, Akt/PKB-induced phosphorylation is regulated by insulin receptor activation of PI-3 kinase. Glucose metabolism is controlled by insulin, which stimulates glycogen synthesis, glucose uptake and lipid storage. The actions of insulin are amplified by the insulin receptor-mediated tyrosine phosphorylation of the β_2-adrenergic receptor and subsequent internalization.

1.3.1e. Role of phosphorylation in arrestin interaction with G protein-coupled receptors

GPCRs can also function as scaffolding proteins that interact with a variety of signaling molecules; these interactions are often influenced by the conformation of the receptor. The general dogma is that agonist binding results in a change in the positions of transmembrane α-helices III and VI, resulting in a re-orientation of the third intracellular loop in a manner that facilitates G-protein coupling[35]. This conformational change is also presumed to unmask potential phosporylation sites on the intracellular domains of GPCRs[36]. The synergistic effects of conformational changes and posttranslational modification allow the receptor to interact with proteins that were previously inaccessible. Additionally, a conformation change of the receptor may lead to the conformational change in interacting proteins and subsequent signal transduction. Arrestins are a prime example of GPCR-interacting proteins that interact with receptors in a phosphorylation-dependent manner.

There are four family members in the arrestin family. Arrestin1 and arrestin4 (visual arrestins) are expressed solely in rod and cone photoreceptor cells and interact with phosphorylated rhodopsin and cone opsins to terminate phototransduction[37]. Arrestin2 and arrestin3 (nonvisual arrestins) are also known as β-arrestin1 and β-arrestin2. These proteins are expressed in a variety of tissues and can regulate hundreds of activated and phosphorylated GPCRs[38]. Generally, the concave surface of arrestins recognize and bind with high affinity to multiple phosphorylated serine and threonine sites on the receptor to inhibit G-protein coupling and desensitize the receptor[39].

Structural studies of arrestin have revealed amino and carboxyl termini composed of antiparellel β-sheets that are linked by an unusual 12-residue polar core[39]. The basal conformation of arrestin is maintained by a network of intramolecular interactions

between charged residues buried within the polar core[38]. The amino terminus of arrestin binds to various GPCR intracellular sequences, whereas positively charged polar core interacts with receptor-associated phosphates. Upon binding to activated and phosphorylated GPCRs, arrestins undergo a conformational change induced by engagement of receptor-associated phosphates with the polar core. Binding is followed by exposure of the arrestin C-terminal domain, which interacts with components of the endocytic machinery, including the clathrin heavy-chain and the β2-adaptin subunit of AP2, and thereby facilitates GPCR internalization[39].

The molecular mechanism of receptor/arrestin binding appears to be conserved between visual and nonvisual arrestins. However, there is a range of receptor/arrestin affinities. Class A receptors have a relatively low affinity for arrestins and the complex dissociates during the trafficking of the receptor to intracellular compartments. Class B receptors have a significantly high affinity for arrestins and the complex remains intact during the trafficking of the receptor to intracellular compartments following agonist stimulation[40-41].

It is also clear that phosphorylation is not a requirement for arrestin binding for all receptors. Phosphorylation-deficient mutants (substance P and leukotriene B4 receptors) still maintain the ability to interact with arrestins[42-43]. In addition, activated oxrexin-1[44] and protease-activated receptor-2 mutants[45] that have mutations in putative phosphorylation sites can still bind arrestins. Thus, it is possible that arrestins interact with multiple sites on the receptor and that these multiple interactions are sufficieint to form a stable complex in the absence of phosphorylation.

1.3.2. G protein-coupled receptor internalization

Agonist-stimulated GPCR internalization into intracellular compartments of the cell is an important mechanism in regulating signal magnitude and duration. Receptor internalization has been intensively and extensively investigated over the past several years and much is now understood regarding the molecular mechanisms involved in regulating internalization. Studies have identified GPCR domains involved in receptor internalization, adaptor proteins, and multiple endocytic mechanisms. The molecular mechanisms of GPCR internalization and intracellular trafficking were initially generalized from data on β_2-adrenergic receptor internalization[46-47] (Figure 1.1). However, recent studies investigating other GPCRs in a number of different cell types have revealed various patterns in GPCR internalization as well as intracellular trafficking. Studies have also revealed that the rates of internalization vary for each receptor[48], further supporting the idea that GPCR internalization can be mediated by multiple endocytic mechanisms. In addition, the different rates of internalization between different GPCRs may be reflective of the binding of different endocytic adaptor proteins to facilitate internalization.

Following internalization, the receptor signaling response is regulated by intracellular sorting. Internalized receptors are typically sorted to recycling or lysosomal pathways, trafficking routes that produce opposite effects on signal duration and magnitude. Endocytic trafficking to lysosomes is a major pathway by which many GPCRs are downregulated after agonist stimulation[49], while the recycling pathway promotes rapid recovery (resensitization) of cellular responsiveness. Alternatively, some

GPCR-arrestin complexes remain intact and continue to signal, or initiate new signaling pathways from the endosomal membrane[45].

Receptor-mediated endocytosis involves multiple steps: receptors bind to complementary adaptor proteins, accumulate in coated pits, and enter the cell as a receptor-adaptor protein complex within vesicles that bud off the plasma membrane. This process is a selective concentrating mechanism that increases the efficiency of internalization of ligands. Most G protein-coupled receptors internalize into cells via clathrin-coated vesicles. Clathrin-mediated endocytosis has been extensively studied and much information is available on the molecular mechanisms of clathrin-mediated endocytosis as well as on the adaptor proteins that regulate internalization. More recently, it has become clear that not all GPCRs internalize in a clathrin-dependent manner, but the molecular mechanisms are not fully understood and there are many adaptor proteins yet to be discovered.

1.3.2a. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is the major route for intergral membrane proteins and cell-surface receptors, including GPCRs. An endocytic-coated vesicle begins with recruitment of clathrin, adaptor, and endocytic accessory proteins to plasma membrane regions enriched in phosphatidylinositol (4,5)-bisphosphate (PIP_2), where they form patches ranging in diameter from 10 nm to more than 500 nm. Electron microscopy has provided images of polygonal clathrin networks at various stages of invagination, which culminate in almost completely closed polyhedral structures that surround coated membrane buds[50]. These images led to the accepted budding model in which the

internalization process starts with predominantly flat hexagonal lattices that turn into curved ones. Release of the clathrin-coated vesicle from the plasma membrane is mediated by the large GTPase dynamin. Eventually, vesicles encased in a clathrin network separate from the plasma membrane by a process referred to as fission. Before fusion with endosomes, clathrin and adaptors are released from the vesicular membrane. The now soluble coat proteins can engage in a fresh round of coated vesicles.

The function of clathrin adaptor proteins is to enrich specific cargo within a forming vesicle[51]. The adaptor protein complex-2 (AP2), the most abundant adaptor protein found in clathrin-coated vesicles at the plasma membrane, recognizes short linear tyrosine- and/or dileucine-based cytoplasmic sequences of cargo proteins[52]. Increasing evidence suggests that monomeric clathrin adaptors that bind clathrin and PIP_2 co-operate with AP2 to recruit cargo and promote clathrin-dependent endocytosis. Recent work has also identified an α-helical motif in several monomeric adaptor proteins, including arrestins, autosomal recessive hypercholesterolemia (ARH) and epsins, that bind the β2-subunit of AP2 with high affinity[53]. Clathrin adaptors interact with short linear sequences, in addition to recognizing phosphorylated and ubiquitinated cargo (Figure 1.4). Clathrin adaptors themselves are also modified by phosphorylation and ubiquitination, suggesting that clathrin adaptor activity is tightly regulated.

The first monomeric adaptor proteins shown to function in clathrin-mediated endocytosis were the nonvisual arrestins. Nonvisual arrestins interact with clathrin and AP2 and facilitate internalization of activated and phosphorylated GPCRs. A second group of monomeric clathrin adaptor proteins include epsins, clathrin assembly lymphoid myeloid leukemia protein (CALM)/AP180 (AP180 is a neuronal ortholog of CALM,

Huntington-interacting protein-1 (HIP1), and HIP1-related protein (HIP1R). These proteins contain an N-terminal PIP_2-binding domain [epsin N-terminal homology (ENTH)/AP180 N-terminal homology (ANTH) domain] and interact with clathrin and AP2. In addition, epsin harbors three ubiquitin-binding domains (UBDs), binds eps15 and eps15-related protein (eps15R), which also contain UBDs, and recruits ubiquitinated cargo to clathrin-coated pits. Both CALM/AP180 and HIP/HIPR lack UBDs and how they function in cargo recruitment is unknown. HIP1 and HIP1R dimerize via their coiled-coil regions and HIP1R binds directly to F-actin linking the actin cytoskeleton to the endocytic machinery.

A third group of monomeric clathrin adaptors include disabled-2 (Dab2), ARH, and numb. Members of this group bind AP2, some interact with clathrin, and all contain a phosphotyrosine-binding (PTB) domain that recognizes FXNNPXY motifs, although tyrosine phosphorylation is not required for binding. Very few studies examining the function of other clathrin adaptors in GPCR endocytosis exist, with the exception of arrestins. A summary of clathrin adaptor proteins and their interactions is provided (Figure 1.2)

1.3.2a.i. G protein-coupled receptor kinases

Depending on the GPCR studied, GRK-mediated phosphorylation is not an absolute requirement for internalization. For example, β_2AR mutants lacking sites for both second messenger-dependent protein kinase- and GRK-mediated receptor phosphorylation internalized comparably to the wildtype receptor[13,54]. In contrast, a protease-activated receptor-1 mutant lacking all possible carboxyl tail phosphorylation

sites failed to internalize[55]. GRK-mediated phosphorylation appears to have a role in GPCR internalization by stabilizing a conformation required to promote interaction of the receptor with adaptor proteins that directly promote receptor internalization. In fact, GRK-phosphorylation increases the binding affinity of GPCRs for non-visual arrestins, which in addition to uncoupling the receptor from heterotrimeric G proteins, act as an endocytic adaptor protein to target GPCRs for clathrin-mediated internalization[8-9]. Thus, the β_2AR mutant that lacks sites for GRK-mediated phosphorylation must be able to interact with an endocytic adaptor protein in the absence of phosphorylation.

1.3.2a.ii. Arrestins

The ubiquitously expressed arrestin2 and arrestin3, also known as β-arrestin1 and β-arrestin2, are best known for their regulatory role in desensitization (by uncoupling GPCRs from the heterotrimeric G-protein complex) and internalization. The mechanism by which arrestins promote GPCR endocytosis is best characterized for the β_2AR[56]. Activation of the β_2AR causes translocation of arrestins to the plasma membrane where the receptor preferentially binds arrestin3 rather than arrestin2. The activated β_2AR-arrestin complex redistrbutes to clathrin-coated pits and arrestin dissociates upon receptor internalization. Arrestin interaction with clathrin and AP2 is essential for agonist-induced β_2AR internalization. The endocytic activity of arrestin is controlled by both phosphorylation and ubiquitination. Arrestins are basally phosphorylated and dephosphorylated upon recruitment to the plasma membrane. Dephosphorylation of arrestins is required for agonist-promoted β_2AR internalization.

In addition to phosphorylation, the ubiquitination status of arrestin is important for its endocytic activity. Arrestins interact with the E3 ubiquitin ligase mouse double minute-2 (Mdm2), which specifically ubiquitinates arrestins bound to activated β_2AR[57]. Ubiquitination of arrestin is essential for β_2AR internalization.

The first reports of arrestin-independent GPCR internalization used dominant-negative arrestin and dynamin mutants. Using these mutants, the m1, m2, m3, and m4 muscarinic acetylcholine receptors[58-59], prostacyclin receptor[60], and 5-HT_{2A} serotonin receptor[61] were shown to internalize through an arrestin-independent, dynamin-dependent pathway. More definitive evidence for an arrestin-independent mechanism for GPCR endocytosis came from the use of mouse embryo fibroblasts (MEFs) derived from arrestin2 and arrestin3 knockout mouse mice[62]. Both constitutive and agonist-induced internalization of PAR1 were shown to proceed through an arrestin-independent but clathrin- and dynamin-dependent pathway in MEFs lacking endogenous arrestins[63]. Subsequent studies using arrestin-null MEFs demonstrated arrestin-independent internalization of the N-formyl peptide receptor[64], the human viral chemokine receptor US28[65] and the urotensin receptor[66]. These studies provide strong evidence that alternate clathrin adaptors function in GPCR endocytosis.

In addition to GPCRs, arrestins also regulate endocytosis of other cell-surface receptors and integral membrane proteins including the insulin-like growth factor-1 receptor, type III transforming growth factor-β receptor, low-density lipoprotein (LDL) receptor, the Na+/H+ exchanger 5 transporter and most recently vascular/endotheilial (VE)-cadherin[67]. In some cases, arrestins facilitate endocytosis by binding to phosphorylated serine/threonine residues in the cytoplasmic regions of these cargo

proteins. Thus, like other clathrin adaptors, arrestins are capable of regulating endocytosis of diverse cargo.

1.3.2.aiii. Adaptor protein complex-2

The finding that several GPCRs internalize independent of arrestins suggests that other clathrin adaptors function as critical regulators of mammalian GPCR endocytosis. One such critical clathrin adaptor is the adaptor protein complex-2. AP2 was the first clathrin adaptor identified and is still the key protein in the model of clathrin-dependent internalization. The heterotetrameric AP2 complex is composed of α (100 kD), β2 (100 kD), μ2 (50 kD), and σ2 (17 kD) subunits. Three subunits directly function in clathrin coat assembly. The amino terminus of the α2 subunit of AP2 binds to PIP_2 to position AP2 on the plasma membrane[68-69], while the β2 subunit has a flexible hinge the contains a clathrin-binding sequence (clathrin box) that binds to the terminal domain of the clathrin heavy chain promoting lattice assembly[70-71]. Cargo proteins bind to directly to AP2 through its μ2 subunit[72] while the primary role of the σ2 subunit is structural[69].

Transmembrane proteins require an internalization signal for rapid endocytosis. AP2 is able to recruit cargo through the μ2 subunit interactions with the tyrosine-based motif, YXXΦ (where X is any amino acid and Φ is a bulky hydrophobic residue). In addition, α2/σ2 subunits and possibly the β2 subunit recognizes acidic dileucine motifs ([DE]XXXL[LI]) on cargo proteins[73]. The endocytic activity of AP2 is regulated by μ2 subunit phosphorylation mediated by the AP2-associated kinase, AAK1, which enhances μ2 affinity for YYXΦ motifs[74].

AP-2 is known to mediate endocytosis of a variety of cell-surface receptors and integral membrane proteins, and a recent study demonstrated that AP2 functions directly in GPCR endocytosis. AP2, and not arrestin2 or -3, is required for constitutive internalization of PAR1 in a clathrin- and dynamin-dependent manner. Constitutive PAR1 internalization creates an intracellular pool of naïve receptor that replenishes the cell surface after thrombin exposure and is required for rapid cellular resensitization independent of *de novo* receptor synthesis. The μ2 subunit of AP2 was shown to directly bind to a tyrosine-based motif (YXXL) in the cytoplasmic tail of PAR1 using Surface Plasmon Resonance. In addition, expression of a PAR1 tyrosine mutant or depletion of AP2 by siRNA led to significant inhibition of PAR1 constitutive internalization, loss of intracellular, uncleaved PAR1, and failure of endothelial cells and other cell types to resensitize to thrombin signaling. Interestingly, internalization of agonist-activated PAR1 is neither dependent on arrestins nor AP2, suggesting that constitutive and agonist-induced internalization requires distinct endocytic machinery[75].

Several GPCRs contain tyrosine- and dileucine-based motifs conforming to canonical and non-canonical AP2-recognition motifs in their cytoplasmic tails[75], suggesting that AP2 might function in trafficking of other GPCRs. The thromboxane β receptor (TPβ) cytoplasmic tail contains a YXXXΦ and displays arrestin-independent constitutive internalization, but the role of AP2 has not been tested[76]. The CXCR2 chemokine receptor in which both cytoplasmic tail dileucine motifs were mutated bound arrestin but failed to interact with AP2 to undergo agonist-induced internalization[77]. The β_2AR and CXCR4 also contain a cytoplasmic tail dileucine motif that appears to function in receptor endocytosis. Mutation of the β_2AR cytoplasmic tail dileucine motif affects

agonist-induced endocytosis[78], but the affects on arrestin and AP2 binding is unknown. Interestingly, agonist-induced CXCR4 internalization is dependent on serine residues and an adjacent dileucine motif in some cell types[79], yet in other cell lines, the serine and dileucine endocytic signals are required for PKC-mediated internalization but not for agonist-induced internalization[80-81].

1.3.2a.iv. Disabled-2, autosomal recessive hypercholesterolemia, and numb

Numb, Dab2, and ARH are clathrin adaptor proteins that contain a phosphotyrosine-binding domain (PTB) domain that recognizes FXNPXY motifs in the cytosolic sequences of cargo. Outside of the PTB domain, the carboxy terminus of Dab2, ARH, and numb are predicted to be disordered, and like arrestins, contain adjacent clathrin box and/or AP2 appendage-binding sequences[82-85]. The *Xenopus* ARH orthologue requires these features to drive the internalization of the vitellogenin receptor, an FXNPXY-containing member of the LDLR family[86]. In addition, in *Drosophila*, there is strong genetic evidence for a numb-AP2 internalization in endocytosis[87]. These data suggest that PTB adaptor proteins can bind cargo, PIP_2, and clathrin in addition to AP2.

ARH and Dab2 have well-established functions in LDL receptor internalization, whereas numb has been shown to regulate endocytosis of a variety of integral membrane proteins including EGFR and notch. A recent study shows that numb is phosphorylated and redistributed to the plasma membrane following activation of the neurokinin-1/substance P receptor[88]. However, numb is not essential for receptor internalization and a role for Dab2 and ARH has not been demonstrated.

1.3.2a.v. Epsin superfamily

An alternate group of adaptors include epsin 1, CALM/AP180, HIP1, and HIP1R[89]. These proteins contain an N-terminal PIP_2-binding ENTH/ANTH domain[90] and all bind to and colocalize with AP2 and clathrin. Ubiquitination is the primary internalization signal in S. *cerevisiae*, and the yeast epsins, Ent1p and Ent2p, use ubiquitin-interaction motifs (UIMs) to promote rapid internalization[91]. Mutant alleles of *Liquid facets*, the *Drosophila* epsin, that disrupt ubiquitin prevent internalization of the transmembrane Notch ligand Delta in compound eye progenitors and consequently, severely malformed eyes develop[92]. Mammalian epsin UIMs may act similarly; however, interpretation of many studies is complex as a variety of proteins are ubiquitinated upon EGFR activation.

Studies suggest that the *C. elegans* AP180 orthologue, UNC-11, may participate in the sorting of synaptobrevin. Genetic disruption of the UNC-11 gene leads to selective mis-sorting of the v-SNARE protein at the presynaptic plasma membrane[93]. In addition, GluR1-containing AMPA receptor endocytosis is defective in neurons from $HIP1^{-/-}$ mice[94]. Currently, the molecular basis for AP180 and HIP1 cargo recognition is unknown. Both proteins lack UIMs, but the similarity between the ANTH and VHS (another cargo recognition module) domains suggest that ubiquitination may function in cargo selection and internalization.

1.3.2a.vi. Actin

A regulatory role for actin in yeast *S. cerevisiae* endocytosis has been clearly established; however its function in mammalian internalization is less clear. Yeast and

mammalian endocytosis not only differ in their requirement for actin but also for dynamin. Although dynamin is essential for release of clathrin-coated pits in mammalian cells, it does not appear to have a direct role in yeast endocytosis. Recently, the dynamics of actin, clathrin, and GPCR recruitment to endocytic sites in yeast were examined using total internal reflection fluorescence (TIRF) microscopy in living cells[95]. These studies used a combination of fluorescently tagged Ste2, a GPCR for the mating α-factor, pharmacological inhibitors, and mutant yeast strains lacking critical actin components to establish more clearly an essential role for actin cytoskeleton in Ste2 endocytosis. In budding yeast, actin appears to function mainly in plasma membrane invagination, constriction, and scission.

In contract to the yeast Ste2 receptor, the role of actin in mammalian GPCR endocytosis is less clear. In mammalian cells, actin appears at the plasma membrane in a transient burst just prior to clathrin-coated pit scission in living cells. However, its precise function in endocytosis is not well understood[96]. Given that many GPCRs interact directly or indirectly with a variety of actin-binding proteins such as EBP50, spinophilin, filaminA/ABP-280, cofilin, and spectrin, endocytosis of at least some GPCRs is likely to be regulated in an actin-dependent manner.

1.3.2a.vii. Ubiquitination

Although arrestins play a major role in desensitization and internalization of many mammalian GPCRs, their function is not evolutionarily conserved. The yeast *S. cerevisiae* do not express arrestins, but the protein Vps26 has a structural fold similar to arrestins[97]. Both biochemical and genetic studies indicate that Vps26 exists as part of the

retromer complex that functions in retrograde trafficking between endosomes and the *trans* Golgi network and Vps26 is unlikely to function in endocytosis. Instead of arrestins, internalization of yeast GPCRs requires ubiquitin, a small 76-amino acid polypeptide that functions as an internalization endocytic-sorting signal[98]. Protein ubiquitination is mediated by the sequential action of a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). E3 ligases are diverse and mediate substrate specificity.

Hicke et al. demonstrated an essential role for ubiquitination in endocytosis of Ste2 using yeast strains that lack specific ubiquitin-modifying enzymes and Ste2 ubiquitin mutants or chimeras[98]. Terrell et al. also showed that mono-ubiquintination is necessary and sufficient for both constitutive and agonist-induced internalization[99]. Although mono-ubiquitination is sufficient for endocytosis, attachment of short ubiquitin chains to lysine-63 of an adjacent ubiquitin facilitates endocytosis of many transmembrane proteins[100]. In addition, phosphorylation of activated Ste2 facilitates ubiquitination and promotes internalization[101]. A role for ubiquitination in endocytosis of Ste3, a GPCR for the mating a-factor, also has been established[102]. A recent study showed that ubiquitination differentially regulates clathrin-dependent internalization of PAR1[103], although the clathrin adaptor that mediates agonist-induced internalization of ubiquitinated PAR1 has yet to be identified. In contrast, several studies have suggested that mammalian GPCR ubiquitination is essential for lysosomal sorting but not for receptor internalizaition[57,104].

Several adaptor proteins, including epsin and eps15, contain ubiquitin-binding domains (UBDs) that recognize ubiquitinated cargo and facilitate internalization through

clathrin-coated pits. The NPF domains of epsin binds the epsin homology (EH) motifs in eps15 and these proteins likely function cooperatively to recruit ubiquitinated cargo to clathrin-coated pits.

Similar to arrestins, the endocytic function of epsin is regulated by phosphorylation and ubiquitination[105]. Epsin is basally phosphorylated and ubiquitinated and an influx of Ca^{2+} initiates rapid dephosphorylation[106]. De-phosphorylated epsin is then de-ubiquinated by the de-ubiquitinating enzyme, FAM/USP9X. In yeast, the epsin homologs, Ent1 and Ent2, as well as the eps15 homolog, Ede1, have been shown to regulate Ste2 internalization[91]. In mammalian cells, epsin has been shown to regulate endocytosis of epidermal growth factor (EGFR) and the epithelial sodium channel[107-109], but its function in mammalian GPCR internalization is unknown. Given that several GPCRs are ubiquitinated and internalize independent of arrestins, it is plausible that certain receptors will use an epsin and/or eps15-dependent mechanism for internalization. In addition, many GPCRs couple to G proteins that activate phospholipase Cβ (PLCβ), resulting in PIP_2 hydrolysis and Ca^{2+} mobilization raising the possibility that activated GPCRs may regulate epsin activity, thus allowing it to function as a clathrin adaptor for mammalian GPCRS.

1.3.2.b. Clathrin-independent endocytosis

In addition to clathrin-coated pits and vesicles, there are other, less well-understood mechanisms by which cells can form vesicles. One of these pathways initiates at caveolae, originally recognized by their ability to transport molecules across endothelial cells. Caveolae are present in the plasma membrane of most cell types, and in

some cell types are seen as deeply invaginated 50-80 nm flasks in electron micrographs. Caveolae are thought to form from lipid rafts, which are patches of plasma membrane that are rich in cholesterol, glycosphinglipids, and GPI-anchored membrane proteins. The major structural protein in caveolae is the integral membrane protein, caveolin.

The large GTPase dynamin was shown to be involved in a clathrin-independent pathway that is mediated by caveolae. Since vesicle scission at the plasma membrane is required for internalization, it was assumed that dynamin might have a role in all forms of endocytosis. However, it was later discovered that some clathrin-independent endocytic pathways are dynamin-independent, based on studies utilizing exogenous expression of mutant dynamin isoforms[110] or from analysis of organisms that are homozygous for mutant dynamin[111]. Thus, clathrin-independent internalization pathways can be categorized based on their dynamin dependence.

Little is known about how cargo is selected for the clathrin-independent endocytosis. In contrast to clathrin-dependent endocytosis, in which specific adaptor molecules have been identified that recruit cargo to coated pits, no such well-defined adaptors for clathrin-independent endocytosis have been identified. One potential mechanism that has been considered is sorting based on the association of cargo with microdomains at the plasma membrane. It should be noted, however, that the association of cargo with detergent-resistant membranes (DRMs) does not guarantee subsequent internalization by a clathrin-independent mechanism[112-114].

It recently has been suggested that ubiquitination could have a role in determining whether a protein is internalized via a clathrin-dependent or –independent mechanism. Ubiquitination of some receptor tyrosine kinases, e.g. the epidermal growth factor (EGF)

receptor, is mediated by c-Cbl, an E3 ubiquitin ligase. Ubiquitin ligases are enzymes that function downstream of or in combination with an ubiquitin-conjugating enzyme (E2) to attach ubiquitin molecules to a target protein, marking the protein for subsequent recognition by ubiquitin-binding domains. Although EGF receptors have been found to be internalized through both clathrin-dependent and –independent pathways, it was recently suggested that ubiquitination might be used to sequester EGF receptors into clathrin-independent routes, despite the fact that they also have clathrin-pit localization signals. For example, high concentrations of EGF promote ubiquitination of EGF receptors, association with DRMs, and endocytosis has a cholesterol-sensitive clathrin-independent mechanism, possibly by caveolae[113].

Mechanistically, it has been difficult to elucidate the sequestration of cargo in caveolae. Some data suggest that EGF receptors are guided into the caveolar pathway by three ubiquitin interacting motif (UIM)-containing proteins: EPS15, epsin, and EPS15R. However, all three proteins are somewhat redundant in their function because a strong inhibitory phenotype is only observed in triple small interfering RNA (siRNA) knock-down studies[113]. These studies were controversial based on the established function of EPS15, epsin, and EPS15R in classic clathrin-dependent internalization.

Clathrin-independent internalization has also been described for GPCRs, including the endothelian A (ETAR) and endothelian B (ETBR) receptors, adenosine A1, β_1-adrenergic, bradykinin B2, and chemokine receptors[115]. To characterize the various endocytic routes used by different receptors, several pharmacological and biochemical tools have been used. These include blockers that do not discriminate between clathrin-dependent or caveolae-mediated internalization (ex. hypertonic sucrose,

low temperature, concanavalin-A, and dominate-negative mutants of dynamin) as well as with inhibitors believed to selectively inhibit clathrin-coated vesicle endocytosis (e.g. monodansylcadaverine, chlorpromazine, as well as dominant-negative mutants of Eps15) or caveolae-mediated endocytosis (e.g. filipin and nystatin)[115-118]. Although these approaches have been useful, interpretation of the data were sometimes difficult because the tools were an indirect measurement.

The above discussion illustrates the importance of developing new tools that allow direct assessment of the molecular steps involved in endocytosis of specific GPCRs. Hamdan et al. took advantage of the fact that, for internalization, arrestin binding to the β2-adaptin subunit of the clathrin AP-2 is needed for arrestin-mediated targeting of GPCRs to clathrin-coated pits. They designed a bioluminescence resonance energy transfer (BRET)-based assay that allows real-time monitoring of the interaction between arrestins and β2-adaptin as a biosensor for arrestin-promoted clathrin-mediated endocytosis. BRET is a non-fluorescence energy transfer that occurs between the energy donor *Renilla* luciferase (*R*luc) and variants of the green fluorescent proteins as energy acceptors only when the two proteins are within 100 Å from one another and situated in favorable orientation, making it an attractive assay for studying both inducible and constitutive protein-protein interactions.

BRET was assessed between arrestin-*R*luc and β2-adaptin-EYFP in response to the activation of 12 different GPCRs, including V1aR, β2AR, ETAR, ETBR, V1P1R, V2R, AT1aR, EP4R, C5aR, CCR5, B2R, and M2R, previously suggested to internalize via different endocytic routes. The data showed that some of the GPCRs previously suggested to internalize via a clathrin-independent pathway were capable of promoting

arrestin interaction with AP2. Thus, these receptors internalize via an arrestin- and clathrin-dependent mechanism.

The ETAR and ETBR receptors are capable of recruiting arrestins but were shown to internalize via caveolae. These receptors, however, did not promote arrestin interaction with β2-adaptin, suggesting an arrestin-dependent, but clathrin-independent endocytic pathway[119]. In addition to providing a new tool to dissect the molecular events involved in GPCR endocytosis, the BRET-based arrestin/β2 adaptin interaction assay can also be used to detect constitutive receptor internalization and to quantify receptor activation in a pharmacologically relevant manner.

1.3.3. G protein-coupled receptor down-regulation

G protein-coupled receptor desensitization is the downregulation of total receptor levels following prolonged agonist treatment. Endocytic sorting to lysosomes is the major pathway by which most GPCRs and other cell surface receptors, including receptor tyrosine kinases, are down-regulated following activation[49,120-122]. When agonist is present for an extended period of time, receptors are sorted to lysosomes and subsequently degraded (Figure 1.1). For example, the β_2AR predominantly recycles after initial agonist-induced internalization, but can undergo significant down-regulation following prolonged exposure to agonist. Such behavior in receptor regulation is thought to be physiologically important and may contribute to the loss of drug potency or effectiveness observed over time in clinical settings[123-124].

1.4. G protein-coupled receptors as therapeutic targets

GPCRs represent the largest family of cell-surface molecules involved in signal transduction by a large margin, accounting for >2% of the total genes encoded by the human genome. These receptors control key physiological functions such as neurotransmission, hormone and enzyme release from endocrine and exocrine glands, and immune responses, and pathological conditions such as thrombosis, atherosclerosis, and inflammation. Their dysfunction contributes to some of the most prevalent human diseases, including cardiovascular diseases and cancer, as reflected by the fact that GPCRs are targets (directly or indirectly) of 50-60% of all current therapeutic agents.

1.4.1. G protein-coupled receptor signaling and thrombosis

Cardiovascular diseases are major causes of death in the developed world, and are becoming an increasing burden in a number of developing countries. In the United States, cardiovascular disease, including high blood pressure, coronary heart disease, stroke, and heart failure, claimed almost one in three deaths in 2004 (American Heart Association, 2008). Thrombosis, which is formation of a blood clot within the blood vessel that results in occlusion of blood flow, is a major problem that triggers both myocardial infarction and stroke. Thrombosis is the result of inappropriate triggering of a normal protective homeostatic mechanism, haemostasis, the function of which is to prevent excessive blood loss following injury.

Platelets form the first line of defense, triggering haemostasis upon encountering damaged tissue. When tissue is damaged, it triggers the release of prothrombotic factors, such as oxidized lipids, and exposure of collagen, which trigger thrombosis. Thus, therapeutic targeting of receptors in platelets that mediate thrombosis is an effective

means to prevent and treat cardiovascular disease. The regulation of platelet function, and therefore haemostasis and thrombosis, is a tightly regulated balance between activation and inhibitory mechanisms that control platelet activation upon exposure to damaged tissues, yet enables platelets to remain quiescent in the undamaged circulation. Characterization of these mechanisms, including agonists, receptors, and signaling pathways involved, is essential for the understanding of platelet function in health and disease and for the development of more effective anti-platelet therapies. Thrombin and purinergic signaling, mediated by G protein-coupled protease-activated (PAR) and P2Y receptors, respectively, are important regulators of platelet biology (Figure 1.3). **Chapter three in this dissertation investigates the mechanisms of regulation of $P2Y_1$ receptor internalization.** These data could ultimately provide insight into the mechanism of platelet function and thrombosis regulation.

1.4.2. G protein-coupled receptor expression and cancer

Emerging experimental and clinical data indicate that GPCRs have a crucial role in cancer progression and metastasis. Malignant cells can hijack the normal physiological functions of GPCRs to proliferate autonomously, evade immune detection, increase their nutrient and oxygen supply, invade their surrounding tissues and disseminate to other organs. Activating mutations of G proteins and GPCRs drive the unregulated growth of some endocrine tumors and constitutively active GPCRs are even expressed from the genomes of human oncogenic DNA-viruses.

GPCRs are the target of key inflammatory mediators, therefore providing a probable link between chronic inflammation and cancer. In addition, GPCRs have a

central role in tumor-induced angiogenesis and tumor metastasis via GPCR-guided migration of cancer cells to their target organs. Ultimately, aberrant overexpression of GPCRs and their autocrine and paracrine activation by agonists released from tumor or stromal cells represents the most frequent tactic used by tumor cells to stimulate GPCRs and their signaling networks. Among such overexpressed GPCRs are protease-activated receptors. **The specific roles of protease-activated receptor 1 (PAR1) and protease-activated receptor 2 (PAR2) in migration and invasion of carcinoma cells will be examined in chapter two.**

1.5. Coagulant proteases and protease-activated receptors

1.5.1. Coagulation and cellular signaling

Blood coagulation is part of an important host defense mechanism termed haemostasis, which is the termination of blood loss from a damaged vessel. Upon vessel injury, platelets adhere to macromolecules in the subendotheilal tissues and then aggregate to form the primary haemostatic plug. Platelets stimulate local activation of plasma coagulation factors, leading to generation of a fibrin clot that reinforces the platelet aggregate. Later, as wound healing occurs, the platelet aggregate and fibrin clot are broken down. Mechanisms that restrict formation of platelet aggregation and fibrin clots to sites of injury are necessary to maintain the fluidity of the blood.

The coagulation cascade has two pathways, the intrinsic or contact activation pathway and the extrinsic or tissue factor pathway that lead to fibrin formation (Figure 1.4). The pathways are a series of reactions, in which a zymogen (inactive enzyme

precursor) of a serine protease and its glycoprotein co-factor are activated to catalyze the next step in the reaction cascade, ultimately resulting in cross-linked fibrin.

The primary pathway for the initiation of blood coagulation is the tissue factor pathway. Its main role is to generate a "thrombin burst" where thrombin, the major effector of the coagulation cascade, is released. The extrinsic pathway is initiated following damage to the blood vessel when the cell surface integral membrane protein tissue factor comes in contact with plasma. Tissue factor is expressed by epithelial cells, macrophages, and other cell types that normally are not in contact with blood and circulating factors. Tissue factor binds to factor VII and activates it by forming the TF-FVIIa complex. TF-VIIa in turn activates factor X which converts prothrombin to thrombin. The contact activation pathway begins when plasma protein factor XII becomes activated (XIIa) once it comes in contact with collagen beneath the endothelium. FXIIa converts FXI into FXIa. Factor XIa activated FIX, which with its co-factor FVIIIa activated FX to FXa.

Thrombin has a variety of functions. Its primary role is the conversion of fibrinogen to fibrin, the building block of a haemostatic plug. In addition, it activates Factors VII and V and their inhibitor protein C (in the presence of thrombomodulin), as well as Factor XIII, which forms covalent bonds that crosslink the fibrin polymers that form from activated monomers. Following activation by the contact factor or tissue factor pathways, the coagulation cascade is maintained in a prothrombic state by the continued activation of FVIII and FIX to form the ternase complex, until it is down-regulated by the anticoagulant pathways. In addition to acting on blood proteins, thrombin also activates protease-activated receptors.

1.5.2. Protease-activated receptors

The coagulant protease thrombin mediates a variety of cellular responses important for haemostasis and thrombosis, as well as atherogenesis, restenosis, and embryonic development. Thrombin is a potent activator of platelets and endothelial cells, and is a mitogen for fibroblasts and vascular smooth muscle cells. Thrombin elicits its actions through the activation of at least three protease-activated G protein-coupled receptors: PAR1, PAR3, and PAR4. A fourth protease-activated receptor, PAR2, is activated by multiple trypsin-like serine proteases, including factor VIIa-Xa (tissue-factor) complex (Table 1.2).

1.5.2a. PAR1

PAR1 is expressed in a wide variety of cell types including platelets, endothelium, epithelium, fibroblasts, myocytes, neurons, and astrocytes. Formerly known as the thrombin receptor, PAR1 was cloned by two laboratories by employing the strategy of expressing RNA from thrombin-responsive cells of humans and hamsters in oocytes from *Xenopus*[125-126]. cDNA libraries from thrombin-responsive megakaryocyte-like HEL and Dami cells were synthesized and transcribed *in vitro*. The resulting cRNA was injected into *Xenopus* oocytes and screened for thrombin-induced calcium responses. Clones were identified that encoded a protein of 425 residues with seven hydrophobic domains typical of a GPCR. The deduced sequence of PAR1 contains an amino-terminal signal sequence, an extracellular amino-terminal domain of 75 residues, and two sites on the extracellular amino-terminus that are important for thrombin binding and activation. The

binding site is a negatively-charged hirudin-like sequence (DKYEPFWEDEE) that interacts with the recognition site of thrombin (Table 1.2).

The mechanism by which thrombin activates PAR1 has been investigated in detail. The interaction between PAR1 and thrombin is believed to induce a conformational change that facilitates the binding of the catalytic site of thrombin and subsequent cleavage after ^{41}Arg (LDPR^{41} ↓ S^{42}FLLRN, where ↓ denotes cleavage). After cleavage, a new tethered ligand, S^{42}FLLRN, is exposed and binds to and activates the receptor, resulting in signal transduction. Several observations support this mechanism of activation. Mutation of the cleavage site prevents thrombin cleavage and signaling, supporting the importance of this site for PAR1 activation[126]. A synthetic peptide that mimics the tethered-ligand domain (S^{42}FLLRNPNDKYEPF) directly interacts with PAR1 without the requirement for hydrolysis by thrombin[126], and peptides as short as six residues (S^{42}FLLRN) are also fully active[127]. In addition to thrombin, several serine proteases, including coagulation proteases factor VIIa and factor Xa, metalloproteases, and plasmin are also capable of cleaving and activating PAR1[128-129].

1.5.2b. PAR2

PAR2 was identified by screening a mouse genomic library with degenerate primers designed from the second and sixth transmembrane domains of the bovine neurokinin 2 receptor[130-131]. A clone was found that encodes a protein of 395 residues with the typical characteristics of a GPCR and with ~30% sequence homology to PAR1. The extracellular amino terminus of 46 resides contains a putative trypsin cleavage site (SKGR^{34}↓S^{35}LIGKV). Similar observations indicated that trypsin cleavage of PAR2 at

$R^{34}\downarrow S^{35}$LIGKV reveals the amino-terminal tethered ligand SLIGKV[130-132]. For example, mutation of the trypsin site inhibits trypsin cleavage and activation of PAR2, and synthetic peptides corresponding to the tethered ligand domain activate PAR2 in the absence of receptor cleavage.

PAR2 is expressed in a variety of cell types including human endothelial, smooth muscle, leukocytes, fibroblasts, and epithelial cells and appears to regulate a broad range of normal physiological responses, including chemotaxis, cell growth, mitogenesis, and angiogenesis[133-137]. PAR2 has also been implicated in various pathological responses including tissue repair, inflammation, and pain[134,138-139]. In addition to trypsin and tryptase, the tissue factor-factor Xa- factor VIIa complex can activate PAR2[135] (Table 2).

1.5.2c. PAR3

The observation that platelets from PAR1-deficient mice still responded to thrombin suggested the existence of additional receptors for thrombin[140]. PAR3 was cloned by using degenerate primers designed from conserved domains of PAR1 and PAR2 to screen a cDNA library from rat platelets[141]. Human and mouse forms of PAR3 were cloned and found to have ~28% sequence homology to human PAR1 and PAR2. The thrombin cleavage site for PAR3 within the extracellular amino terminus is at $LPIK^{38}\downarrow T^{39}$FRGAP. Cleavage by thrombin exposes a new tethered ligand (T^{39}FRGAP) that is thought to interact intramolecularly with the receptor. However, in contrast to PAR1, PAR2, and PAR4, synthetic peptides corresponding to the putative tethered ligand do not activate PAR3. Another unexpected observation was that mouse PAR3 did not signal when expressed in the absence of other PARs. Expressing mouse PAR4 alone in

COS-7 cells resulted in thrombin-promoted phosphoinositide hydrolysis; however, co-expression of mouse PAR4 with mouse PAR3 decreased the EC_{50} for thrombin-stimulated phosphoinositide hydrolysis by 10-fold[142]. Thus, it is assumed that mouse PAR3 acts as a cofactor for thrombin cleavage and activation of mouse PAR4. Thrombin is thought to remain transiently tethered to PAR3 via the hirudin-like sequence to cleave and activate PAR4 *in trans*. PAR3 expression is observed on endothelial cells, myocytes, and astrocytes (Table 1.2).

1.5.2d. PAR4

PAR4, a 385-amino acid protein with a potential cleavage site for thrombin and trypsin in the extracellular amino-terminal domain, was found through searching expressed sequence tag (EST) libraries[143-144]. Thrombin and trypsin cleave PAR4 at $PAPR^{47}\downarrow G^{48}YPGQV$, and peptides corresponding to the tethered-ligand domain GYPGQV can directly activate PAR4 (Table 1.2). PAR4 is ~33% homologous to the other human PARs, but has distinct differences in the amino- and carboxyl-terminal domains. Interestingly, PAR4 does not have a hirudin-like thrombin-binding domain like PAR1 and PAR3[143-144]. Consequently, activation of PAR4 requires ~50-fold higher thrombin concentrations than that required to activate PAR1. The agonist peptide concentration required for PAR4 activation is also substantially higher than those for PAR1 and PAR2.

In addition, PAR4 displays a slower and more sustained signaling response compared to PAR1[145]. In transfected fibroblasts, activated PAR4 promoted substantially higher phosphoinositide hydrolysis per activated receptor than activated PAR1 and was

shut off more slowly. For PAR1, agonist-induced shutoff and internalization depends upon phosphorylation of the carboxyl-tail. PAR4 phosphorylation is undetectable, however, and agonist-induced internalization was much slower than that observed for PAR1[145].

1.5.3. Regulation of protease-activated receptor signaling

PAR1, the prototype of this family, couples to multiple G-protein subtypes including G_q, G_i, G_o, and $G_{12/13}$ to elicit a variety of cellular responses, including cytoskeletal changes, adhesion, migration, gene transcription, and proliferation. Because proteolytic activation creates a tethered ligand that cannot diffuse away, the mechanisms that contribute to termination of receptor signaling are critical in determining the magnitude and duration of thrombin responses in cells.

Despite the irreversible proteolytic mechanism of PAR1 activation, signaling by the receptor to G protein activation is rapidly terminated by a phosphorylation- and arrestin-dependent mechanism. Activated PAR1 is then internalized, sorted directly to lysosomes, and rapidly degraded[49]. Thus, in contrast to most GPCRs, internalization and lysosomal sorting of activated PAR1 is required for termination of signaling. PAR1 utilizes a clathrin- and dynamin-dependent pathway for internalization based on observations that activated PAR1 was rapidly recruited to clathrin-coated pits and internalization was blocked by dominant-negative dynamin and clathrin hub mutants in both fibroblasts and HeLa cells[55]. In striking contrast to most GPCRs, activated PAR1 internalizes independent of arrestins[63].

1.6. Role of PARs in physiology

1.6.1. Embryonic development

Protease-activated receptors have an important function in embryonic development through their contribution to normal blood vessel development. At mid-gestation, approximately half of PAR1-deficient embryos die with pericardial edema, bleeding, and delayed maturation of yolk sack vasculature[140,146]. It is difficult to attribute the bleeding and death of PAR1-deficient embryos to a platelet function defect because PAR1 does not function in mouse platelets and mice that lack platelets or platelet responses to thrombin develop normally. PAR1 is expressed most abundantly in endocardial and endothelial cells at midgestation, and the endothelial-specific Tie2 promoter drives PAR1 expression to prevent the death of PAR1-deficient embryos[146]. Thus, it seems as though PAR1 expression and signaling in endothelial cells is required for blood vessel development. Interestingly, knockout of several coagulation factors causes abnormal vascular development[147-150], suggesting that coagulant proteases signal through PAR1 in the embryo.

1.6.2. Cardiovascular system

Endothelium-dependent relaxation or contraction, depending on the type of blood vessel, is regulated by PAR1. In endothelial cells, PAR1 also controls angiogenesis and alters the expression of multiple genes, including chemokines, cytokines, and cell adhesion molecules. In smooth muscle cells, activation of PAR1 regulates contraction, hypertrophy, production of the extracellular matrix (ECM), cellular migration, and proliferation.

In addition to PAR1, other protease-activated receptors have a role in regulating the vasculature. PAR2 also controls relaxation and angiogenesis in endothelial cells and in smooth muscle cells regulate the contraction, cell migration, proliferation, production of ECM, and hypertrophy. PAR4, along with PAR3 as a cofactor, has been reported to induce nitric oxide (NO) production in endothelial cells, and endothelium-dependent relaxation[151]. The role of PAR4 in smooth muscle cell regulation has yet to be defined.

1.6.3. Platelet activation

PAR knockout mice and blocking antibodies have been invaluable in understanding the function of protease-activated receptors in platelet activation by thrombin. Thrombin is generated at the sites of vascular injury when tissue factor is exposed to plasma coagulant proteases. Platelet activation by thrombin induces shape change, release of ADP and thromboxane A2 (TxA_2), and integrin-mediated aggregation. ADP and TxA_2 further act on platelets to create a positive feedback loop to amplify platelet activation. Activated platelets also provide a surface for assembly of the ternase and prothrombinase complexes to produce more thrombin.

In platelets, PAR1 couples to several G proteins to regulate signaling pathways important for activation. PAR1 coupling to $G\alpha_q$ is required for platelet shape change, secretion and aggregation, $G\alpha_{13}$ contributes to shape change and $G\alpha_{z/i}$ synergize signaling by other platelet agonists. PAR4 also couples to $G\alpha_q$ and possibly $G\alpha_{13}$, but not to $G\alpha_i$ in fibroblasts. The importance of differential G protein coupling between PAR1 and PAR4 has yet to be determined for platelet function.

Interestingly, there are species-specific differences in platelet PAR expression. PAR1 and PAR4 mediated thrombin signaling in human platelets whereas PAR3 and PAR4 are functional receptors in mouse platelets. In human platelets, low thrombin concentration is sufficient to induce secretion and aggregation through PAR1 activation. In contrast, high thrombin concentrations activate PAR4 in the absence of PAR1, suggesting redundancy in this important system. Additionally, it is possible that PAR4, which lacks a thrombin-binding hirudin-like sequence, is activated by proteases other than thrombin. Platelet activation by cathepsin G, a granzyme released by activated neturophils, is mediated by PAR4; however the importance of this phenomenon *in vivo* is unknown.

1.7. Pathology of PARs and therapeutics

1.7.1. Hemostasis and thrombosis

Thrombosis is a pathologic process in which a platelet aggregate and/or a fibrin clot forms in the lumen of an intact blood vessel or in a chamber of the heart. If thrombosis occurs in an artery, the tissue supplied by the artery may undergo ischemic necrosis (i.e., myocardial infarction due to thrombosis of a coronary artery). If thrombosis occurs in a vein, the tissues drained by the vein may become inflamed. Thrombosis of a deep vein in a lower extremity may be complicated by pulmonary embolism, in which all or a portion of the thrombus breaks loose, is carried in the bloodstream through the vena cava and the right side of the heart, and becomes lodged in a pulmonary artery. Massive pulmonary embolism can cause hypoxemia, shock, and death.

Thrombin-mediated activation of platelets and endothelial cells via PAR1 plays an important function in regulating haemostasis. Therefore, developing therapies aimed at inhibiting thrombin function have garnered much attention. However, data from mouse studies suggest that thrombin is absolutely necessary for haemostasis, and in man a relatively narrow therapeutic index with these agents is observed[142-143]. A significant portion of prothrombin-deficient mice die at midgestation due to cardiovascular collapse and the surviving mice die from uncontrollable bleeding. In addition, clinical studies in human patients show that inhibiting thrombin increases bleeding risks, suggesting that thrombin is not a viable therapeutic target.

Studies in platelets from *PAR4*-/- mice, which are not activated by thrombin, suggest that PARs are viable therapeutic targets[143]. However, because of the species difference in PAR expression in platelets, antagonizing PARs in humans for anti-coagulant therapy is more complicated than in mice. In humans, it may be necessary to antagonize both PAR1 and PAR4 for therapeutics aimed at inhibiting thrombin activation of platelets, since both receptors mediate thrombin-stimulated platelet activation. Additionally, thrombin triggers fibrin formation and protein C activation in addition to activating PARs on platelets. Thus, PAR antagonists have the potential to block thrombin-mediated platelet activation without affecting these other functions of thrombin.

1.7.2. Cancer

A function for PAR1 in breast carcinoma cell invasion is suggested by increased PAR1 mRNA and protein expression detected in highly invasive breast carcinoma cell lines and in infiltrating ductal carcinomas of breast tissue specimens. In contrast, PAR1

expression is minimal or absent in noninvasive cell lines, premalignant atypical hyperplasia, and normal breast epithelial tissue specimens. Importantly, the addition of thrombin further increases invasiveness of certain breast carcinoma cells. Antisense inhibition of PAR1 expression reduces invasion *in vitro* in response to thrombin and fibroblast conditioned medium[152].

In a study quantifying PAR1 protein expression in human mammary carcinoma cell lines, thrombin-induced invasive potential was determined by examining the ability of cells to invade through a reconstituted basement membrane *in vitro*. Increased PAR1 protein expression correlated with the degree of cellular invasiveness exhibited by the various breast carcinoma cell lines[152]. These findings strongly suggest that activation of PAR1 is required for thrombin-mediated breast carcinoma cell invasion, and raise many questions regarding the molecular basis for these effects.

In highly invasive breast carcinoma cells, activated PAR1 fails to efficiently down-regulate, whereas the receptor is rapidly degraded in normal noninvasive human mammary epithelial cells (HMECs) that ectopically overexpress PAR1. The failure of activated PAR1 to be efficiently down-regulated was due to a decrease in PAR1 internalization and increase of recycling in breast carcinoma cells compared to HMECs[152]. Thus, activated PAR1 is not sorted to lysosomes and degraded in invasive breast carcinoma cells and could explain the increased expression of PAR1 protein. As a result of aberrant sorting to lysosomes, proteolytically activated PAR1 caused sustained activation of phosphoinositide hydrolysis and extracelluar signal-related kinase (ERK 1/2) signaling in highly invasive cells. ERK1/2 not only promotes cell cycle progression, but also contributes to cellular transformation, migration, and survival. No gene

mutations were detected in the endogenous PAR1 coding sequence and the mechanism(s) responsible for abnormal PAR1 trafficking or signaling in invasive breast carcinoma cells is not known.

In addition to thrombin, which has a known role in tumor metastasis, it is possible that other coagulation proteases, upstream of thrombin generation, contribute to cellular responses in the tumor microenvironment via activation of PAR1 and PAR2. Tissue factor is highly overexpressed in invasive carcinoma cells and has been shown to promote cellular signaling, tumor growth, and angiogenesis. Tissue factor also plays an important role in metastasis by generating active coagulant proteases factor VIIa and Xa.

1.8 Purinergic signaling and receptors

1.8.1. Purinergic signaling

The concept of purinergic signaling was introduced in 1929 by Drury and Szent-Györgyi when ATP was discovered as an extracellular signaling molecule in the heart and blood vessels. However, ATP was known as an intracellular energy source and the notion that such a ubiquitous molecule would be an extracellular messenger was considered unlikely by many. Evidence that ATP was a neurotransmitter in nonadrenergic, noncholinergic (NANC) nerves supplying the gut was presented in 1970, and in 1972 the word "purinergic" was coined. In 1972, Burnstock proposed the purinergic neurotransmission hypothesis. Key points from the hypothesis were: 1) purinergic inhibitory neurons are present in the stomach of fish, amphibians, reptiles, and birds and that the neurons in the stomach and distal rectum are controlled by preganglionic cholinergic fibers in the vagus and pelvic nerves, respectively. In

mammals, however, purinergic inhibitory neurons extend throughout the alimentary tract and are controlled by intramural cholinergic neurons; 2) ATP mimicks a prominent feature in the autonomic nervous control system, the excitatory action of postganglionic, non-cholinergic excitatory nerves in the gut of lower vertebrates, fish, amphibians, and mammals.

More recent studies show that ATP is a co-transmitter in the peripheral and central nervous systems and further illustrate the roles of nucleotides as extracellular signaling molecules to non-neuronal cells including endothelial, exocrine and endocrine, immune, inflammatory, and secretory cells. In fact, clear signaling roles for ATP, ADP, UTP, and UDP have been established in several tissues to regulate various functions such as i) ionotropic, chronotropic, and arrhythmogenic effects in the myocardium, ii) gastrointestinal and liver function, iii) regulation of epithelial cellular responses, iv) blood flow distribution and oxygen delivery, v) immune responses and control of leukocyte trafficking between blood and tissues, and vi) activation and aggregation of platelets at sites of vascular injury. Nucleotides also appear to have potent roles in cellular proliferation and growth, induction of apoptosis and anticancer activity, atherosclerotic plaque formation, wound healing, and bone formation and resorption.

1.8.2. Mechanisms of nucleotide release

The release of endogenous nucleotides represents a critical component for initiating signal transduction. Upon cell lysis, massive leaking of nucleotides could occur, but this nonspecific mechanism is restricted to organ injury, traumatic shock, or

certain inflammatory conditions. A non-lytic efflux of nucleotides also represents an important nucleotide release mechanism. Since ATP is a neurotransmitter, ATP is released from excitatory cells into the extracellular space where ATP (and its break down products) can activate cell surface purinergic receptors. One study shows that in addition to adenine nucleotides, other nucleotides are released[153]. In addition, cells other than excitatory cells can release nucleotides. For example, most resting cells release ATP in a basal, constitutive manner, which results in low steady-state concentrations in the extracellular milieu due to the competing actions of ATP release and subsequent hydrolysis. Very little is understood regarding the mechanisms of nucleotide release; however, the diversity of conditions in which the cells release ATP and/or ADP implies multiple nucleotide-releasing pathways.

1.8.2a. Exocytotic release

Various excitatory/secretory tissues such as nerve terminals, platelets, chromaffin, mast, and pancreatic acinar cells store ATP and ADP with other neurotransmitters and extracellular mediators in specialized granules. The release of nucleotide-containing vesicles in a Ca^{2+}-dependent manner is regulated via exocytosis. In neurons specifically, the regulated exocytosis of ATP is a widely-studied process.

1.8.2b. Mechanical stimulation

Nucleotide release also occurs from non-excitotory tissues, including epithelial and endothelial cells, astrocytes, fibroblasts, hepatocytes, bone cells, cardiomyocytes, neutrophils, macrophages, and other hematopoietic cells. These cells have been shown to

release ATP transiently under certain mechanical and other stimuli, such as shear stress, hypotonic cell swelling, and hypoxia. Moreover, cells release low nanomolar concentrations of ATP at basal rates. The molecular mechanism of mechanical stimulation to release nucleotides from nonexcitatory cells is not fully understood. However, the process may involve ATP-binding cassette transporters, plasmalemmal voltage-dependent ion channels, and/or connexin hemichannels.

1.8.2c. Agonist-dependent nucleotide release

An increase in extracellular ATP concentrations in endothelial cells has been observed upon addition of various GPCR agonists, including thrombin, serotonin, acetylcholine, bradykinin, and other Ca^{2+}-mobilizing pharmacological agonists[154]. The molecular mechanisms of agonist-dependent nucleotide release in the response to agonists are poorly understood.

1.8.3. Extracellular nucleotide metabolism

The release of endogenous nucleotides represents a critical component for initiating a signaling cascade; thus, extracellular nucleotides need to be rapidly inactivated subsequent to signal transduction. General schemes of extracellular nucleotide metabolism include a role for 1) ecto-nucleoside triphosphate diphosphohydrolases, 2) ecto-nucleoside pyrophosphatases, 3) alkaline phosphatases, and 4) ecto-5'-nucleotidases.

1.8.3a. Ecto-nucleoside triphosphate diphosphohydrolase family

Ecto-nucleoside triphosphate diphosphohydrolases (E-NTDPase) hydrolyze nucleoside tri- and/or diphosphates, but not monophosphates, and require millimolar Ca^{2+} and Mg^{2+} concentrations for maximal activity. Initially, it was assumed that the sequential hydrolysis of ATP to ADP and AMP occurred in two steps requiring ATPase and ADPase activity. However, more recent studies suggest that a single ATP-diphosphohydrolase can hydrolyze both ATP and ADP. Current nomenclature now refers to these enzymes as NTPDases, based on the broad substrate specificity of ecto-ATPases/ADPases towards different purine and pyrimidine nucleoside tri- and diphosphates.

There are eight members of the NTPDase protein family. NTPDase 1, 2, 3, and 8 are expressed on the cell surface. These enzymes are highly glycosylated with molecular masses ranging from ~70-80 kDa. NTPDases 1, 2, 3, and 8 show close immunological cross-reactivity and may exist in either monomeric or dimeric to tetrameric states. These proteins contain predicted transmembrane domains at the amino and carboxy termini and a large extracellular loop containing a central hydrophobic region with five highly conserved sequence domains known as the apyrase conserved regions (ACR). ACR1 and ACR4 share a common sequence homology with members of the actin/HSP90/sugar kinase superfamily.

NTPDases 5 and 6 are intracellular and undergo secretion after heterologous expression, while NTPDases 4 and 7 are entirely intracellular, facing the lumen of cytoplasmic organelles. NTPDase proteins can hydrolyze a variety of nucleoside di-and triphosphates, although preferences vary among enzymes. Specifically, the ATP:ADP hydrolysis ratios for NTPDases 1, 2, 3, and 8 are ~1-1.5:1, 10-40:1, 3-4:1, and 2:1,

respectively. In addition to their catalytic properties, NTPDase may also function in intracellular interactions with the membrane scaffolding protein RanBMP to modulate ERK/Ras signaling pathways.

1.8.3b. Ecto-nucleoside pyrophosphatase family

The ecto-nucleoside pyrophosphatase (E-NPP) family consists of seven structurally related family members. This enzyme family exhibits broad substrate specificity capable of hydrolyzing pyrophosphate and phosphodiester bonds in nucleotide triphosphates, dinucleotides, nucleic acids, nucleotide sugars, choline phosphate esters, and lysophospholipids. NPP1, 2, and 3 are glycosylated proteins with a molecular mass of 110 to 125 kDa that share a highly homologous extracellular domain with two vibronectin domains and a highly conserved catalytic site. All three of these NPPs were initially thought to be transmembrane proteins with an intracellular amino-terminus, but more recent studies have shown that NPP2 is not a transmembrane protein, but is synthesized as a pre-pro-enzyme. Following the removal of the signal peptide and further cleavage by a furine-type protease, NPP2 is secreted.

NNP1, 2, and 3 are the only family members able to hydrolyze various nucleotides while NPP6 and 7 are only able to hydrolyze phosphodiester bonds in lysophospholipids or other choline phosphodiesters. The substrates for NPP4 and 5 are currently unknown.

1.8.3c. Alkaline phosphatase family

Alkaline phosphatases (AP) are ubiquitously expressed and evolutionarily conserved from bacteria to humans. Most APs are homodimeric and each catalytic site contains one Mg^{2+} and two Zn^{2+} ions, which are necessary for enzymatic activity. Mammalian APs display broad substrate specificity towards different phosphomonoesters and other phosphorylated compounds, including adenine nucleotides, pyrophosphate, inorganic polyphosphates, and bis(p-nitophenyl) phosphate. Each catalytic reaction releases inorganic phosphate and has an optimum pH range from eight to eleven. Three isozymes, intestinal AP, placental AP, and germ cell AP are tissue-specific and are 90-98% identical. A fourth isozyme, TNAP, is 50% homologus to the other three isozymes and has high expression levels in the bone, liver, and kidney.

1.8.3d. Ecto-5'-nucleotidase family

The ecto-5'-nucleotidase family is expressed in a variety of tissues, including colon, kidney, brain, liver, heart, and lung and consists of seven members: five of the enzymes are localized in the cytosol, one in the mitochondrial matrix, and one is attached to the outer plasma membrane. The intracellular nucleotidases dephosphorylate 5'-ribonucleoside monophosphates with low affinity, hydrolyze 5'-dNMPs, and participate in salvage pathways and/or de novo nucleotide synthesis.

Surface-associated ecto-5'-nucleotidase is also known as CD73 and contains two glycoprotein subunits tethered by non-covalent bonds and has a molecular mass of ~60-70 kDa. CD73 binds zinc and other divalent metal ions at the amino-terminus and is anchored to the plasma membrane at its carboxy terminus by glycosyl-phosphatidylinositol (GPI). This enzyme belongs to a large superfamily of dinuclear

metallophosphoesterases acting on various substrates such as Ser/Thr phosphoproteins, nucleotides, and sphingomyelin.

1.8.4. Purinergic receptors

The cellular effects of purines and pyrimidine nucleotides are mediated via a family of nucleotide-selective receptors. Based on pharmacological, functional, and cloning data, two major receptor subfamilies, P1 and P2, were described. The P1 subfamily of receptors is sensitive to adenosine while the P2 subfamily of receptors is activated by ADP, ATP, UDP, and UTP.

P2 receptors were originally classified based on the rank-order potency of nucleotides and nucleotide analogs. Most of these receptors have been cloned and their functions characterized in various heterologous expression systems. However, their functional characterization in native tissues and animals has been limited by the lack of agonist and antagonist receptor ligands. All of the known P2 agonist ligands are analogues of ATP, UTP, and ADP and show varying degrees of intrinsic activity and susceptibility to extracellular degradation.

1.8.4.a. Adenosine (P1) receptors

Four adenosine-selective P1 receptors, A_1, A_{2A}, A_{2B}, and A_3 have been cloned. Interestingly, all four receptors show species-specific pharmacology. A_1 and A_3 receptors couple to G_i and G_o, with the A2 receptors couple to G_s. Adenosine receptors are heterogeneously expressed in a variety of mammalian tissues; including heart, smooth muscle, kidney, testis, platelets, leukocytes, and adipocytes.

The A_1 receptor is expressed in the central nervous system (CNS) and is functionally coupled to inhibition of cyclic AMP (cAMP) formation, stimulation of potassium conductance, inhibition of N-channel-mediated calcium conductance, stimulation of phospholipase C production, and modulation of nitric oxide production.

The A_2 receptor is categorized into two subtypes, grouped together based on their capacity to activate adenylate cyclase. The A_{2A} receptor has a high affinity for adenosine, signals through N- and P- type Ca^{2+} channels, and is localized in the striatum, nucleus accumbens, and olfactory tubercle regions of the mammalian brain. The A_{2B} receptor has a lower affinity for adenosine and is more ubiquitously expressed in the CNS. The functional significance of A_{2B} expression in the brain, however, is unknown due to a lack of receptor-specific ligands.

The A_3 receptor was the first P1 receptor identified by cloning, rather than by pharmacologic properties. Activation of the A_3 receptor leads to inhibition of adenylyl cyclase and elevation of inositol-1,4,5-triphosphate (IP_3) levels and intracellular Ca^{2+}. A_3 receptors are involved in the regulation of mast cell function, eosinophil apoptosis, and preconditioning that occurs during ischemic reperfusion of the heart that protects against myocardial infarction.

1.8.4b. P2X receptors

The P2X receptors are ATP-gated ion channels that form a nonselective pore permeable to Ca^{2+}, K^+, and Na^+ and mediate rapid neurotransmission events. There are seven receptor subtypes: $P2X_1$-$P2X_7$. Each receptor shares a common structure of intracellular N- and C-termini, two transmembrane-spanning regions (2TM), one at each

terminus, and a large extracellular domain. A functional P2-receptor channel consists of three subunits, which can be homomeric or heteromeric combinations of the various P2X subunits. To date, $P2X_{1/5}$, $P2X_{2/3}$, and $P2X_{4/6}$ have been identified as functional heteromers. Whereas most P2X subunits can form functional homomers, $P2X_5$ and $P2X_6$ receptors do not, and instead appear to function only as heteromers with other P2X receptor subtypes. Currently, little information is available on the agonist (ATP) binding site on P2X receptor constructs or on ancillary sites that may modulate receptor function.

1.8.4c. P2Y receptors

P2Y receptors are G protein-coupled receptors that are activated by purine or pyrimidine nucleotides. P2Y receptors were first cloned and characterized in the early 1990s. Once the first P2Y receptor, termed $P2Y_1$, was cloned, additional members were subsequently identified through sequence homology searches, expression cloning techniques, or library screening with probes of previously identified P2Y receptors. There are eight verified family members, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$. These receptors are further subdivided into the $P2Y_1$ and the $P2Y_{12}$ receptor subfamilies, based on their sequence homology and G-protein coupling properties (see below and Table 1.3). On numerous occasions, orphan receptors were wrongly identified as P2Y receptors, resulting in gaps in the P2Y receptor nomenclature.

The P2Y receptor family differs from other GPCR families in that their members have low sequence identity despite similar pharmacological profiles. For example, the human $P2Y_1$ and $P2Y_{11}$ receptors, both of which are coupled to Gq and are activated primarily by adenine nucleotides, share only 33% homology. The regions with the

highest degree of identity between P2Y receptors lay within the TM 3, 6, and 7 domains of the receptors close to the intracellular loops. Interestingly, these TM domains are likely to contribute to the binding of agonists and may subsequently be involved in the signal transduction of the heterotrimeric G protein. The conserved sequence of TM 3, SILFLTCIS, is found in all cloned and functionally defined P2Y receptors with the exception of the $P2Y_4$ (SVLFLTCIS) and $P2Y_{11}$ (SVIFLTCIS) receptors.

1.8.4i. $P2Y_1$ receptor subfamily

The $P2Y_1$ receptor subfamily consists of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors. These receptors couple to $G\alpha_q$ to activate phospholipase C beta (Table 1.3). It should be noted that the $P2Y_{11}$ receptor is dually coupled to both Gq and Gs, resulting in dual stimulation of phospholipase C and adenylyl cyclase.

$P2Y_1$ receptor

The prototypical $P2Y_1$ receptor was the first family member identified, and was originally cloned from chicken brain. Subsequent homologues were cloned and characterized from turkey, cow, rodent, human, and skate. $P2Y_1$ receptors are widely distributed in various tissues, including brain, lung, platelets, and endothelia, and are activated potently by adenine dinucleotides and by adenine trinucleotides to a lesser extent. One primary function of the $P2Y_1$ receptor is regulation of platelet aggregation, in which it plays an essential role in ADP-induced shape change. Platelet aggregation in *$P2Y_1$ -/-* mice is inhibited[155]. In addition, prolonged bleeding time and protection from collagen- and ADP-induced thromboembolism is observed. The $P2Y_1$ receptor is also

involved in ion channel regulation, renal function, and vasodilation via stimulation of nitric oxide.

$P2Y_2$ receptor

The second cloned P2Y receptor, $P2Y_2$, was the first family member identified that was activated by uracil nucleotides. The receptor was cloned by expression cloning in *Xenopus* oocytes and monitoring for nucleotide-promoted Ca^{2+}-activated Cl^- currents. The receptor is expressed in various tissues including lung, kidney, and hematopoietic cells. In addition to being activated by UTP, the $P2Y_2$ receptor is equipotently activated by ATP. This unique activation by UTP and ATP has been used identify this receptor. A role for $P2Y_2$ receptors in regulation of Cl^- secretion in airway epithelia has been established[156]. Subsequently, a similar physiological role for $P2Y_2$ receptors in nasal epithelia and in cells found in the lacrimal gland and gastrointestinal tract was identified.

Cystic fibrosis (CF) is a genetic disease that results in an absent or malfunctioning cystic fibrosis transmembrane regulator (CFTR) protein. A defective or missing CFTR results in abnormal Cl^- conductance on the apical membrane of the epithelial cell and ultimately, airway surface liquid depletion. Because airway surface liquid is essential to support ciliary stability and function, ciliary collapse and decreased mucociliary transport occur as a result, leading to phlegm retention, infection, and inflammation.

Since $P2Y_2$ regulates Cl^- secretion by activating a Ca^{2+}-activated Cl^- current independent of the mechanism used by the CFTR, $P2Y_2$ may be used as a therapeutic CF target. A $P2Y_2$ agonist is currently in clinical trials for the treatment of CF to test the hypothesis that activating $P2Y_2$ would lead to downstream Cl^- channel activation and

subsequent restoration of the aqueous layer in the lumen of airway cells[156]. Restoration is normally facilitated by the cystic fibrosis transmembrane regulator (CFTR), but is defective in CF patients.

$P2Y_4$ receptor

The $P2Y_4$ receptor was the second uracil nucleotide-activated P2Y receptor identified. This receptor is distributed in smooth muscle, lung, and jejunum tissue. Interestingly, the $P2Y_4$ pharmacological profile is species-dependent. For example, the human orthologue is activated by UTP but not ATP, with ATP acting as an antagonist. In rodent orthologues, however, UTP and ATP are equally potent agonists. Recently it has been shown that the $P2Y_4$ receptor is involved in differentiation and cell death of human neuroblastoma cells[157].

$P2Y_6$ receptor

The $P2Y_6$ receptor was originally cloned from rodent tissue, followed by identification of the human homologue, and is the third receptor to be activated by uracil nucleotides. UDP is a potent activator of this receptor, and it is weakly activated if at all by ADP, ATP, and UTP. This receptor is expressed in a variety of tissue, including the aorta, kidney, brain, and lung. $P2Y_6$ receptors are also expressed in T-cells and are implicated in regulating the infiltration of these cells into the colon, leading to damage of the epithelial lining and inflammatory bowel disease. In addition, the $P2Y_6$ receptor was recently found to be up-regulated in neurons when are damaged, and could function as a

sensor for phagocytosis by sensing diffusible UDP signals[153,158]. This is a previously unknown pathophysiological function of P2Y receptors in microglia.

$P2Y_{11}$ receptor

Unlike the other P2Y family members, $P2Y_{11}$ can couple to both $G\alpha_q$ and $G\alpha_s$ to activate both PLC and adenylyl cyclase signaling pathways. However, the receptor is coupled to activation of PLC at much lower agonist concentrations compared to adenylyl cylcase activation. Interestingly, a rodent orthologue does not exist. However, a canine $P2Y_{11}$ receptor was cloned and it also couples to both PLC and adenylyl cyclase activation. While there is 70% homology between species, different pharmacologic properties between the human and canine receptors exist. The human $P2Y_{11}$ receptor is more potently activated by ATP than ADP and is insensitive to 2-thioether substitutions in the adenine ring. In contrast, the canine $P2Y_{11}$ receptor is activated more potently by ADP than ATP and is sensitive to the 2-thioether substitution. The differences in ADP versus ATP potencies was shown to be due primarily to a single amino acid substitution of Arg-265, located at the juxtaposition of TM6 and the third extracellular loop, in the human receptor to Gln in the canine receptor, while the amino acid(s) responsible for the sensitivity to 2-thioether substitution was not identified. The $P2Y_{11}$ receptor regulates several physiologic responses, including cardiac function, platelet aggregation and smooth-muscle cell proliferation.

1.8.4ii. $P2Y_{12}$ receptor subfamily

The $P2Y_{12}$ receptor subfamily consists of the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors. These receptors all couple to $G\alpha_i$ to inhibit adenylyl cyclase and activate potassium channels (Table 1.3).

$P2Y_{12}$ receptor

In the early 1960s, studies showed that adding ADP to platelets induced aggregation via an unidentified receptor referred to as the P2T receptor. Later studies showed that in platelets, ADP promoted the inhibition of adenylyl cyclase and an increase of intracellular calcium, suggesting either that there was one receptor coupled to two signaling pathways, or that there were two receptors activated by ADP coupled to two different signaling pathways. While the $P2Y_1$ receptor was eventually identified as the receptor coupled to PLC stimulation and increased intracellular Ca^{2+} mobilization, the receptor leading to inhibition of ADP-promoted adenylyl cyclase remained unknown.

The $P2Y_{12}$ receptor (also referred to as $P2Y_{ADP}$, $P2Y_{AC}$, $P2Y_{CYC}$, and $P2T_{AC}$) was ultimately identified as the $G\alpha_i$-coupled receptor involved in platelet aggregation in 2001. In contrast to the ubiquitous expression of other P2Y receptors, $P2Y_{12}$ receptor expression is limited to platelets and the brain. In addition to activation by ADP, 2MeSADP is also an agonist while adenosine monophosphates such as 2MeSAMP are antagonists.

$P2Y_{13}$ receptor

The function of the ADP-activated $P2Y_{13}$ receptor is poorly understood due to the lack of studies available for the $G\alpha_i$-coupled receptor. $P2Y_{13}$ receptors are distributed in

the brain, lymph nodes, bone marrow, spleen, and liver. This distribution suggests that the $P2Y_{13}$ receptor may have a role in regulating immune responses and/or haematopoiesis. For example, one study suggests that the $P2Y_{13}$ receptor may be a potential therapeutic target in the treatment of atherosclerosis through its regulatory role in the regulation of cholesterol homeostasis. A recent study shows that RhoA/ROCK I signaling downstream of the $P2Y_{13}$ receptor controls HDL endocytosis in human hepatocytes.

$P2Y_{14}$

The $P2Y_{14}$ receptor is unique from the other P2Y receptors because it is activated by UDP sugars (UDP-glucose, UDP-galactose, and UDP-N-acetylglucosamine). This receptor is found in various tissues, including moderate expression in lung, spleen, brain, and heart, and high expression in neutrophils, placenta, adipose tissue, intestine, and stomach and skeletal muscle. The physiological function of the $P2Y_{14}$ receptor is not clearly understood, although increasing evidence supports a role in immune function.

1.9. Role of purinergic receptors in platelet activation

Purinergic receptors regulate a variety of physiological processes in multiple cell types, including ion transport and stress response in epithelial cells, vascular smooth muscle relaxation, and platelet activation. Vascular injury leads to the exposure of sub-endothelial collagen, activation of platelets, and the initiation of the coagulation cascade. Platelet activation is characterized by rapid shape change, release of granular contents, generation of lipid mediators, and aggregation. Secondary mediators, such as ADP

released from granules and thromboxane A_2, activate other resting platelets, resulting in amplification of initial physiological haemostatic response.

Platelet activation is achieved through various surface receptors that include GPCRs, integrins, and glycoprotein receptors. Adenosine nucleotides that are secreted following platelet activation signal through purinergic receptors on the platelet membrane (Figure 1.3). The P2 receptors expressed on the cell surface of platelets are the $P2Y_1$, $P2Y_{12}$, and $P2X_1$ subtypes, whose agonists are ADP, ADP, and ATP, respectively. Agonist activation of these receptors initiates a complex signaling cascade that ultimately results in platelet activation and thrombus formation.

1.9.1. $P2Y_1$ receptor

The $P2Y_1$ receptor is required for ADP-induced platelet shape change and aggregation. Platelets from $P2Y_1$ receptor $^{(-/-)}$ mice display severely impaired platelet aggregation upon exposure to collagen and agonists for the TPα and protease-activated receptors. These platelets also show a loss in ADP-mediated intracellular calcium mobilization, a key step in platelet signaling. In addition, transgenic mice that over-express $P2Y_1$ receptors exhibit a decrease in bleeding time *in vitro* and *in vivo*.

The $P2Y_1$ receptor also is important for $G\alpha_q$ signaling. Activation of the $G\alpha_q$-coupled serotonin receptor in the presence of $P2Y_1$ receptor antagonists results in platelet aggregation[155]. Additionally, although serotonin cannot cause platelet activation alone, it can restore the ADP response in $P2Y_1$ receptor-deficient platelets by promoting Ca^{2+} mobilization and thereby functionally substituting for the $P2Y_1$ receptor.

1.9.2. $P2Y_{12}$ receptor

The role of the $G\alpha_i$-coupled $P2Y_{12}$ receptor in platelet function was investigated using pharmacological and mouse models. $P2Y_{12}$ receptor antagonists include AR-C compounds, 2MeSAMP, and metabolites of thienopyridines, clopidogrel, ticlopidine, and CS-747. AR-C compounds showed that the $P2Y_{12}$ receptor does not contribute to platelet shape change, but enhances other agonist-induced dense granule release. The ADP-induced $G\alpha_i$ signaling is necessary but not sufficient for fibrinogen receptor activation and platelet aggregation, and thromboxane generation.

Platelets from *$P2Y_{12}$ -/-* mice display severely inhibited ADP-induced rates of aggregation, are insensitive to clopidogrel treatment, and show prolonged bleeding times, with no effects on $P2Y_1$ receptor-mediated shape change or intracellular Ca^{2+} mobilization[159]. Thrombin-induced platelet aggregation is also compromised. Most importantly, patients with dysfunctional $P2Y_{12}$ receptor expression suffer from a severe defect in ADP-induced adenylyl cyclase inhibition and platelet aggregation, but retain normal platelet shape change.

The $P2Y_{12}$ receptor is the therapeutic target of ticlopidine and clopidogrel, two platelet aggregation inhibitors used for prevention and treatment of arterial thrombosis. Several experimental models show that clopidogrel (PLAVIX) is a potent anti-aggregant and antithrombotic drug. Clopidogrel was released into the market following a successful clinical trial showing superior efficacy versus aspirin in preventing myocardial infarction, stroke, and vascular death.

Clopidogrel is a pro-drug that is oxidized by hepatic P450 enzymes to the activate metabolite, which irreversibly inhibits ADP-promoted platelet aggregation by binding to

the $P2Y_{12}$ receptor and covalently reacting with a cysteine residue in the receptor that is required for activity[160-161]. ADP-promoted platelet shape change is not affected by clopidogrel, indicating that this response is regulated by the $P2Y_1$ receptor[160,162-163].

1.9.3. $P2X_1$ receptor

Ubiquitously expressed $P2X_1$ receptors are the third category of purinergic receptors implicated in the regulation of platelet function. These receptors are non-selective cation channels primarily activated by physiological agonists such as extracellular ATP. Activation of $P2X_1$ receptors leads to a transient shape change, but fails to induce the formation of long filopodial extensions. This is due to transient influx of calcium resulting from receptor activation. In addition to shape change, activated $P2X_1$ receptors cause centralization of granules, but do not lead to the release of granular contents. The relevance of the granular movements remains unclear. Finally, animals deficient in $P2X_1$ display normal bleeding times, but display increased resistance to thrombosis. Conversely, animals overexpressing the $P2X_1$ receptor show increased thrombin formation in response to injections of collagen and epinephrine.

1.9.4. Regulation of purinergic receptors in platelets

Regulation of the ADP response in platelets is critical in understanding platelet activation. It is established that ADP released from isolated platelets causes them to become refractory to activation by ADP in vitro[159]. A recent study demonstrated that in platelets, the $P2Y_1$ receptor was rapidly desensitized upon addition of ADP, while the $P2Y_{12}$ was refractory to desensitization[164]. It has also been reported that the $P2Y_1$

receptor was rapidly internalized in both platelets and 1321N1 cells in an agonist-dependent manner[165]. Finally, in a study quantifying the kinetics of desensitization, the data show that the $P2Y_1$-selective agonist MRS2365 causes very rapid desensitization of subsequent ADP-promoted platelet aggregation, with a half-life of desensitization of 18 sec[166]. Thus, it is clear that the desensitization and internalization of the $P2Y_1$ receptor is an important means for regulating platelet function.

Table 1.1. Examples of top-selling drugs targeting G protein-coupled receptors (GPCRs).

Trade Name (Generic Name)	**Therapeutic Use**	**Mechanism of Action**	**Name of Company**	**World Sales** (USD millions)
Allegra (fexofenadine)	Allergies	H_1 antagonist	Sanofi-Aventis	1792
Imigran (sumatriptan)	Migraines	$5HT_{1D}$ agonist	GlaxoSmithKline	1454
Plavix (clopidogrel)	Stroke	$P2Y_{12}$ antagonist	Bristol-Myers Squibb	5277
Serevent (salmeterol)	Asthma	B_1 agonist	GlaxoSmithKline	679

GPCR drugs cover many therapeutic uses and represent a substantial population of today's market. Source: IMS Knowledge Link; reported world sales are 12 months to the end of the first quarter of the 2005 fiscal year.

Table 1.2. Summary of protease-activated receptor (PAR) activating proteases, activating peptides, tissue distribution, and PAR-deficient mice phenotype.

Receptor	Activating proteases	Synthetic/activating peptide (AP)	Tissue distribution	Knockout mouse phenotype
PAR1	Thrombin, FXa, FVIIa, APC, MMP, plasminogen	SFLLRN, TFLLRN	Human platelets, fibroblasts, neurons, astrocytes, endothelium, epithelium	Partial embryonic lethality
PAR2	Trypsin, tryptase, FVIIa, FXa	SLIGKV	Fibroblasts, myocytes, neurons, astrocytes, epithelium, endothelium	Impaired leukocyte migration; impaired allergic inflammation of airway, joints, kidney
PAR3	Thrombin	None	Mouse platelets, endothelium, myocytes, astrocytes	Protection against thrombus formation/pulmonary embolism
PAR4	Thrombin	AYPGKF, GYPGQV	Human platelets, endothelium, myoctes, astrocytes	Protection against thrombus formation/pulmonary embolism

APC, activated protein C; MMP, matrix metalloprotease

Table 1.3. Summary of P2Y receptor signaling and tissue expression

Receptor	Nucleotide selectivity	G protein-coupling	Effectors	Tissue distribution
$P2Y_1$	2 MeSADP > 2 MeSATP ≃ ADP	Gq	↑PLC	Brain, lung, platelets, endothelia
$P2Y_2$	UTP ≃ ATP	Gq	↑PLC	Lung, kidney, hemato-poietic cells
$P2Y_4$	UTP	Gq	↑PLC	Smooth muscle, lung, jejunum
$P2Y_6$	UDP >> UTP	Gq	↑PLC	Aorta, kidney, brain, lung
$P2Y_{11}$	ATP >> ADP	Gq and Gs	↑PLC, ↑AC	Spleen, mono-cytes, kidney
$P2Y_{12}$	2MeSADP > ADP	Gi	↓AC, ↑K+ channels?	Platelets, brain
$P2Y_{13}$	2MeSADP ≃ ADP	Gi	↓AC, ↑K+ channels?	Brain
$P2Y_{14}$	UDP-glucose > UDP-galactose	Gi	↓AC, ↑K+ channels?	Lung, neutrophils

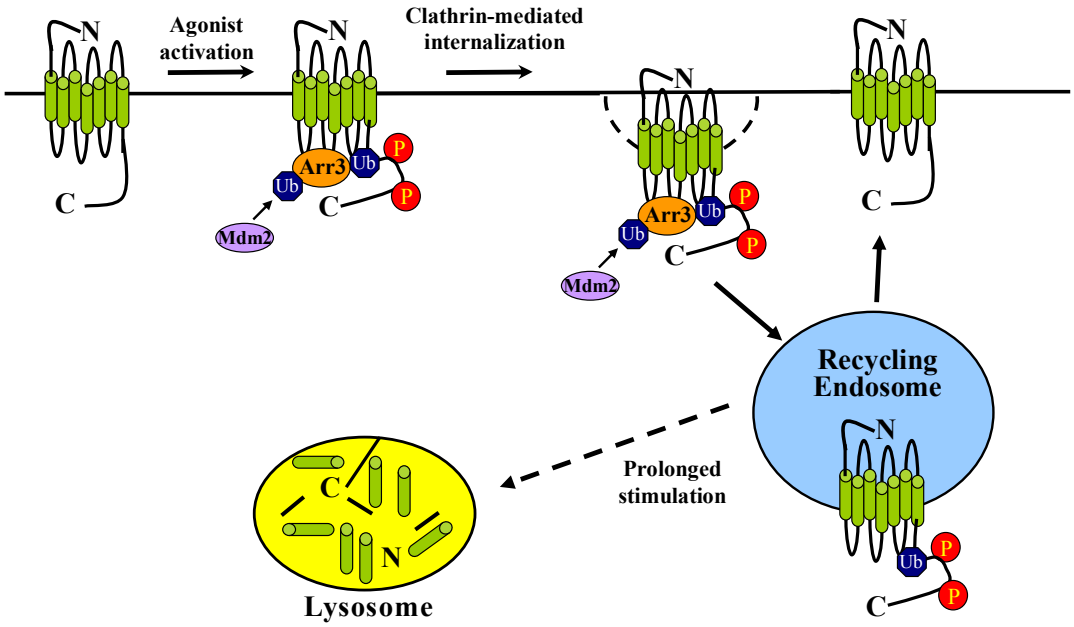

Figure 1.1. Classical G protein-coupled receptor internalization and trafficking paradigm. The general dogma of GPCR internalization and trafficking is based off β_2AR studies. Upon agonist stimulation, the receptor is rapidly phosphorylated (P) to recruit arrestin3 (Arr3). In addition, the receptor and arrestin protein are ubiquitinated (Ub) via Mdm2, internalized via clathrin coated pits, and sorted to recycling endosomes. Upon short agonist stimulation, the receptor is recycled back to the plasma membrane and is ready for re-activation. However, upon prolonged exposure to agonist, the β_2AR is sorted to lysosomes and degraded.

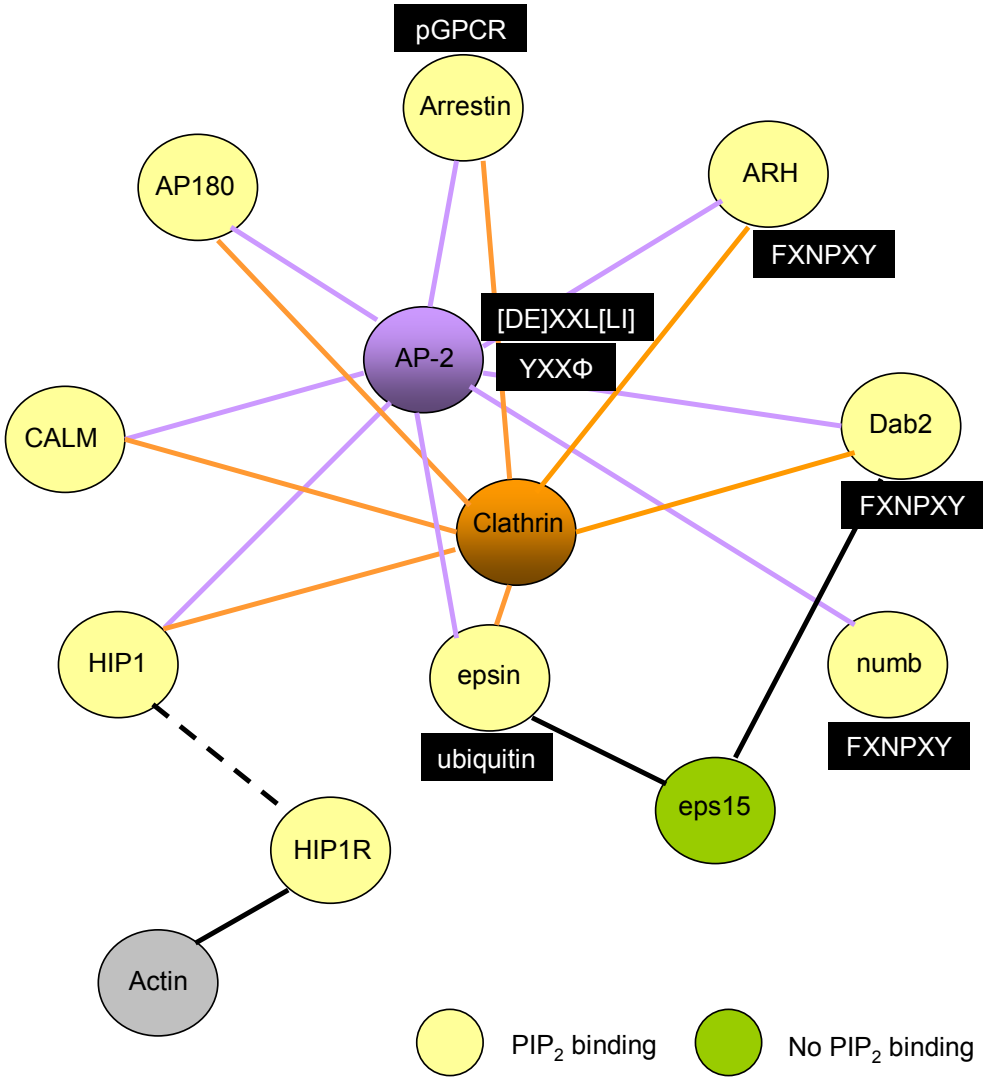

Figure 1.2. The clathrin adaptor protein interaction web. A schematic representation of the protein-protein interactions between clathrin, AP-2, and endocytic adaptor proteins. The sorting signal or putative cargo types recognized by the different adaptors are boxed in black. pGPCR, phosphorylated G protein-coupled receptor; PIP_2, PtdIns(4,5)P_2.

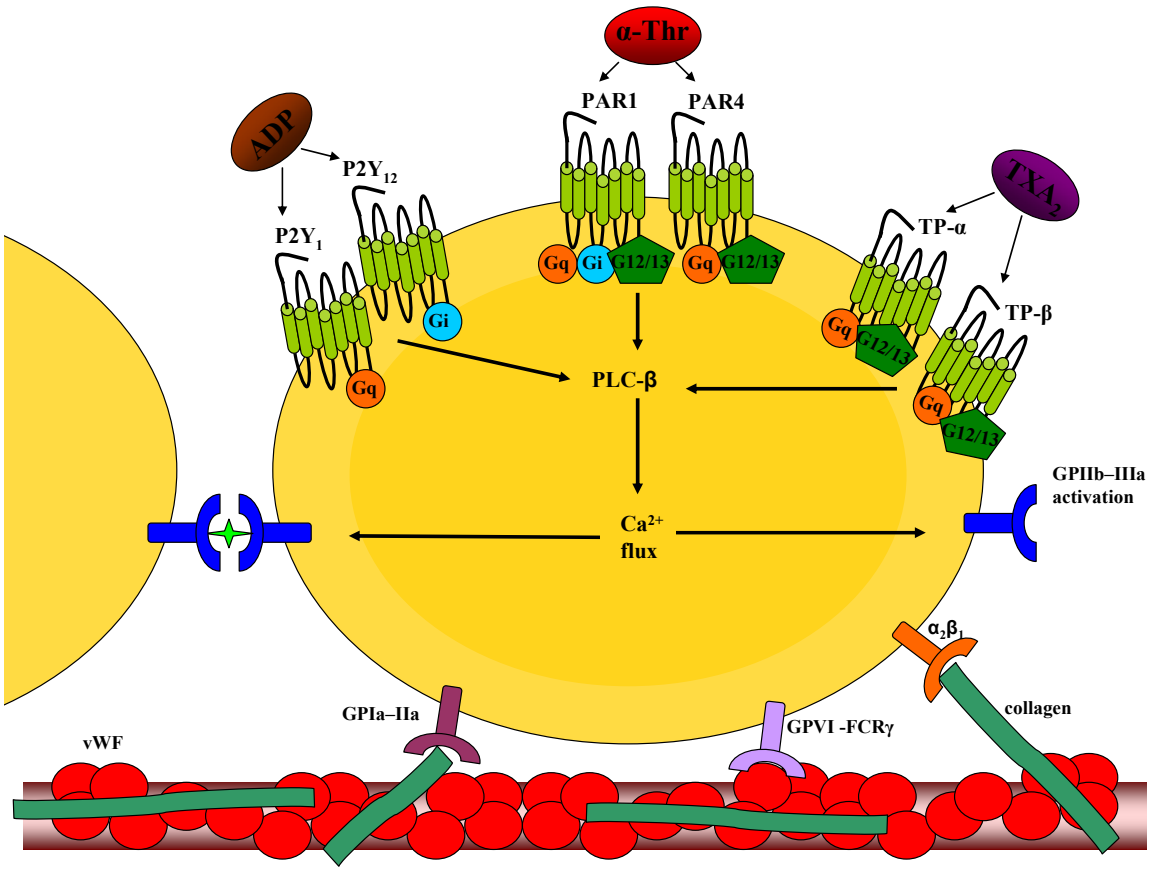

Figure 1.3. Schematic of the major adhesion and agonist receptors on the surface of platelets. Platelets have a variety of cell-surface receptors that mediate their activation, adhesion to the blood vessel wall, and their aggregation with each other. Three G protein-coupled receptor families exist on the platelet surface and are linked to one or more different G proteins. ADP receptors: $P2Y_1$ and $P2Y_{12}$ which are linked to G_q and G_i, respectively. Thrombin receptors: PAR1 and PAR4, which are linked to $G_{i,q,12/13}$ and $G_{q,12/13}$, respectively. TXA_2 receptors: TP-α and TP-β, each of which is linked to G_q and $G_{12/13}$. All of these receptors activate specific signalling cascades that are ultimately linked to the mobilization of calcium from intracellular stores. GPIIb–IIIa functions in a range of platelet responses, including platelet aggregation, spreading and clot retraction. GPIa–IIa and $\alpha_2\beta_1$ integrins support platelet adhesion on collagen. The GPVI receptor FcR γ-chain complex supports platelet adhesion and activation on fibrillar collagens. PAR, protease-activated receptors; PLC, phospholipase C; TXA_2, thromboxane A_2; TP, thromboxane/prostanoid receptors; vWf, von Willebrand factor.

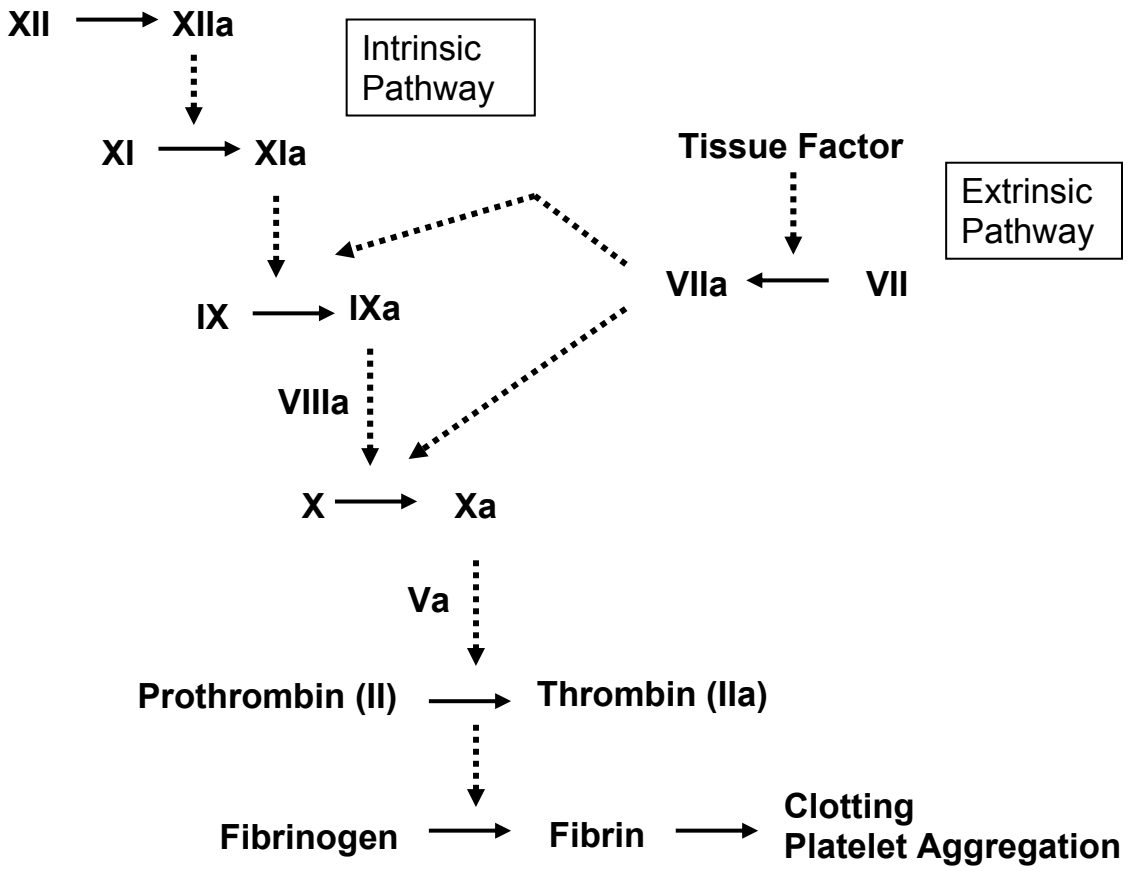

Figure 1.4. Coagulation cascade. The coagulation cascade has two pathways, intrinsic and extrinsic pathways that lead to fibrin formation, clotting, and platelet aggregation. The pathways are a series of reactions, in which a serine protease zymogen is activated to become an active component that then catalyzes the next reaction in the cascade.

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Chapter 2

Protease-activated Receptor 2 is Essential for Factor VIIa and Xa-Induced Signaling, Migration, and Invasion of Breast Cancer Cells

2.1. Abstract

Protease-activated receptors (PARs) are G protein-coupled receptors (GPCRs) that function as cell surface sensors for coagulant proteases, as well as other proteases associated with the tumor microenvironment. PAR1 is activated by thrombin, whereas the upstream coagulant protease VIIa bound to tissue factor and Xa can activate both PAR1 and PAR2. PAR1 has been implicated in tumor cell growth, migration and invasion, whereas the function of PAR2 in these processes is largely unknown. Towards defining the functional importance of PAR2 in cancer cells, we used small interfering RNAs (siRNAs) to deplete highly invasive breast cancer cells of endogenous PAR proteins. Our findings strongly suggest that PAR2 is critical for MDA-MB-231 and BT549 breast cancer cell migration and invasion towards NIH-3T3 fibroblast conditioned medium. To define the relative importance of PAR1 versus PAR2 in mediating factors VIIa and Xa responses, we assessed signaling in cancer cells lacking either endogenous PAR1 or PAR2 proteins. Strikingly, in MDA-MB-231 cells depleted of PAR2, we observed a marked inhibition of VIIa and Xa signaling to phosphoinositide hydrolysis and ERK1/2 activation, whereas signaling by VIIa and Xa remained intact in PAR1 deficient cells. Factors VIIa and Xa-induced cellular migration was also impaired in MDA-MB-231 cells deficient in PAR2 but not in cells lacking PAR1. Together these studies reveal the novel findings that PAR2, a second protease-activated

GPCR, has a critical role in breast cancer cell migration and invasion and functions as the endogenous receptor for coagulant proteases VIIa and Xa in these cells.

2.2. Introduction

A link between malignant cancer and hyperactivation of the coagulation system is well documented in the literature [1-4]. However, the mechanism by which coagulant activity contributes to tumor cell invasion and metastasis remains poorly understood. The expression of tissue factor, the principal cellular activator of the coagulation cascade, on cancer cells leads to generation of active coagulant proteases on the cell surface [5]. Thrombin, the main effector protease of the coagulation cascade, is thought to promote metastasis, at least in part, by facilitating tumor cell interactions with host vascular cells including platelets and endothelial cells lining blood vessels [6-8]. Thrombin and upstream coagulant proteases are also generated in the tumor microenvironment independent of blood coagulation [2], and potentially contribute to cancer cell migration and invasion by acting directly on tumor cells. Thus, coagulant proteases are likely to facilitate tumor cell metastasis by modulating a number of host vascular cell responses, as well as acting directly on tumor cells themselves.

Thrombin is formed during a series of zymogen conversions that are triggered when coagulant proteases contact tissue factor. Tissue factor, a single spanning integral membrane protein, complexed with VIIa cleaves factor X to generate the active protease Xa. The formation of Xa is localized to the cell surface and triggers proteolytic conversion of prothrombin to thrombin. In addition to cleaving fibrinogen, thrombin activates cells through at least three G protein-coupled protease-activated receptors (PARs): PAR1, PAR3 and PAR4. In contrast, PAR2 is activated by multiple trypsin-like serine proteases, including the upstream coagulant proteases VIIa and Xa, but not by thrombin. Thrombin is the main physiological activator of PAR1; however, activation of PAR1 is not restricted to this protease. PAR1 can be cleaved and activated by tissue factor-VIIa and Xa [9-10], plasmin [11],

activated protein C [12], and metalloproteases [13]. Clearly, multiple proteases can cleave and activate these receptors, and thus the particular PAR and protease that function in a specific cellular setting remains largely undefined.

PAR1 has been implicated in tumor cell growth, invasion and metastasis of several types of human malignant cancers, including breast. PAR1 expression has been directly correlated with the degree of invasiveness exhibited by primary human breast tissue specimens and established cancer cell lines, whereas PAR1 expression is minimal or absent in benign and normal breast tissue and non-invasive carcinoma [14-17]. Ectopic expression of PAR1 in mouse mammary gland epithelia induces an oncogenic phenotype [18], and PAR1 also has the capacity to transform NIH-3T3 fibroblasts, further suggesting a role for this receptor in oncogenesis [19]. Moreover, expression of PAR1 in non-invasive breast carcinoma is sufficient to promote growth and invasion of breast cancer cells in a xenograft nude mouse model [13]. PAR2 is also expressed in malignant breast tissue and in highly invasive breast carcinoma cell lines [15,20]. However, in contrast to PAR1, the functional importance of PAR2 in breast cancer cells is largely unknown. While activation of PAR1 with thrombin or matrix metalloproteinase-1 occurs on breast carcinoma [13-14,21], the actual protease responsible for PAR2 activation remains to be determined. Interestingly, the upstream coagulant proteases VIIa and Xa have also been reported to stimulate signaling and migration of certain breast carcinoma cells [22-23]; however, whether this involves direct activation of PAR1 and/or PAR2 has not been clearly defined.

We hypothesize that besides thrombin, other tumor cell-generated proteases activate PAR1, and perhaps PAR2, to promote breast cancer cell invasion and metastasis. Tissue factor is highly overexpressed in invasive tumor cells and is responsible for generation of

active coagulant proteases VIIa and Xa. Factors VIIa and Xa potentially make important contributions to cancer cell invasion and metastasis via activation of PAR1 and/or PAR2. In the present study, we used small interfering RNAs (siRNAs) to knockdown endogenous PAR2 protein in an effort to define the importance of this receptor in breast cancer cells. Our findings strongly suggest that PAR2 is an important mediator of breast cancer cell migration and invasion. Our studies further demonstrate an essential role for PAR2 in mediating coagulant protease VIIa and Xa-induced signaling and migration of MDA-MB-231 breast cancer cells. These findings are the first to demonstrate a critical role for PAR2 in mediating cancer cell migration and invasion and also define a new role for PAR2 as the endogenous receptor for factors VIIa and Xa in invasive breast cancer cells. Moreover, these studies are the first to demonstrate the relative contribution of PAR1 *versus* PAR2 in mediating coagulant protease effects on breast cancer cells and provide important information for the design and use of protease inhibitors in breast cancer disease.

2.3. Materials and methods

2.3.1. Reagents and antibodies

Human α-thrombin, plasma-derived factor Xa, and factor VIIa were purchased from Enzyme Research Laboratories (South Bend, IN). Collagen type IV, laminin, and α-trypsin, treated with tosylamide-2-phenylethyl chloro-methyl ketone, were purchased from Sigma-Aldrich (St. Louis, MO). PAR1-selective agonist peptide (TFLLRNPNDK) and PAR2-selective agonist peptide (SLIGKV) were synthesized as the carboxyl amide and purified by reverse-phase high-pressure liquid chromatography (University of North Carolina Peptide Facility, Chapel Hill, NC).

A rabbit polyclonal anti-PAR1 antibody was generated against the hirudin-like domain peptide sequence YEPFWEDEEKNESGLTEYC as previously described [24]. The monoclonal anti-PAR2 antibody SAM11 was generously provided by Dr. Lawrence Brass (University of Pennsylvania, PA). Anti-actin antibody was purchased from Sigma-Aldrich. Monoclonal anti-phospho-p44/42 mitogen-activated kinase (MAPK); extracellular signal-related kinase (ERK) 1/2 antibody and polyclonal anti-p44/42 MAPK (ERK1/2) antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit antibodies were from Bio-Rad (Hercules, CA).

2.3.2. Cell lines

MDA-MB-231 and BT549 cell lines were purchased from American Type Culture Collection (Manassas, VA). MDA-MB-231 cells were cultured in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS). BT549 cells were grown in RPMI 1640 medium supplemented with 10% FBS and 10 μg/mL insulin.

2.3.3. Phosphoinositide hydrolysis

MDA-MB-231 cells were seeded at 1.6 x 10^5 cells per well in 24-well dishes. Cells were labeled with 2 μCi/mL of *myo*-[^{3}H]inositol in inositol- and serum-free medium containing 1 mg/mL bovine serum albumin (BSA) overnight. Cells were then washed and incubated in the absence or presence of agonists diluted in medium containing 1 mg/mL BSA and 20 mM lithium chloride for 1 hour at 37ºC. Cell medium was removed and total cellular [^{3}H]inositol phosphates were extracted, isolated, and quantified as described [25].

2.3.4. siRNA electroporation

MDA-MB-231 and BT549 cells (1 x 10^6) were electroporated with 600 nM of PAR1-specific (5'-AGAUUAGUCUCCAUCAAUA-3'), PAR2-specific (5'-GGAAGAAGCCUUAUUGGUA-3'), or nonspecific control siRNA (5'-GGCUACGUCCAGGAGCGCACC-3') using a system developed by Amaxa, Inc. (Gaithersburg, MD). Cells were then plated in normal growth media. PAR-specific siRNAs were synthesized by Dharmacon, Inc. (Lafayette, CO).

2.3.5. Immunoblotting for PAR expression

To assess PAR1 or PAR2 protein expression, MDA-MB-231 cells were seeded at 1 x 10^6 cells/well in a six-well plated and grown for 24, 48, and 72 hours after siRNA electroporation. Cells were then lysed in 1% Triton X-100 lysis buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 50 mM NaF, and 10 mM sodium pyrophosphate with protease inhibitors. Equal amounts of protein lysates (~30 μg) were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with anti-PAR1 or anti-PAR2 antibodies. Membranes were then stripped and re-probed with a monoclonal anti-actin antibody. Immunoblots were developed with enhanced chemiluminescence plus (Amersham Biosciences, Inc., Piscataway, NJ) and imaged by autoradiography.

2.3.6. Cell-surface ELISA

MDA-MB-231 cells electroporated with siRNAs were seeded on fibronectin-coated 24-well plates at 1.6 x 10^6 cells/well. After 72 hours, cells were incubated with anti-PAR1, anti-PAR2 antibody, or pre-immune serum for 1 hour at 4ºC. Cells were then washed to remove unbound antibody, fixed with 4% paraformaldehyde, and then incubated with goat-anti rabbit antibody conjugated to HRP for 1 hour at 25ºC. Cells were then washed and incubated with HRP substrate, One-Step ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, Pierce). The optical density was determined at 405 nm using a Molecular Devices (Sunnyvale, CA) SpectraMax Plus microplate reader.

2.3.7. ERK1/2 activation

MDA-MB-231 cells were seeded at a density of 1.6 x 10^5 cells/well. Cells were then serum-starved overnight and incubated in the absence or presence of agonists for 30 minutes at 37ºC. Following incubation, cells were lysed in 2x SDS-gel loading buffer [100 mM Tris-HCL (pH 6.8), 20% glycerol, 4% SDS, 0.02% bromophenol blue]. Cell lysates were resolved by SDS-PAGE, transferred to membranes, and immunoblotted with an anti-phospho-p44/42 MAPK (ERK1/2) antibody. To detect total p44/42 MAPK (ERK1/2), membranes were stripped and re-probed with an anti-p44/42 MAPK (ERK1/2) antibody. Immunoblots were developed and imaged by autoradiography.

2.3.8. Cellular Migration

Cell migration was assessed using a 24-well format containing a transwell insert (8-μm pore size polycarbonate filter) (BD Biosciences) that was coated with 7.5 μg/ml collagen type IV for MDA-MB-231 cells or 10 μg/ml laminin for BT549 cells. After 72 hours of

electroporation with siRNAs, serum-deprived MDA-MB-231 and BT549 cells (5 X 104 cells) were diluted in 100 µl of DMEM containing 0.1% BSA and 10 mM HEPES, pH 7.4 and added to the upper chamber of the transwell. Cells were then allowed to migrate in response to the addition of 600 µl of either serum-free media, NIH-3T3 fibroblast-conditioned medium or to various agonists added to the lower chamber for 3 h at 37°C. Non-migrated cells in the upper chamber were removed by wiping with a cotton swab, while the attached migrated cells were fixed and stained with 0.1% crystal violet diluted in 0.1 M borate, pH 9.0 and 2% ethanol. Three different fields of cells were photographed with IX81 Olympus microscope fitted with a 20X objective and the number of migrated cells in each field was counted.

2.3.9. Cellular invasion

After 72 hours of electroporation with siRNAs, serum-starved MDA-MB-231 cells (1 x 10^5) or BT549 cells (5 x 10^4) diluted in 100 µl of DMEM (without phenol red) containing 0.1% BSA, 10 mM HEPES (pH 7.4) were added to the upper well of a 24-well transwell insert coated with matrigel (BD Biosciences). The lower well contained 600 µl of NIH 3T3 fibroblast conditioned medium prepared as previously described [26]. Cells were then incubated for 24 hours at 37ºC. Cells remaining in the upper chamber were removed and invaded cells were fixed, stained, and counted as described above.

2.3.10. Data analysis

Data were analyzed using Prism 3.0 software and statistical significance was determined using InStat 3.0 (GraphPad, San Diego, CA).

2.4. Results

2.4.1. siRNAs targeted to PARs specifically and effectively cause loss of receptor expression

Protease-activated receptors are capable of mediating tumor cell responses to a variety of extracellular proteases associated with the tumor microenvironment including coagulant proteases. Towards defining the relative importance of the individual PARs in mediating extracellular protease responses in cancer cells, we generated PAR-specific siRNAs to deplete highly invasive breast cancer cells of endogenous PAR proteins. Characterization of PAR family members expressed in MDA-MB-231 breast carcinoma using reverse-transcriptase polymerase chain reaction (RT-PCR) revealed the presence of PAR1 and PAR2 mRNA transcripts (data not shown), consistent with the high-level of receptor protein expression previously reported [16,20]. We first determined whether siRNAs targeted against PAR1 and PAR2 mRNA transcripts were effective at reducing expression of these proteins. MDA-MB-231 cells were electroporated with PAR-specific siRNAs and after 24, 48, and 72 h of siRNA incubation, cell lysates were prepared and the amounts of endogenous PAR1 and PAR2 protein remaining were determined by immunoblotting. A significant decrease in both PAR1 and PAR2 protein expression was observed after 72 h of siRNA incubation (Fig. 2.1A). Small interfering RNAs directed against specific PAR mRNA sequences also caused a significant and selective loss of cell surface expression of the targeted receptor after 72 h of incubation (Fig. 2.1B). These findings suggest that siRNAs targeted to specific PAR mRNA transcripts are effective and selective at causing loss of receptor protein.

2.4.2. Loss of PAR expression by siRNAs results in loss of agonist-induced signaling

We then determined whether siRNA-mediated depletion of specific PAR proteins resulted in loss of functional responses by examining agonist-induced phosphoinositide (PI) hydrolysis. PAR1 and PAR2 stimulate $G\alpha_q$-mediated PI hydrolysis primarily through the activation of phospholipase C-β [27-28]. In control non-specific siRNA treated cells, thrombin and TFLLRNPNDK induced an ~2-fold increase in [^{3}H]IP accumulation following 60 min of agonist exposure at 37°C (Fig. 2.2.A). In contrast, activated PAR1-stimulated PI hydrolysis was markedly impaired in cells depleted of PAR1 by siRNA, whereas signaling by trypsin in the same cells was unaffected (Fig. 2.2.A). Similarly, activation of PAR2 with trypsin or SLIGKV caused an ~2-fold increase in signaling in control siRNA cells that was virtually abolished in cells depleted of PAR2 by siRNA (Fig. 2.2.A). Thrombin signaling remained intact in PAR2-deficient cells (Fig. 2.2.A). These results suggest that depletion of either PAR1 or PAR2 protein by siRNA is sufficient to ablate receptor-stimulated signaling to PI hydrolysis in highly invasive breast cancer cells.

We next evaluated whether siRNA-mediated knockdown of PAR proteins affected agonist-induced ERK1/2 activation, an important mitogenic signaling pathway. In cells treated with non-specific siRNA, activation of PAR1 with TFLLRNPNDK and of PAR2 with SLIGKV caused ERK1/2 activation. However, PAR1-mediated activated of ERK1/2 was abolished in cells depleted of PAR1 protein by siRNA. In contrast to loss of PAR1 signaling, PAR2-specific ERK1/2 activation was unaffected. Additionally, depleting cells of PAR2 protein using siRNA resulted in loss of PAR2 agonist-stimulated ERK1/2 activation while activation with PAR1-specific agonist peptide TFLLRNPNDK resulted in ERK1/2 activation

(Fig. 2.2.B). Together, these findings strongly suggest that siRNAs targeting specific PAR mRNA sequences are effective and selective at depleting highly invasive breast carcinoma cells of endogenous PAR protein and inhibiting the corresponding functional responses.

2.4.3. PAR2 is a critical mediator of MDA-MB-231 and BT549 cellular migration and invasion

The functional importance of PAR2 in mediating breast cancer cell migration and invasion has not been previously determined. BT549 cells express endogenous PAR1 and PAR2 proteins and PAR-specific siRNAs were effective and selective at reducing endogenous receptor function in these cells (data not shown). Control siRNA treated MDA-MB-231 and BT549 breast cancer cells exhibited a high degree of basal migration; however, the addition of NIH-3T3 fibroblast conditioned medium caused an even greater ~2-fold increase in migratory responses. In contrast, cells lacking PAR2 protein showed a substantial ~50% inhibition of migration towards conditioned medium comparable to that observed in cells depleted of PAR1, whereas basal migration was modestly reduced (Fig. 2.3. A and B). These findings suggest that in addition to PAR1, PAR2 is an important mediator of MDA-MB-231 and BT549 cell migration induced by NIH-3T3 cell-conditioned medium.

To determine a role for PAR2 in cellular invasion, we evaluated the ability of MDA-MB-231 and BT549 cells to migrate and invade towards NIH-3T3 fibroblast-conditioned medium. Knockdown of PAR1 expression by siRNA resulted in significant inhibition of breast cancer cell invasion; only ~37% of MDA-MB-231 cells invaded compared to control siRNA treated cells (Fig. 2.3 C). Depletion of endogenous PAR1 protein by siRNA in BT549 cells caused a similar reduction of migration and invasion (Fig. 2.3 D). These findings are

consistent with a recent study showing loss of MDA-MB-231 cell invasion towards NIH-3T3 cell conditioned medium after treatment with PAR1-specific siRNAs [13] and strongly suggest that PAR1 is an important mediator of breast cancer cell invasion. Strikingly, in cells depleted of PAR2 we also observed a significant loss of MDA-MB-231 and BT549 cell invasion, with only ~50% of cells exhibiting an ability to invade through matrigel in response to NIH-3T3 cell-conditioned medium (Fig. 2.3 C and D). The simultaneous knockdown of both PAR1 and PAR2 proteins also resulted in significant inhibition of MDA-MB-231 and BT549 cellular invasion (Fig. 2.3 C and D). These findings are the first to demonstrate a critical role for PAR2 in mediating breast cancer cell migration and invasion.

2.4.4. In PAR2-depleted cells, coagulant protease VIIa and Xa-induced migration is inhibited

Tissue factor is expressed in many invasive carcinomas including MDA-MB-231 cells but not in BT549 breast cancer cells and is critical for the formation and activity of upstream coagulant proteases VIIa and Xa. Tissue factor-bound VIIa and Xa can activate PAR1 and PAR2, and hence the mechanism by which these upstream coagulant proteases contribute to breast cancer cell migration and invasion remains poorly defined. Towards understanding the function of factors VIIa and Xa in breast cancer cell migration, we assessed the effect of PAR1 *versus* PAR2 knockdown on coagulant protease-induced breast cancer cell migration. We initially examined the ability of MDA-MB-231 cells to migrate in response to various concentrations of factor Xa. MDA-MB-231 cells exhibited a high-level of migration in the absence of agonist and a sub-maximal 52 nM concentration of factor Xa was most effective at stimulating cell migration (Fig. 2.4.A). The highly migratory MDA-

MB-231 cells were then electroporated with PAR-specific or non-specific siRNAs and allowed to migrate in response to low concentrations of either VIIa, Xa or VIIa and Xa together for 3 h at 37°C. In cells depleted of PAR1, ~80-90% of cells retained the capacity to migrate in response to factors VIIa and/or Xa compared to control siRNA-treated cells (Fig. 2.4.B). In contrast, cells lacking PAR2 showed a substantially greater inhibition of cell migration, with only ~70% of cells exhibiting migratory responses to coagulant proteases (Fig. 2.4.B). These findings indicate that the majority of MDA-MB-231 migratory responses occur independent of coagulant protease stimulation. However, activation of PAR2 by factors VIIa and Xa is capable of enhancing migration of a subpopulation of MDA-MB-231 breast cancer cells. The MDA-MB-231 cell line is heterogeneous with different subpopulations of cells displaying distinct growth and metastatic abilities due to discrete variations in gene expression patterns [29]. Together, these studies strongly suggest a critical function for PAR2 in mediating breast cancer cell migration and invasion and reveal a new role for PAR2 as the endogenous receptor for coagulant proteases VIIa and Xa in highly invasive breast cancer cells.

2.5. Discussion

A link between coagulation and several types of human malignant cancers is well established. Tissue factor and coagulant proteases are likely to promote cancer cell invasion and metastasis by modulating host vascular cell responses, as well as acting directly on tumor cells themselves. Thrombin is the major effector for PAR1, whereas tissue factor-VIIa and Xa can activate both PAR1 and PAR2. PAR1 promotes breast cancer cell migration and invasion [13-14,21], whereas the function of PAR2 in these processes remains poorly defined.

Towards understanding the mechanism by which coagulant proteases and PARs promote cancer cell invasion and metastasis, we sought to determine the functional importance of PAR2 in breast cancer cells. Depletion of PAR2 protein significantly reduced MDA-MB-231 and BT549 cell migration and invasion towards NIH-3T3 fibroblast conditioned medium. These results strongly suggest that PAR2 is a critical mediator of breast cancer cell migration and invasion. Moreover, our findings strongly suggest that PAR2 is the endogenous receptor for upstream coagulant proteases VIIa and Xa, whereas thrombin acts mainly at PAR1 in breast cancer cells.

In addition to PAR1, we show for the first time that PAR2 makes equally important contributions to breast cancer cell migration and invasion, as a reduction of PAR2 protein by siRNA caused significant decreases in cellular migration and invasion towards NIH-3T3 cell conditioned medium comparable to that observed with loss of PAR1. The depletion of PARs by siRNA provided an opportunity to rigorously define the role of these receptors in breast cancer cell migration and invasion. Previous studies have relied upon the use of blocking antibodies, cross desensitization experiments and antagonists that lack PAR selectivity. Our siRNA approaches have proven to be highly effective and selective in targeting PAR degradation resulting in loss of receptor-specific functional responses in breast cancer cells (Figs. 2.1 and 2.2). The transwell migration and invasion assays used in our experiments provide a reliable measure of *in vitro* cellular invasiveness. However, tumor cell metastasis involves complex interactions between blood cells, endothelial cells, mesenchymal cells and extracellular matrix that cannot be accurately reproduced *ex vivo*. Thus, future studies are necessary to definitively determine the importance of PAR2 in mediating breast cancer cell migration and invasion and metastasis *in vivo*.

Our studies strongly suggest that PAR2 is the endogenous receptor for tissue factor-VIIa and Xa-mediated invasion in invasive breast cancer cells. Whether tumor cells or surrounding stromal cells generate endogenous factors VIIa and Xa has not been determined. Tissue factor-VIIa alone appears to be sufficient to stimulate PAR2 signaling, however, tissue factor-VIIa-generated endogenous Xa might also contribute to signaling. In contrast, Xa activation of PAR2 occurs independent of tissue factor, indicating that formation of a ternary tissue factor-VIIa-Xa complex is not essential for signaling in breast cancer cells. Consistent with our findings, PAR2 is the major effector for Xa-elicited responses in endothelial cells [30]. However, in fibroblasts PAR1 is responsible for virtually all Xa-induced signaling responses [9,30]. The ability of tissue factor-VIIa and Xa to preferentially cleave and activate PAR2, as opposed to PAR1, is likely due to surface expressed cofactors that localize and regulate coagulant protease activity and specificity. Indeed, substrate recognition and cleavage by coagulant proteases is facilitated by cofactors, which function to properly position the substrate scissile bond to the relative membrane-associated protease [31]. Clearly, tissue factor serves this function for VIIa; however, the cofactor and/or mechanism that confers Xa's preferential recognition and cleavage of PAR2 *versus* PAR1 in cancer cells remains to be determined. The amino-terminal cleavage and activation domain of PAR2 lacks obvious protease recognition features, besides an adjoining positively charged arginine residue essential for serine protease cleavage of the receptor. However, the receptor is not cleaved by thrombin, indicating that the scissile bond may confer some specificity. The tumor microenvironment is replete with serine proteases as well as zinc-dependent metalloproteases, raising the possibility that PAR2 is likely to be cleaved and activated by other proteases released by tumor, activated stromal, infiltrating immune or endothelial cells.

The mechanism by which PAR2 promotes cancer cell migration and invasion is poorly understood. Activated PAR2 stimulates PI hydrolysis and mobilization of Ca^{2+} in a variety of cell types, suggesting that the receptor is capable of coupling to G_q and/or G_i signaling pathways [28,32]. However, whether PAR2 regulates important effectors of malignant progression such as Ras- and Rho-GTPases in cancer cells has not been determined. In addition, activation of PAR2 leads to prolonged increases in ERK1/2 activity, an important mitogenic signaling pathway. The early transient increase in ERK1/2 activity induced by PAR2 is likely mediated by a G-protein dependent pathway, whereas the slower and more persistent ERK1/2 activation involves receptor-interaction with arrestins on endocytic vesicles [28,33]. Arrestins are multifunctional proteins that act as scaffolds and transducers of mitogen-activated protein kinase (MAPK) signaling cascades. Indeed, PAR2-mediated ERK1/2 activation and cell migration are significantly inhibited in MDA-MB-231 cells lacking arrestins [34]. However, whether the spatial and/or temporal activation of ERK1/2 by PAR2 is particularly dysregulated in cancer cells has not been determined. Moreover, PAR2 simulates secretion of angiogenic factors and is capable of regulating the angiogenic process [22,35]. Thus, PAR2 may facilitate tumor cell invasion and metastasis at least in part by contributing to tumor angiogenesis.

In conclusion, there is increasing evidence for the role of aberrant PAR activation in cancer cell invasion and metastasis. PARs appear to be the target of a variety of extracellular proteases associated with the tumor microenvironment including serine proteases as well as metalloproteases. Previous studies have identified thrombin and metalloproteinase-1 as effectors of PAR1 activation on tumor cells [13-14,21]. In this study, we report that PAR2, a second protease-activated GPCR, has important functions in mediating cancer cell migration

and invasion towards NIH-3T3 fibroblast conditioned medium. The actual proteases responsible for PAR2 cleavage and activation present in conditioned medium remains to be defined. However, our studies also reveal a new role for PAR2 as the endogenous receptor for factors VIIa and Xa signal transduction in breast cancer cells. Whether dysregulated PAR2 activation and signaling promotes breast cancer cell migration and invasion will be important to determine.

2.6. Acknowledgements

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2.7. Footnotes

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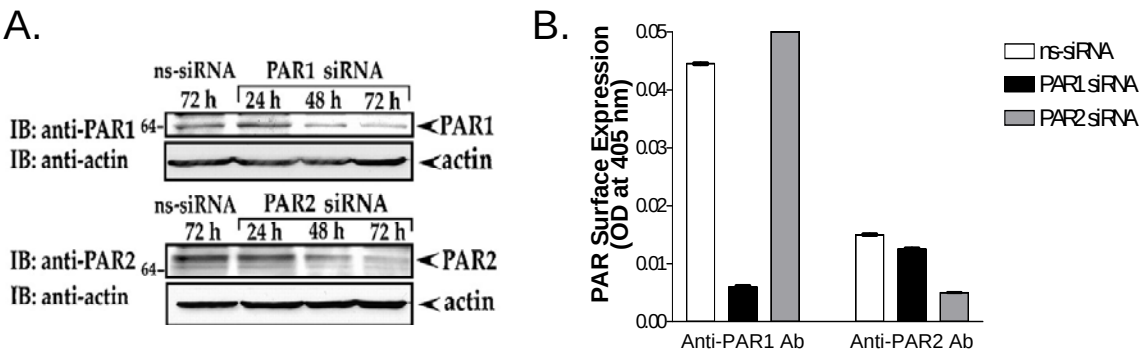

Figure 2.1. PAR-specific siRNAs cause selective and effective loss of receptor expression. A. MDA-MB-231 cells were electroporated with siRNAs targeted to PAR1, PAR2 mRNA sequences, or non-specific (ns) control siRNA and after 24, 48 or 72 h cell lysates were prepared. Equivalent amounts of protein lysates (~30 μg) were resolved by SDS-PAGE, transferred and immunoblotted with anti-PAR1 or anti-PAR2 antibodies. The membrane was stripped and reprobed with anti-actin antibody to control for loading. B. MDA-MB-231 cells electroporated with either ns-siRNA and PAR-specific siRNAs were incubated with either anti-PAR1 antibody or anti-PAR2 antibody (Ab), fixed and the amount of antibody bound to the cell surface was determined by ELISA. The data shown (mean ± S.D.; n=3) are representative of at least three separate experiments.

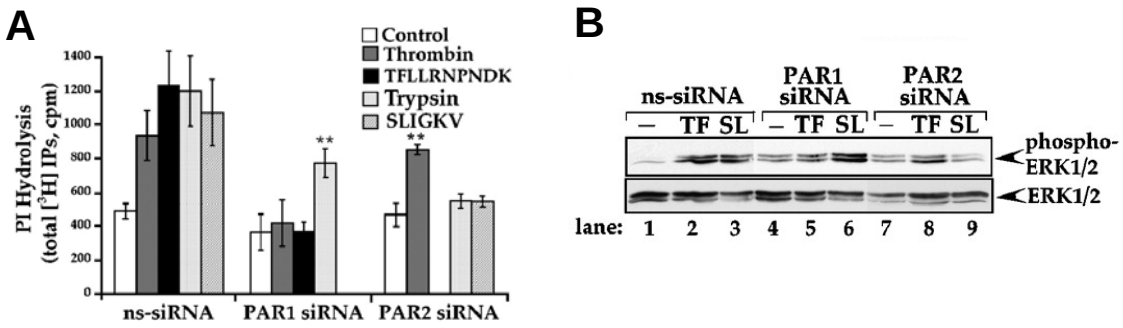

Figure 2.2. PAR-specific siRNAs cause effective loss of receptor-mediated signaling. A. MDA-MB-231 cells were electroporated with ns-siRNA or PAR-specific siRNAs and after 72 h of electroporation, cells labeled with myo-[3H]inositol were incubated in the absence or presence of PAR1 specific agonists (10 nM α-thrombin or 100 μM TFLLRNPNDK) or PAR2 agonists (10 nM trypsin or 100 μM SLIGKV) for 60 min at 37°C and amounts of [3H]IPs generated were measured. The data (mean ± S.D.; n=3) are representative of three independent experiments. In PAR1 siRNA treated cells, a significant difference (**, $p<0.01$) was observed in trypsin-induced signaling compared to untreated control cells. Similarly, thrombin signaling compared to control PAR2-siRNA cells was significantly different (**, $p<0.01$). Statistical analysis was determined using an unpaired Student's t test. B. MDA-MB-231 cells electroporated with ns-siRNA or PAR specific siRNAs, were serum-deprived overnight and then incubated in the absence (-) or presence of 100 μM TFLLRNPNDK (TF) or 100 μM SLIGKV (SL) for 30 min at 37°C, and immunoblotted for activated ERK1/2 using anti-phospho-p44/42 MAPK antibodies. Membranes were stripped and reprobed with anti-p44/42 MAPK antibody to control for loading.

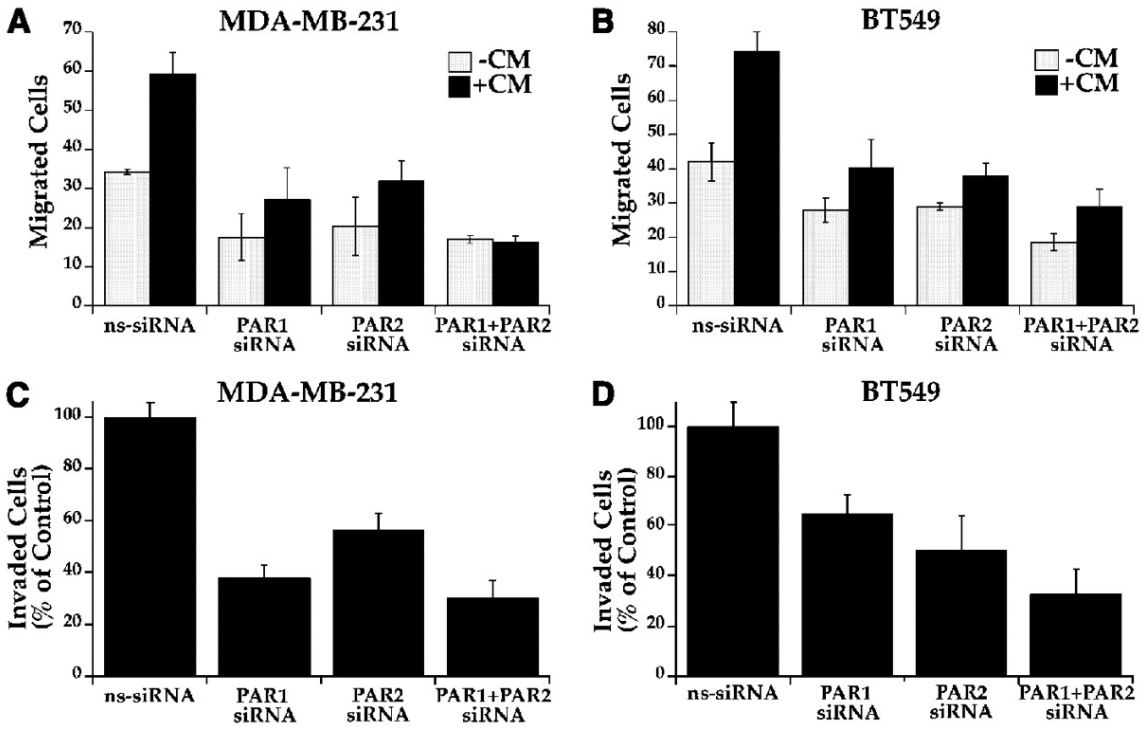

Figure 2.3. PAR2 is a critical mediator of MDA-MB-231 and BT549 cellular migration and invasion. A and B. MDA-MB-231 and BT549 cells were electroporated with PAR1-, PAR2- specific or non-specific (ns) siRNAs and after 72 h cell migration was assessed using transwells coated with collagen IV (MDA-MB-231) or laminin (BT549) containing either serum-free medium or conditioned medium prepared from NIH-3T3 fibroblasts as described in "Materials and Methods". The data (mean ± S.D.; n=3) shown are the average number of cells migrated determined from three different fields and is a representative experiment of at least three independent experiments. C and D. After 72 h of siRNA electroporation, MDA-MB-231 and BT549 cell migration and invasion towards NIH-3T3 cell conditioned medium was examined as described in "Materials and Methods". The data shown (mean ± S.E.) are expressed as the percentage of invaded cells compared to ns-siRNA treated control cells performed in duplicate and are the average of three independent experiments.

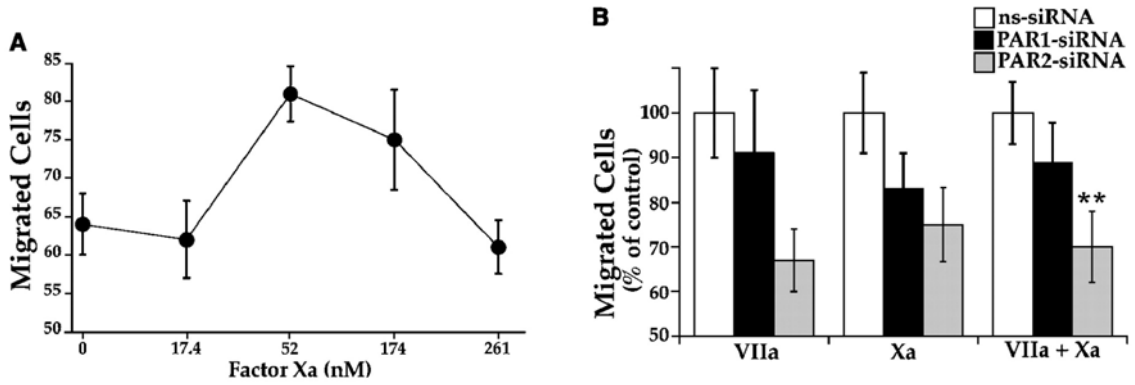

Figure 2.4. Coagulant protease VIIa and Xa-induced migration is inhibited in PAR2-depleted cells. A. Serum-deprived MDA-MB-231 cells were allowed to migrate in response to varying concentrations of factor Xa for 3 h at 37°C and the extent of cellular migration was assessed as described in "Materials and Methods". The data shown (mean ± S.D., n=3) are the average number of cells migrated determined from three different fields from one experiment is representative of three independent experiments. B. MDA-MB-231 cells were electroporated with PAR-specific or non-specific (ns) siRNAs. After 72 h the ability of serum-starved cells to migrate towards 3 nM VIIa, 52 nM Xa or VIIa and Xa together was then evaluated. The data (mean ± S.D., n=3) are expressed as the percentage of cells migrated compared to VIIa, Xa or VIIa and Xa treated ns-siRNA control cells. The difference in the number of PAR2 siRNA treated cells migrating towards VIIa and Xa compared to ns-siRNA treated cells was significant (**, $p<0.01$). Statistical analysis was determined using an unpaired Student's t test.

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Chapter 3

Ser^{336} in the C-terminal Tail of the $P2Y_1$ Receptor Regulates Agonist-promoted Internalization

3.1. Abstract

We examined the role of a conserved motif, $S^{336}RAT^{339}$, in the membrane-proximal region of the C-terminal tail in agonist-promoted internalization of the $P2Y_1$ receptor in MDCK (II) cells. Mutation of Ser336, but not Arg337 or Thr339, to alanine markedly increased both the rate and extent of agonist-promoted internalization. Whereas mutation to alanine of all serine and threonine residues C-terminal to the SRAT motif in the $P2Y_1$ receptor abrogated agonist-promoted internalization, further mutation of Ser336 and Thr339 to alanine resulted in a receptor that internalized similarly to wild type receptors in response to agonist. Agonist-promoted [^{32}P]phosphate incorporation into both $P2Y_1$ wild type and SRAT → ARAA receptors was similar (~3-fold over basal), indicating that increased phosphorylation is not the cause of enhanced internalization. Both $P2Y_1$ wild type and -ARAA receptors mobilized arrestin3-GFP in an agonist-dependent manner, and internalization studies in arrestin 2 & 3 double knockout mouse embryonic fibroblasts demonstrated that both receptors required arrestins to undergo agonist-promoted internalization. We propose a model in which Ser336 is a critical residue in the transition between arrestin-unreceptive (basal) and –receptive (agonist bound) conformational states of

the $P2Y_1$ receptor. This model predicts that mutation of Ser336 to Ala increases the rate of agonist-promoted conversion to its arrestin-receptive conformational state, resulting in increased binding of arrestin and enhanced internalization. These studies provide important information on the internalization and regulation of the $P2Y_1$ receptor and may increase our understanding of a broad range of physiological processes.

3.2. Introduction

Adenine and uridine di- and triphosphates and nucleotide sugars are released extracellularly from virtually all cells, where they activate P2-purinergic receptors in an autocrine or paracrine manner. There are two families of P2 receptors, ligand-gated P2X receptors and G-protein coupled P2Y receptors, which are broadly distributed and regulate myriad physiological processes. P2Y receptors are further classified into two subfamilies based on sequence homology and G-protein coupling. The $P2Y_1$ receptor subfamily, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$, couples to $G\alpha_q$ to stimulate phospholipase C activity and activate protein kinase C and mobilize intracellular Ca^{2+}. The $P2Y_{11}$ receptor additionally couples to $G\alpha s$ to stimulate adenylyl cyclase activity. The $P2Y_{12}$ receptor family, encompassing $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors, couples to $G\alpha_i$ to inhibit adenylyl cyclase and activate potassium channels[1-2].

$P2Y_1$ and $P2Y_{12}$ receptors are both expressed in platelets and mediate ADP-promoted platelet aggregation[3-7]. $P2Y_1$ activation regulates platelet shape change while activation of $P2Y_{12}$ is necessary for adhesion. Activation of both of these receptors is required for stable formation of platelet aggregates, and inhibition of either receptor by selective antagonists is effective in blocking stable platelet aggregation. Clopidogrel, commonly known as Plavix, is a $P2Y_{12}$ receptor antagonist that is the second highest selling prescription drug in the U.S. Clopidogrel is a pro-drug that is oxidized by hepatic P450 enzymes to the activate metabolite, which irreversibly inhibits ADP-promoted platelet aggregation by binding to the $P2Y_{12}$ receptor and covalently reacting with a cysteine residue in the receptor that is required for activity[8-9]. ADP-promoted platelet shape change is not affected by clopidogrel, indicating that this response is regulated by the $P2Y_1$ receptor[4,8,10]. The $P2Y_1$ receptor is a target for

antiplatelet compounds as well. Antagonists MRS2179[1] and MRS2500[11] are available for non-commercial use, and studies have shown that these antagonists also block ADP-promoted platelet aggregation[11-12]; however, to date no $P2Y_1$ receptor antagonist has been approved for clinical use.

Regulation of the ADP response in platelets is the subject of study in a number of laboratories. It has been known for some time that ADP released from isolated platelets causes them to become refractory to activation by ADP in vitro[13]. More recently, Gachet and co-workers reported that in platelets, the $P2Y_1$ receptor was rapidly desensitized upon addition of ADP, while the $P2Y_{12}$ was refractory to desensitization[14] . This same group also reported that the $P2Y_1$ receptor was rapidly internalized in both platelets and 1321N1 cells in an agonist-dependent manner and resided in the open canalicular system[15]. Robson and colleagues have shown that deletion of CD39 (eNTPDase-1) results in prolonged bleeding times due to desensitization of the platelet $P2Y_1$ receptor, presumably by preventing scavenging of ADP by the NTPDase[16]. Bordon et al., in a study quantifying the kinetics of desensitization, showed that the $P2Y_1$-selective agonist MRS2365 causes very rapid desensitization of subsequent ADP-promoted platelet aggregation, with a half-life of desensitization of 18 sec[11]. Thus, it is clear that the desensitization and internalization of the $P2Y_1$ receptor is an important means for regulating platelet function.

Our laboratory has focused on the mechanisms of agonist-promoted internalization of the $P2Y_1$ receptor. We have utilized MDCK (II) epithelial cells, which express an endogenous $P2Y_1$ receptor that internalizes in response to ADP challenge with identical kinetics and to the same extent as exogenously expressed $P2Y_1$ receptors. These studies have suggested a role for the C-terminal tail of $P2Y_1$ in regulating agonist-promoted

internalization, and have established that two Ser residues, Ser352 and Ser354, are critical for agonist-promoted internalization[17]. Here, we extend these studies by investigating the mechanism and defining critical residues involved in the regulation of agonist-promoted $P2Y_1$ internalization. We show that Ser336, located within a conserved motif in the membrane-proximal region of the C-terminal tail, is a critical regulator of agonist-promoted internalization.

3.3. Materials and methods

3.3.1. Reagents and antibodies

2-(Methylthio) adenosine 5'-diphosphate (ADP) was purchased from Sigma (St. Louis, MO). Monoclonal anti-HA.11 was purchased from Covance (Trenton, New Jersey). ^{32}P-orthophosphate and I^{125}-conjugated goat anti-mouse were purchased from Perkin Elmer (Waltham, Massachusetts). GFP-tagged arrestin2 and GFP-tagged arrestin3 were generous gifts from Dr. JoAnn Trejo (University of California at San Diego).

3.3.2. Cell lines

MDCK (II) cells were obtained from the Tissue Culture Facility at the University of North Carolina at Chapel Hill. Wildtype and arrestin2/arrestin3-null murine embryonic fibroblast (MEFs) cells were generous gifts from Drs. Bryan Roth (University of North Carolina at Chapel Hill) and Joann Trejo. PA317 retroviral packaging cells were kindly provided by Dr. John Olson at the UNC Cystic Fibrosis Center. MDCK (II) cells were cultured in 50/50 DMEM/F12 medium supplemented with 5% fetal bovine serum (FBS).

1321N1, MEFs, and PA317 cells were maintained in DMEM medium supplemented with 10% FBS.

3.3.3. Retroviral infections and stable expression of receptor constructs

Stable expression of $P2Y_1$ wild type and mutant receptors in MDCK (II), 1321N1, and MEF cells was achieved by retroviral infection. Receptor constructs cloned into pLXSN or pLX-PIH retroviral expression vectors were used to produce virus. Wild type and mutant human $P2Y_1$ receptor constructs harbored an amino-terminal HA-epitope tag, YPYDVPDYA, following the initiating methionine residue. Previous studies showed that addition of an amino-terminal HA epitope tag does not affect P2Y receptor function[18]. PA317 retroviral packaging cells were seeded at a density of 1 x 10^6 cells in a 25 cm^2 tissue culture flask and transfected 24 hours later. Using the calcium phosphate transfection method, each flask was transfected with 14 μg of the receptor construct and 6 μg of VSV-G plasmid. Transfected cells were incubated overnight at 37ºC, the medium was replaced with tissue culture medium containing 5 mM sodium butyrate, and cells were incubated for an additional 48 hours at 32ºC. Cell medium containing virus was harvested, filtered, supplemented with 8 μg polybrene (Sigma, St. Louis, MO), and added to MDCK (II), 1321N1, or MEF cells at 70% confluence. The cells were incubated twice with virus for two hours each. Following infection, the virus-containing medium was replaced with normal cell culture medium and cells were incubated at 37ºC for 48 hours. Retrovirally infected cells

were selected in cell medium supplemented with 0.6 μg/mL G418 (Gibco, Bethesda, MD) or 0.3μg/mL hygromycin (Roche, Indianapolis, IN). After the death of uninfected control cells, MDCK (II) and 1321N1 cells were maintained in normal medium and used for experiments.

3.3.4. Site-directed mutagenesis

Truncation and point mutations were constructed as previously described (Houston thesis). The P2Y**1**-334Z and -339Z truncation mutants were constructed using PCR amplification with 5′ primers containing an *EcoRI* restriction site and 3′ primers containing a stop codon after Arg-334 (334Z) and Thr-339 (339Z). A *XhoI* restriction site enabled cloning into a similarly digested pLXSN. $P2Y_1$-0P (all Ser/Thr residues in the C-terminal tail mutated to Ala) and -SRAT-0P (all Ser/Thr residues C-terminal to the SRAT motif mutated to Ala) mutant receptors were constructed using long primers up to 60 bases in length that overlap by approximately 18 bases. The sense primer contained a *XhoI* restriction site and the antisense primer contained a *BamHI* restriction site. The primers were annealed and filled in with the Klenow fragment of DNA polymerase followed by digestion and ligation into the $P2Y_1$-339Z truncation mutant containing silent mutations to incorporate the necessary restriction sites. The individual point mutations S336A/T339A, T339A, S336A, R337A, and S336D/T339D were constructed by four-primer PCR with HA-$P2Y_1$ in pLXSN as template and reverse complementary primers with substitutions at the indicated sites[19]. The amplified products were digested with *Eco*RI and *Xho*I restriction enzymes and inserted into similarly digested pLXSN, pLX-PIH or pcDNA3.1 vectors. The resulting plasmids were purified using the Invitrogen Concert maxiprep kit (Carlsbad, CA) and sequences were confirmed at the UNC DNA Sequencing facility.

3.3.5. Internalization assays

Cells were seeded at a density of 5 x 10^4 cells/well in a 24-well plate. After 48 hours, cell medium was replaced with 200 µl DMEM supplemented with 50 mM HEPES (pH 7.4) and placed in a water bath at 37ºC for 1 hour. At the indicated times, 50 µl of 50 µM 2MeSADP (10 µM, final concentration) was added to each well. To stop the assay, the plate was added to an ice-cold water bath and cells were fixed with 4% paraformaldehyde. Receptors remaining on the cell surface were labeled with anti-HA.11 monoclonal antibody followed by I^{125}-labeled goat-anti mouse secondary antibody. Cells were then solubilized in 1 M NaOH overnight, following which cell lysates were collected and counted in a gamma counter.

3.3.6. Immunofluorescence confocal microscopy

Cells were seeded on fibronectin-coated glass coverslips at a density of 5 x 10^4 cells/well in a 12-well plate. After 48 hours, surface receptors were pre-labeled with anti-HA.11 monoclonal antibody at 4°C for 1 hour. Cells were stimulated with 2MeSADP (30 µM) for 30 min, fixed with 4% paraformaldehyde, and permeablized with methanol. Receptor internalization was assessed using a goat-anti mouse secondary antibody conjugated to Alexa-488 or -594 (Molecular Probes, Carlsbad, CA). Images were acquired using an Olympus Fluoview 300 laser scanning confocal imaging system configured with an Olympus IX70 fluorescence microscope fitted with a PlanApo 60X oil objective. Confocal XY images sectioned at 0.28 µm were collected sequentially at 800 X 600 resolution with 2X optical zoom.

3.3.7. ^{32}P Incorporation assay

MDCK (II) cells were seeded at a density of $2.5x10^6$ cells/10-cm dish. After 48 hours, cells were incubated in phosphate-free DMEM for 1 hour at 37°C and incubated with 1 mCi of [^{32}P]-orthophosphate for 3 hours at 37°C. The cells were then challenged with either buffer or 10 μM 2MeSADP for 5 min and immediately put on ice. Cells were lysed in RIPA buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails. Receptor protein was immunoprecipitated with anti-HA.11 monoclonal antibody and Protein A/G beads, and then solubilized with SDS gel loading buffer. A portion (80%) of the sample was electrophoresed on a 10% SDS-PAGE gel, and ^{32}P incorporation into the receptor was detected by exposing the fixed and dried gel to X-OMAT film for 6-18 hrs at -80^{o}C. The remaining 20% was electrophoresed on a 10% SDS PAGE gel and receptor protein was detected by western blotting.

3.3.8. Phosphoinositide hydrolysis assay

1321N1 cells were seeded at a density of 5×10^4 cells/well in a 24-well plate 72 hours prior to assay. Inositol lipids were labeled by adding 2 μCi/ml of [^{3}H]-myo-D-inositol to the cells in inositol-free medium 14-20 hours prior to assay. The indicated concentrations of 2MeSADP in 50 mM LiCl were added to individual wells and incubated for 10 min at 37°C. Cells were then lysed in 50 mM formic acid overnight and neutralized with 150 mM ammonium hydroxide. Samples were loaded onto AG10X8 columns and washed

sequentially with water and 50 mM ammonium formate. [^{3}H]Inositol phosphates were eluted with 1.2 M ammonium formate/0.1 M formic acid and quantified by scintillation counting.

3.3.9. GFP-tagged arrestin translocation assay

MDCK (II) cells were nucleofected with HA-tagged wild type or mutant $P2Y_1$ receptors and GFP-tagged arrestin2 or arrestin 3 using Kit T, program U-023 according to manufacturer's instructions (Lonza, Basel, Switzerland), and then seeded on fibronectin-coated glass coverslips. After 48 hours, surface receptors were pre-labeled using anti-HA.11 monoclonal antibody at 4°C for one hour. Cells were then stimulated for 5 min at 37°C with isoprotenol (30 μM) or 2MeSADP (30 μM). GFP-arrestin translocation was assessed by immunofluorescence confocal microscopy as described above.

3.3.10. Data analysis

All experiments were performed in triplicate and carried out at least three times. Data were analyzed using Prism 3.0 software and InStat 3.0 (GraphPad, San Diego, CA).

3.4. Results

3.4.1. The highly conserved $S^{336}RAT^{339}$ sequence motif is involved in regulation of agonist-promoted $P2Y_1$ receptor internalization

The C-terminal tails of GPCRs commonly contain regulatory signals for agonist-promoted desensitization and internalization. Our previous data showed that $P2Y_1$ receptor internalization is also regulated through its C-terminal terminal tail. In particular, two Ser residues, Ser352 and Ser354, are phosphorylated upon agonist addition and are critical for

receptor internalization (Houston thesis). During the course of these studies, we quantified agonist-promoted internalization of the wild type $P2Y_1$ receptor, and $P2Y_1$ receptors lacking either the entire C-terminal tail ($P2Y_1$-334Z) or the C-terminal tail immediately past a highly conserved $S^{336}RAT^{339}$ sequence motif ($P2Y_1$-339Z) (Fig. 3.1.A). Wild type $P2Y_1$ receptors internalized in response to agonist with a $t_{1/2}$ ~9.25 min, whereas agonist-promoted internalization of the $P2Y_1$-339Z receptor was essentially abolished (Fig. 3.1.B). Surprisingly, the $P2Y_1$-334Z receptor internalized both with a similar time course and to the same steady-state level as the wild type receptor (Fig. 3.1.B).

The main difference between the two truncated receptors is the absence of a highly conserved sequence motif, $S^{336}RAT^{339}$, in the 334Z mutant (green bar in Fig. 3.1A). To investigate whether this motif is responsible for the marked differences in internalization of the two truncated receptors, we mutated both Ser336 and Thr339 to Ala (ARAA) in the full length receptor (Fig. 3.2.A) and quantified changes in cell-surface expression in MDCK (II) cells upon agonist addition. Surprisingly, the ARAA mutant receptor displayed enhanced agonist-promoted internalization, with faster kinetics ($t_{1/2}$~ 6.78 min) and a greater extent of internalization (Fig. 3.2.B). These data, taken together with data from the truncated receptor, suggest that one or more amino acids within the SRAT sequence regulate agonist-promoted $P2Y_1$ receptor internalization.

Both Ser and Thr residues are capable of being phosphorylated (and dephosphorylated) during the activation cycle of the receptor. To address the role of agonist-promoted phosphorylation in $P2Y_1$-R internalization, all potential phosphorylation sites, either in the entire C-terminal tail (0P) or only those downstream of the SRAT sequence (SRAT-0P), were mutated to alanine residues and agonist-promoted internalization was

quantified in MDCK (II) cells. Consistent with our studies showing that Ser352 and Ser354 are required for agonist-promoted internalization and phosphorylation, agonist-promoted internalization of the SRAT-0P receptor was abolished, even after 60 min of agonist stimulation (Fig. 3.2.B). In contrast, the $P2Y_1$-0P receptor internalized in response to agonist with kinetics similar to wild-type receptor (Fig. 3.2.B). To ensure that phosphorylation of other residues in the receptor was not occurring, we also quantified agonist-promoted internalization of the $P2Y_1$ receptor in which all cytoplasmic Ser and Thr residues, including those in the three intracellular loops, were mutated to Ala ($P2Y_1$-Pan0P). In response to agonist, the Pan0P receptor internalized with kinetics identical to the $P2Y_1$-0P receptor (data not shown). These data suggest that phosphorylation is not required for agonist-promoted internalization of the $P2Y_1$ receptor. The contradictory data with $P2Y_1$-SRAT-0P and $P2Y_1$-0P receptors suggest a complex regulation of agonist-promoted $P2Y_1$ receptor internalization involving the SRAT sequence and agonist-promoted phosphorylation of the C-terminal tail.

To test if potential phosphorylation of the SRAT sequence plays any role in agonist-promoted internalization, we generated a phospho-mimetic DRAD mutant receptor that theoretically imitates a receptor that is permanently phosphorylated, and following expression in MDCK (II) cells, assessed agonist-promoted internalization. Even after 60 min of agonist stimulation, the DRAD receptor failed to internalize (Fig. 3.3..B).

3.4.2. Ser^{336} is the key residue regulating agonist-induced $P2Y_1$ internalization

To address which residues within the SRAT sequence regulate agonist-stimulated $P2Y_1$ receptor internalization, we mutated Ser336, Arg337, and Thr339 individually in the context of the full length receptor (Fig. 3.3.A) and quantified surface expression of wildtype

or mutant receptors in MDCK (II) cells following activation with 10 μM 2MeSADP. The kinetics and extent of internalization of both the SRAA and SAAT mutant receptors were identical to wild type, whereas those of the ARAT mutant receptor were identical to the ARAA receptor (Fig. 3.3.B). These results implicate Ser336 as the critical residue within this sequence for regulation of internalization.

3.4.3. Ser336 regulates agonist-induced $P2Y_1$ internalization in 1321N1 human astrocytoma cells

In our quest to find a cell line in which to investigate agonist-promoted internalization of the $P2Y_1$ receptor, we observed that exogenously expressed $P2Y_1$ receptors did not internalize in most common cell lines tested (e.g. HEK293, HeLa, C6 glioma, and 1321N1 astrocytoma cells; data not shown), whereas other P2Y receptors, i.e. $P2Y_2$ and $P2Y_4$ receptors, did undergo agonist-promoted internalization, indicating that the inability to observe agonist-promoted $P2Y_1$ receptor internalization was not due to a defect in the endocytic machinery of the cells. In contrast, exogenously expressed $P2Y_1$ receptors in MDCK cells internalized in response to agonist with identical kinetics and to the same extent as endogenously expressed $P2Y_1$ receptors (Houston thesis), indicating that the $P2Y_1$ receptor internalization may be differentially regulated depending on its cellular context.

To test whether the SRAT sequence has a role in the inability of the receptor to undergo internalization in these cell lines, we generated 1321N1 cells stably expressing HA-tagged $P2Y_1$ wild type, -ARAA, -SRAA, or -ARAT receptors and assessed agonist-promoted internalization. Consistent with our past observations, the wild type $P2Y_1$ receptor did not internalize after 60 min of agonist stimulation (Fig. 3.4.A). In contrast, after 60 min of

agonist-stimulation, ARAA and ARAT mutant receptors internalize with a $t_{1/2}$ ~19.0 min, whereas the SRAA mutant receptor did not internalize (Fig. 3.4.A). Internalization data also was confirmed by immunofluorescence confocal microscopy (Fig. 3.4.B). These data support our findings that Ser336 is a critical regulator of $P2Y_1$ internalization, although the reasons for the absence of agonist-promoted internalization of the wild type $P2Y_1$ receptor in 1321N1 cells remain unknown.

3.4.4. Phosphorylation of $P2Y_1$ receptor SRAT mutants

One explanation for the increased internalization of the SRAT mutants is that they are more heavily phosphorylated in response to agonist. To directly assess agonist-induced ^{32}P incorporation of wild type and SRAT mutants, MDCK (II) cells expressing various mutant receptor constructs were labeled with [^{32}P]orthophosphate and both basal and agonist-promoted [^{32}P]phosphate incorporation into each receptor was determined. Both $P2Y_1$ wild type and -ARAA receptors incorporated similar levels of basal and agonist-promoted [^{32}P]phosphate, with phosphorylation increasing ~3-fold over basal (Fig. 3.5.A). These data suggest that the increased internalization observed with $P2Y_1$-ARAA (and –ARAT) receptors is not due to increased agonist-promoted phosphorylation. In contrast, $P2Y_1$-SRAT-0P, CT-0P and –Pan-0P receptors did not show increased agonist-dependent phosphorylation (Fig. 3.5.B and data not shown), suggesting that Ser336 and Thr339 are not phosphorylated in an agonist-dependent manner. $P2Y_1$-DRAD receptors, which do not undergo agonist-promoted internalization, appeared highly phosphorylated in the basal state, and phosphorylation increased ~3-fold upon agonist addition. These data indicate that phosphorylation by itself is not sufficient for internalization.

Lastly, we examined agonist-promoted phosphorylation of the $P2Y_1$ receptor in 1321N1 human astrocytoma cells. As seen in the right panel of Fig. 3.5.A, the $P2Y_1$ receptor, which does not internalize in these cells, is phosphorylated in an agonist-dependent manner. These data further confirm that $P2Y_1$ receptor phosphorylation is not sufficient for agonist-promoted internalization.

3.4.5. $P2Y_1$ mutant receptors are functional

One possibility to explain the lack of internalization of some of the mutant receptors is that these receptors are inactive and incapable of coupling to G proteins. To investigate this possibility, we examined the signaling properties of the mutant receptors by quantifying inositol lipid hydrolysis in 1321N1 cells. 1321N1 cells are the cell line of choice when quantifying P2Y receptor activation, since they lack endogenous P2Y receptors. As shown in Fig 3.5.B, all of the P2Y receptor mutants examined displayed robust agonist-promoted inositol phosphate accumulation. Moreover, the EC_{50} values of 2MeSADP for wild type and mutant receptors were comparable, indicating that the mutations have no effect on coupling to Gq.

3.4.6. Translocation of GFP-tagged arrestin2 and arrestin3 following $P2Y_1$ receptor activation

Addition of agonists to a broad range of GPCRs results in translocation of co-expressed GFP-tagged arrestins to the plasma membrane[20-22], which is often used as a measure of receptor-arrestin interaction. To address the role of the SRAT sequence in $P2Y_1$ receptor-mediated arrestin activation, agonist-promoted GFP-tagged arrestin mobilization was determined in MDCK (II) cells expressing wild type and mutant receptor constructs. MDCK (II) cells were co-nucleofected with HA-β2AR (control) or with P2Y receptor mutants, together with either GFP-arrestin2 or GFP-arrestin3, and seeded on glass coverslips

for immunoflorescence microscopy. Surface receptors were pre-labeled and stimulated for 5 min with either 30 μM isoprotenol (β2AR) or 2MeSADP ($P2Y_1$-R).

Distribution of the β2AR went from primarily plasma membrane-bound to punctate, internalized vesicles and GFP-arrestin3 localization changed from diffuse cytoplasmic staining under control conditions to punctate staining following agonist treatment. This GFP-arrestin mobilization suggests that GFP-arrestin3 is translocated from the cytoplasm to the activated β2AR and functions in receptor internalization. Consistent with literature demonstrating no role for arrestin2 in β_2AR internalization[23-24], GFP-arrestin2 did not translocate despite receptor internalization (data not shown). Following 5 min of stimulation with 2MeSADP (30 μM), both GFP-arrestin2 (Fig. 3.6.A, panel E) and GFP-arrestin3 (Fig. 3.6.B, panel E) translocated and colocalized with internalized $P2Y_1$ receptors, as indicated by the yellow punctate staining in Figs. 3.6.A and 3.6.B, panel F. Colocalization of the $P2Y_1$ receptor with GFP-arrestin3 was more robust than with GFP-arrestin2, which was consistent with published studies on the roles of both arrestin2 and arrestin3 in $P2Y_1$ internalization[25].

To test the function of the SRAT sequence on GFP-tagged arrestin3 activation and translocation in MDCK (II) cells, we co-expressed GFP-arrestin3 with the $P2Y_1$-ARAT (Fig. 3.7.A), -0P (Fig. 3.7.E), -334Z (Fig. 3.7.I), and -SRAT-0P (Fig. 3.7.M) receptors. Upon agonist stimulation of the $P2Y_1$-ARAT and $P2Y_1$-0P receptors, GFP-tagged arrestin3 translocated to internalized puncta together with the receptor (Fig. 3.7, panels C and G), suggesting that mutation of the SRAT sequence does not inhibit $P2Y_1$ receptor-mediated arrestin translocation. Interestingly, these data also suggest that translocation of GFP-arrestin3 occurs *independent* of carboxyl-tail phosphorylation, provided that Ser336 is mutated to Ala. GFP-arrestin2 translocation was also observed following agonist

stimulation, although always to a lesser extent than GFP-arrestin3 (data not shown). In contrast, agonist addition to cells expressing the $P2Y_1$ SRAT-0P receptor neither promoted internalization (Fig. 3.7.N) nor resulted in translocation of GFP-arrestin3 (Fig. 3.77) or GFP-arrestin2 (data not shown). Interestingly, agonist stimulation of the $P2Y_1$-334Z receptor leads to internalization (Fig. 3.7.J), but not GFP-arrestin3 translocation (Fig. 3.7.K). These data suggest that in the absence of the carboxyl-terminus, $P2Y_1$ internalization is through an alternate, arrestin-*independent* mode of internalization.

3.4.7. $P2Y_1$ wild type and SRAT receptors fail to internalize in arrestin2,3 knock-out murine embryonic fibroblast cells

Although our results demonstrate that agonist activation of the $P2Y_1$ receptor causes the translocation of GFP-arrestins and internalization of the receptor, they do not address whether the arrestins are *required* for receptor internalization. To confirm whether arrestins were necessary for receptor internalization, we expressed $P2Y_1$ wild type, -ARAT, and -SRAT-0P receptors in both normal mouse embryonic fibroblasts (MEFs) and arrestin2/arrestin3-knockout MEFs and quantified agonist-promoted internalization in the two cell lines. Consistent with our data in MDCK (II) cells, following 30 min of agonist treatment both $P2Y_1$ wild type and -ARAT receptors internalized in normal MEFs, whereas $P2Y_1$-SRAT-0P receptor internalization was not observed (Fig. 3.8.A). In arrestin2,3 knockout cells, however, agonist-promoted $P2Y_1$ wild type and -ARAT receptor internalization was completely blocked (Fig. 3.8.B). These data indicate that arrestins are required for $P2Y_1$ receptor internalization and that mutation of the SRAT sequence does not prevent $P2Y_1$ receptor-mediated arrestin translocation.

3.5. Discussion

Previous work from our laboratory examining the mechanisms involved in agonist-promoted internalization of the $P2Y_1$ receptor established that two Ser residues, Ser352 and Ser354 are phosphorylated upon agonist addition and are the primary residues that mediate receptor internalization in MDCK (II) cells. In the course of this study, we observed that receptors truncated after Thr339 ($P2Y_1$-339Z) did not undergo agonist-promoted internalization, whereas receptors missing an additional five amino acids ($P2Y_1$-334Z) internalized similarly to wild type receptors (Fig. 3.1.B). These data focused attention on the apparent importance of a conserved sequence motif, $S^{336}RAT^{339}$, in regulating agonist-promoted internalization of the receptor. We show here that mutation of Ser336, but not Arg337 or Thr339 within this motif alters both the rate and extent of agonist-promoted internalization of the $P2Y_1$ receptor (Figs. 3.2.B, 3.3.B, data not shown). Mutation of Ser336 to Ala increased internalization, whereas mutation of both Ser336 and Thr339 to Asp ablated internalization (Fig. 3.2.B). Additionally, whereas wild type $P2Y_1$ receptors do not internalize in 1321N1 astrocytoma cells, mutation of both Ser336 to Ala imparted the ability of the $P2Y_1$ receptor to undergo agonist-promoted internalization in these cells (Fig. 3.4). Mutation of Ser336 does not interfere with arrestin binding to agonist-bound receptors, and internalization of both $P2Y_1$-ARAT and $P2Y_1$-0P receptors is arrestin-dependent (Fig. 3.8). Taken together, our data strongly support a role of Ser336 as a key residue in determining the kinetics and extent of internalization of the $P2Y_1$ receptor.

Based on our data, we propose that $P2Y_1$ internalization is a bi-phasic process. In a basal state, the carboxyl-tail of the $P2Y_1$ receptor interacts with the intracellular body of the receptor through the SRAT sequence. Following agonist binding, residues Ser352 and Ser354 are phosphorylated, resulting in the SRAT sequence being released from the body of the receptor. Arrestins can then bind to the receptor body to facilitate internalization (Fig. 3.9).

Based on our model, the $P2Y_1$-339Z mutant does not internalize (Fig. 1B) because it is lacking agonist-promoted phosphorylation at Ser352 and Ser354. Thus, the SRAT sequence is not released from the receptor body to allow arrestin binding and subsequent internalization. We will address the mode of internalization of the $P2Y_1$-334Z mutant according to the model later in the discussion. When Ser336 is mutated to Ala, as in the case of $P2Y_1$-ARAA and –ARAT receptors, we believe that interaction between the carboxyl-tail (via the SRAT sequence) and the body of the receptor is decreased, leading to an increase in internalization (Fig 3.3.B).

Curiously, we do not see agonist-promoted $P2Y_1$ receptor internalization in several common cell lines, including HEK293, CHO, C6, and 1321N1 astrocytoma cells. In HEK293 cells, which endogenously express the $P2Y_1$ receptor, neither the native nor exogenously expressed receptors internalize in response to agonist (data not shown). The reason for the lack of internalization is not currently understood, although we do observe agonist-promoted phosphorylation and conclude that a defect in phosphorylation is probably not involved (Fig. 3.5). However, the $P2Y_1$- ARAA and –ARAT mutant receptors did internalize following agonist treatment. These data support our model that Ser336 is a critical regulator of $P2Y_1$ internalization (Fig. 4).

Phosphorylation of GPCRs is a key post-translational modification that occurs upon activation of receptors by agonist and precedes internalization of the receptor. To examine the role of phosphorylation in agonist-promoted $P2Y_1$ receptor internalization in MDCK (II) cells, we quantified internalization of mutant receptors. Because serines and threonines are potential phosphorylation sites, we also examined the internalization of mutant receptors that are lacking all potential phosphorylation sites in the absence or presence of the SRAT sequence. We find that the SRAT-0P mutant receptor does not internalize following agonist treatment, suggesting that carboxyl-tail phosphorylation downstream of the SRAT sequence is required (Fig. 3.2.B). In the context of our model, SRAT-0P internalization is inhibited because Ser352 and Ser354 are not phosphorylated and the SRAT sequence is not released from the body of the receptor to allow arrestin biding and subsequent internalization.

We also find that the $P2Y_1$-0P receptor, which lacks all potential carboxyl-terminal phosphorylation sites, internalizes comparably to the wild type receptor (Fig. 3.2.B). These data can be explained by our model as well. Although there is no agonist-promoted phosphorylation of the mutant receptor (Fig. 3.5.A), the SRAT sequence is not interacting with the body of the receptor due to the mutations of the Ser and Thr of the SRAT sequence to Ala. Consequently, arrestin can freely bind to the receptor following treatment to facilitate internalization.

The possibility that phosphorylation of either Ser336 or Thr339 was involved in the increased internalization of the SRAT mutants was not supported by ^{32}P phosphorylation experiments. Paradoxically, we observed hyper basal phosphorylation of the $P2Y_1$-DRAD receptor, but equal fold increase in phosphorylation following agonist treatment compared to the wild type receptor (Fig. 3.5.A). Based on the model, we believe that the hyper

phosphorylation causes the receptor c-tail to more tightly interact with the body of the receptor and that we do not observe $P2Y_1$-DRAD internalization following agonist treatment because Ser352 and Ser354 phosphorylation is not sufficient to release the DRAD sequence from the body of the receptor.

Lastly, we show that activation of the $P2Y_1$ receptor mobilizes GFP-arrestin3, and to a lesser extent, GFP-arrestin2 in a SRAT and carboxyl-tail phosphorylation-*independent* manner (Figs. 3.6 and 3.7). Studies using the $P2Y_1$-334Z receptor, which lacks the entire carboxyl-terminal tail, reveal that the tail is required for arrestin mobilization. This mutant internalizes upon agonist treatment, but cannot mobilize GFP-arrestin, suggesting an arrestin-*independent* mechanism of internalization (Fig. 3.7 panels I, J, K, and L). Thus, our model is not applicable to this mutant.

Our internalization experiments in arrestin2/arrestin3 null MEFs confirm published observations that arrestins are required for $P2Y_1$ internalization (Fig. 3.8). Other GPCRs, such as δ-opioid and receptors, have been shown to activate arrestins independent of phosphorylation[26]. For several GPCRs, including the CXCR4 receptor, phosphorylation of the third intracellular loop is also a site of regulation for agonist-promoted internalization[27]. Further studies should focus on the third intracellular loop for $P2Y_1$ to determine the region and/or residues required for arrestin interactions.

3.6. Acknowledgements

We would like to thank members of Nicholas, Harden, and Lazarowski laboratories for technical assistance and helpful suggestions.

A.

B.

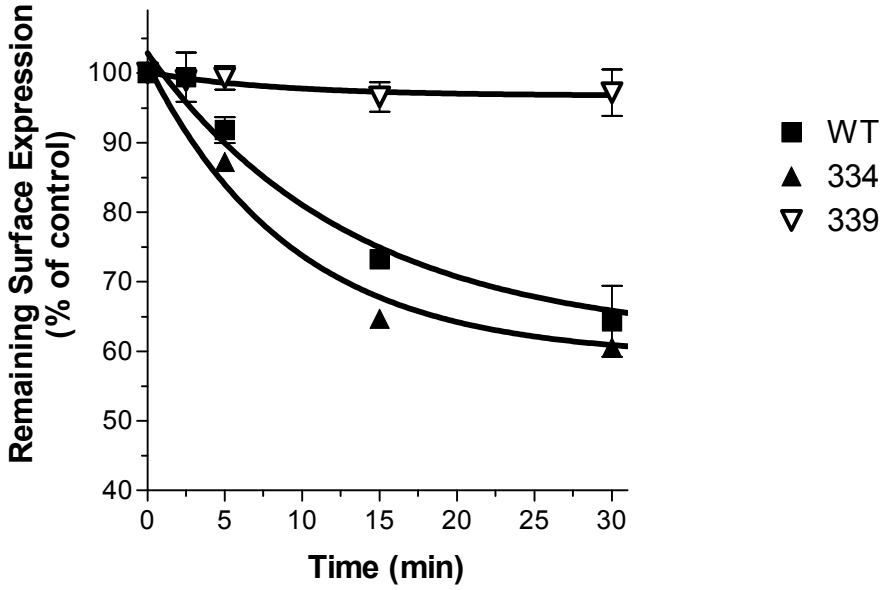


Fig. 3.1. Importance of the SRAT sequence in $P2Y_1$ regulation is illustrated by carboxyl-tail truncation mutants. A. The SRAT motif (green bar) in the $P2Y_1$ receptor C-terminal tail is evolutionarily conserved. Arrows indicate the last amino acids of the $P2Y_1$-334Z and -339Z truncation mutants, and the two critical residues that are phosphorylated in response to agonist and mediate internalization are denoted by an *. B. Serum-deprived MDCK (II) cells stably expressing $P2Y_1$ wildtype, -334Z or -339Z receptors were stimulated with 10μM 2MeSADP for the indicated times. The levels of receptors remaining at the cell surface were quantified as described in "Materials and methods" and reported as remaining cell surface expression compared to control (0 min) cells.

A.

```
P2Y1:   RLSRATRKASRRSEANLQSKSEDMTLNILPEFKQNGDTSL
ARAA:    RLARAARKASRRSEANLQSKSEDMTLNILPEFKQNGDTSL
DRAD:    RLDRADRKASRRSEANLQSKSEDMTLNILPEFKQNGDTSL
SRAT-0P:  RLSRATRKAARRAEANLQAKAEDMALNILPEFKQNGDAAL
0P:      RLARAARKAARRAEANLQAKAEDMALNILPEFKQNGDAAL
```

B.

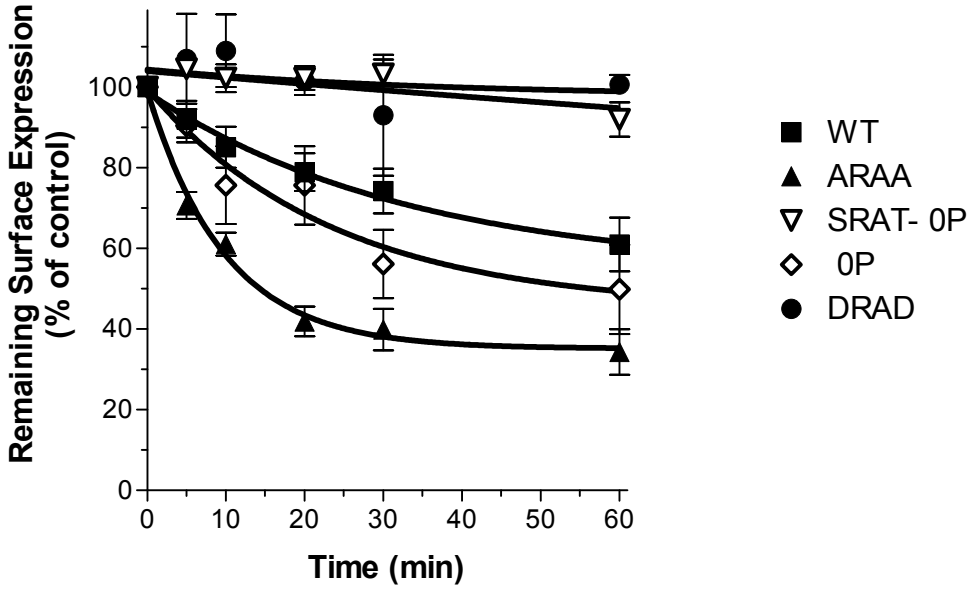


Fig. 3.2. SRAT sequence regulates agonist-promoted $P2Y_1$ internalization. A. Ser and Thr residues in the C-terminal tail of the wild type receptor are highlighted in red, and residues mutated to Ala are highlighted in green. B. Serum-starved MDCK (II) cells stably expressing $P2Y_1$ wild type, -ARAA, -SRAT-0P, -0P, or -DRAD receptors were stimulated with 10μM 2MeSADP for the indicated times. The levels of receptors remaining at the cell surface were quantified as described in "Materials and methods" and reported as remaining cell surface expression compared to control (0 min) cells.

A.

```
P2Y1:   RLSRATRKASRRSEANLQSKSEDMTLNILPEFKQNGDTSL
ARAA:   RLARAARKASRRSEANLQSKSEDMTLNILPEFKQNGDTSL
ARAT:   RLARATRKASRRSEANLQSKSEDMTLNILPEFKQNGDTSL
SRAA:   RLSRAARKASRRSEANLQSKSEDMTLNILPEFKQNGDTSL
```

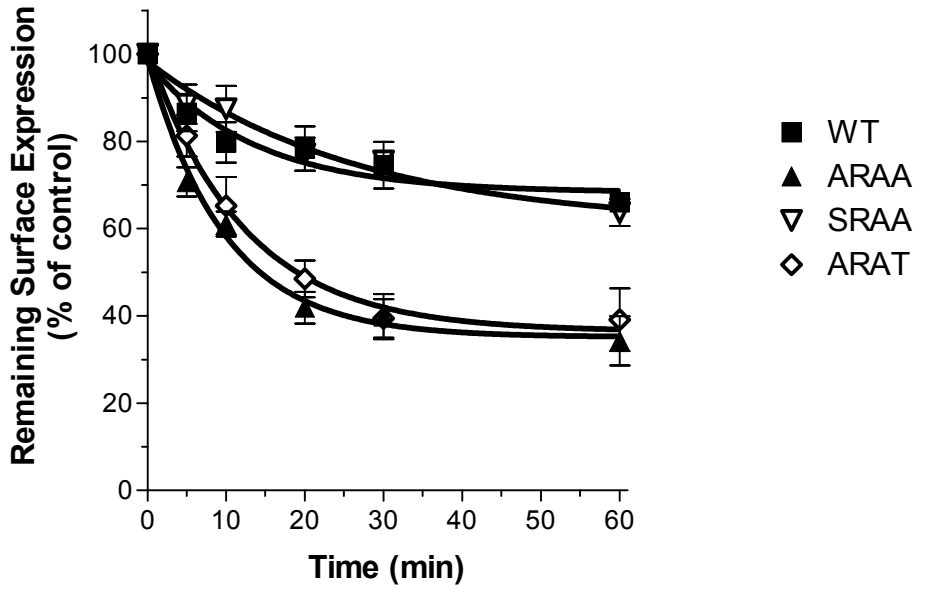
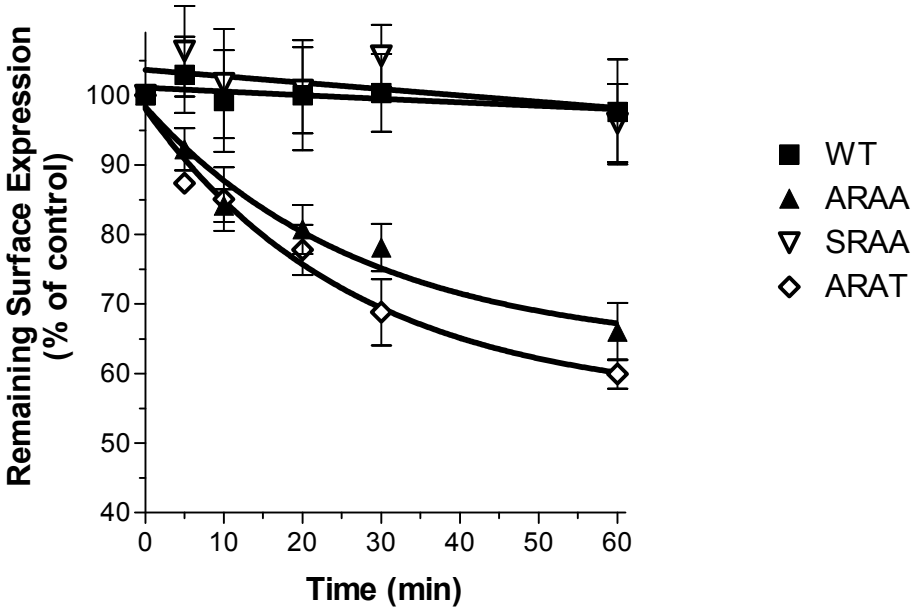


Fig. 3.3. Ser^{336} is the critical residue in regulation of agonist-promoted P2Y$_1$ receptor internalization. A. SRAT mutants (green) are shown. B. Serum-starved MDCK (II) cells stably expressing P2Y$_1$ wildtype, -SRAT, -ARAT, and -SRAA receptors were stimulated with 10μM 2MeSADP for the indicated times. The levels of receptors remaining at the cell surface were quantified as described in "Materials and ,methods" and reported as remaining cell surface expression compared to control (0 min) cells.

A.



B.

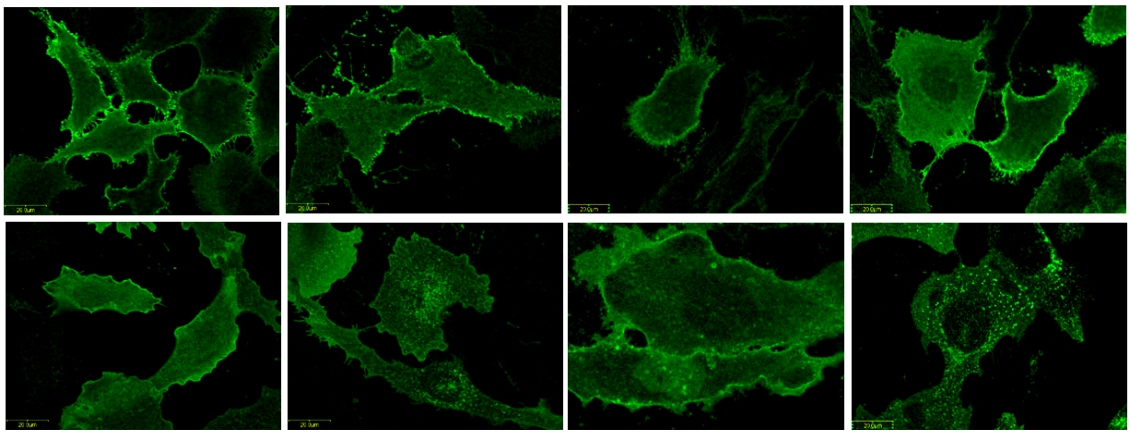
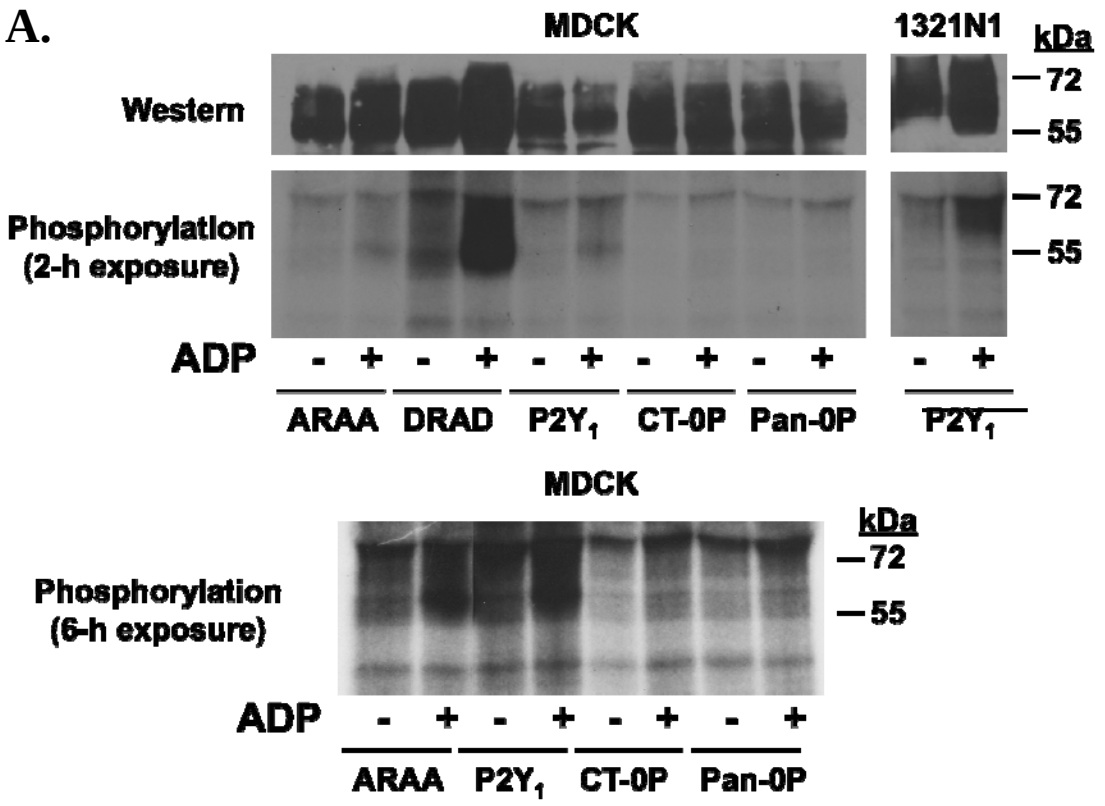


Fig. 3.4. Mutation of Ser336 to Ala allows agonist-induced $P2Y_1$ internalization in 1321N1 cells. A. Serum-deprived 1321N1 cells stably expressing $P2Y_1$ wildtype, -ARAA, -SRAA, and -ARAT mutant receptors were treated with 10μM 2MeSADP for the indicated times. The levels of receptors remaining at the cell surface were quantified as described in "Materials and methods" and reported as remaining cell surface expression compared to control (0 min) cells. B. Confocal images of 1321N1 cells showing location of the indicated $P2Y_1$ receptors in the basal state and following agonist stimulation. Surface HA-tagged receptors were prelabeled with anti-HA.11 monoclonal antibody at 4ºC for 60 min, followed by agonist-stimulation for 30 min at 37ºC. Cells were then fixed with 4% PFA, permeabilized with methanol, and incubated with goat anti-mouse secondary antibody.


A.
MDCK
1321N1
kDa
Western
72
55
Phosphorylation
(2-h exposure)
72
55
ADP
- + - + - + - + - + - +
ARAA
DRAD
P2Y1
CT-0P
Pan-0P
P2Y1
MDCK
kDa
Phosphorylation
(6-h exposure)
72
55
ADP
- + - + - + - +
ARAA
P2Y1
CT-0P
Pan-0P

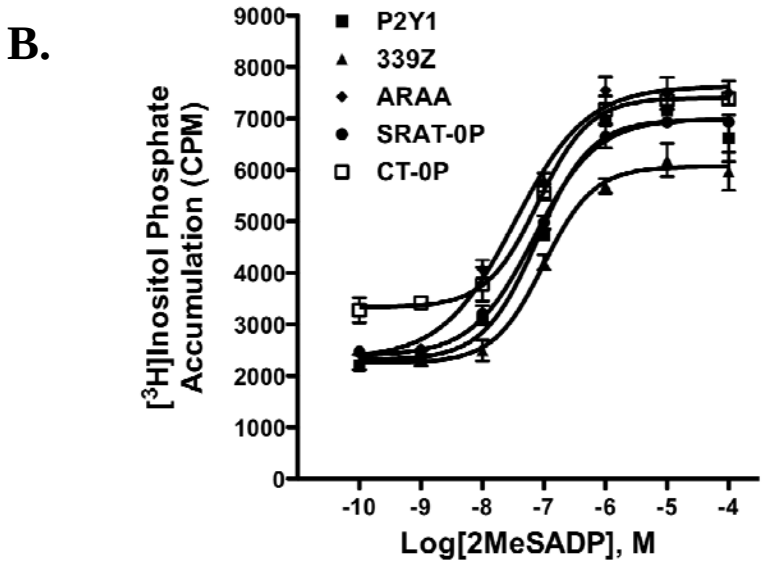

B.
P2Y1
339Z
ARAA
SRAT-0P
CT-0P
[3H]Inositol Phosphate
Accumulation (CPM)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-10
-9
-8
-7
-6
-5
-4
Log[2MeSADP], M

Fig. 3.5. [^{32}P]Phosphate incorporation into P2Y$_1$ wild type and SRAT mutant receptors. A. MDCK (II) and 1321N1 cells expressing P2Y$_1$ wild type and SRAT mutant receptors were incubated with 1 mCi [^{32}P]phosphate to label the ATP pool. After 3 hrs, cells were either not challenged or incubated with 30 μM ADP for 5 min, the HA-tagged receptors were immunoprecipitated, and the eluted samples were split 20/80 for Western blotting/autoradiography. Top panel, Western blot. Middle panel, autoradiograph from 2 hr exposure. Bottom panel, autoradiograph from 6 hr exposure (DRAD lane removed). B. 1321N1 cells expressing P2Y$_1$ wild type and SRAT mutant receptors were labeled with 2 μCi/ml of [^{3}H]-inositiol and stimulated with the indicated concentrations of 2MeSADP for 10 min at 37°C in the presence of 50 mM LiCl. [^{3}H]Inositol phosphate accumulation was quantified as described in Materials and methods. Data courtesy of Aidong Qi, Ph.D.

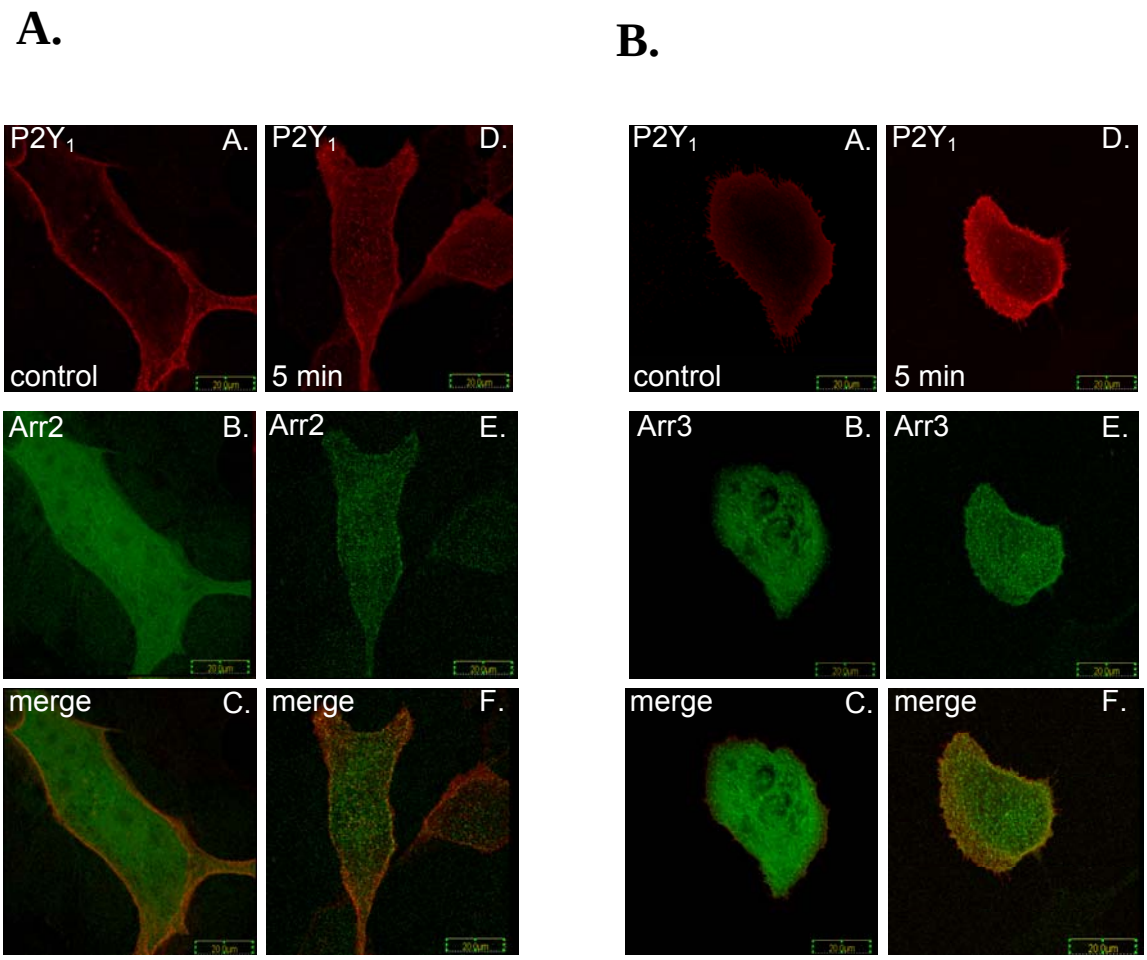


Fig. 3.6. Arrestins are mobilized following $P2Y_1$ receptor activation. MDCK (II) cells were co-nucleofected with HA- $P2Y_1$ receptor and either arrestin2-GFP (A) or arrestin3-GFP (B) and after 2 days in culture were either left untreated (control) or treated for 5 min with 10 μM 2MeSADP. Cells were processed and imaged by confocal microscopy as described in the legend to Fig. 4.

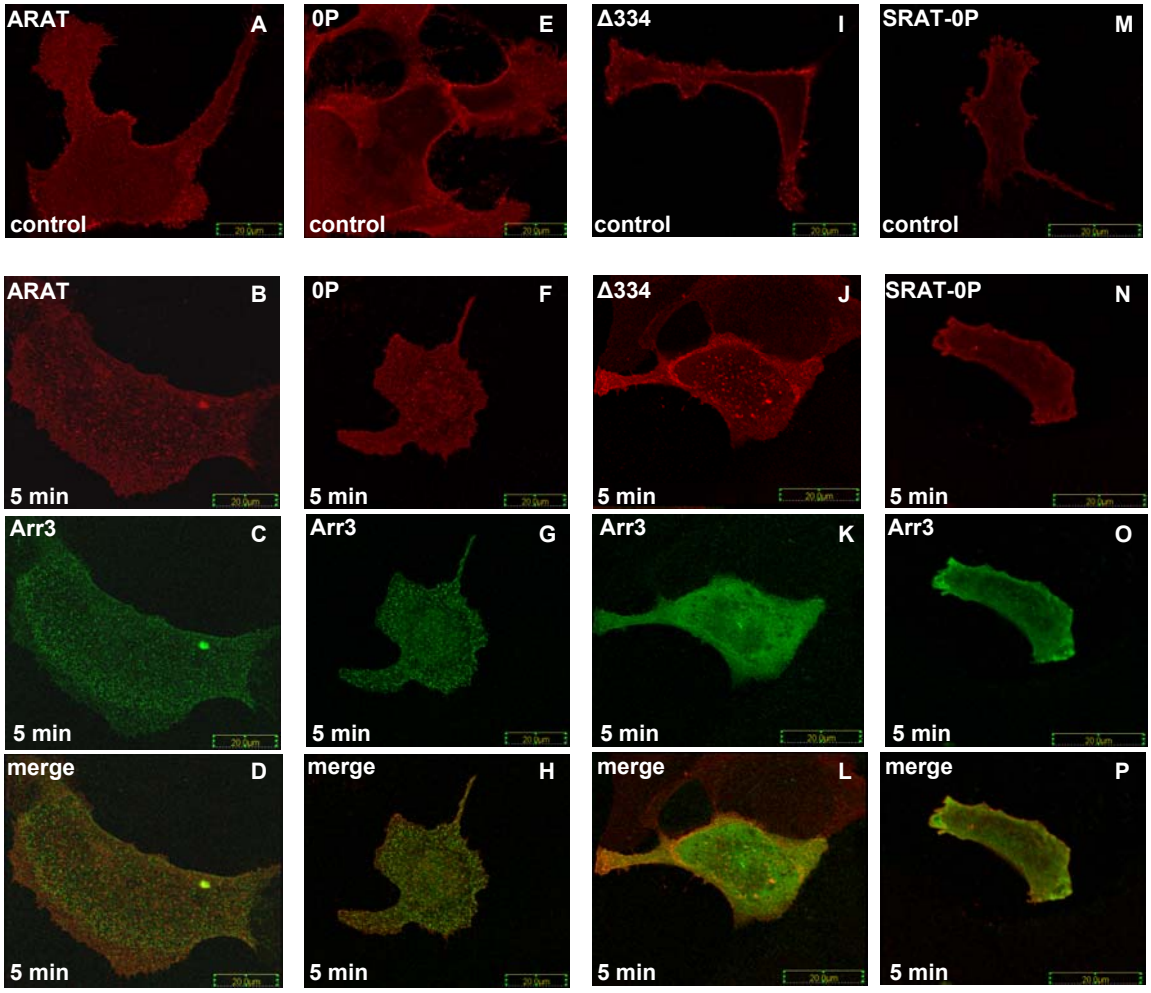
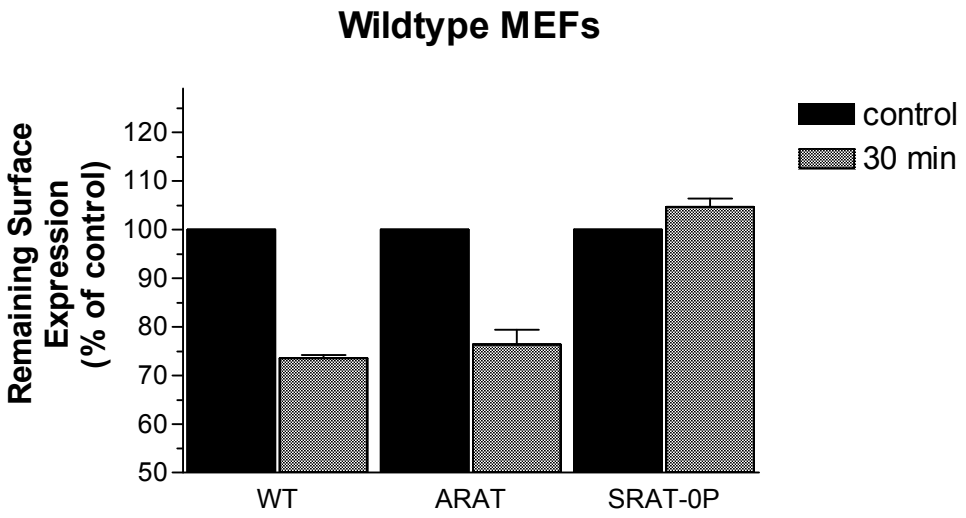


Fig. 3.7. Agonist-promoted mobilization of Arr3-GFP following activation of $P2Y_1$ receptors is SRAT- and phosphorylation-*independent*. MDCK (II) cells were co-nucleofected with GFP-tagged arrestin 3 and HA-tagged $P2Y_1$-ARAT (A), $P2Y_1$-0P (E), $P2Y_1$-334 (I), or $P2Y_1$-SRAT-0P (M) receptors as described in "Materials and methods." Cells were processed and imaged by confocal microscopy as described in the legend to Fig. 3.4.

A.



B.

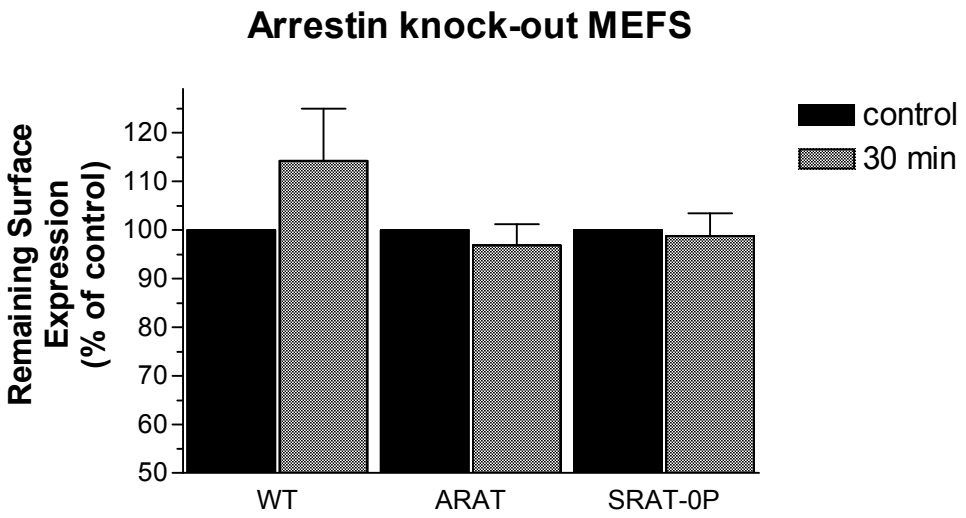


Fig. 3.8. $P2Y_1$ wildtype and ARAA receptors fail to internalize in arrestin knock-out MEFs. Serum-deprived wildtype (A) and arrestin knock-out (B) MEF cell lines stably expressing $P2Y_1$ wildtype, ARAT, SRAT-0P or 0P receptors were stimulated with 2MeSADP (10μM) for 30 min. Internalization was quantified as described in "Materials and methods."

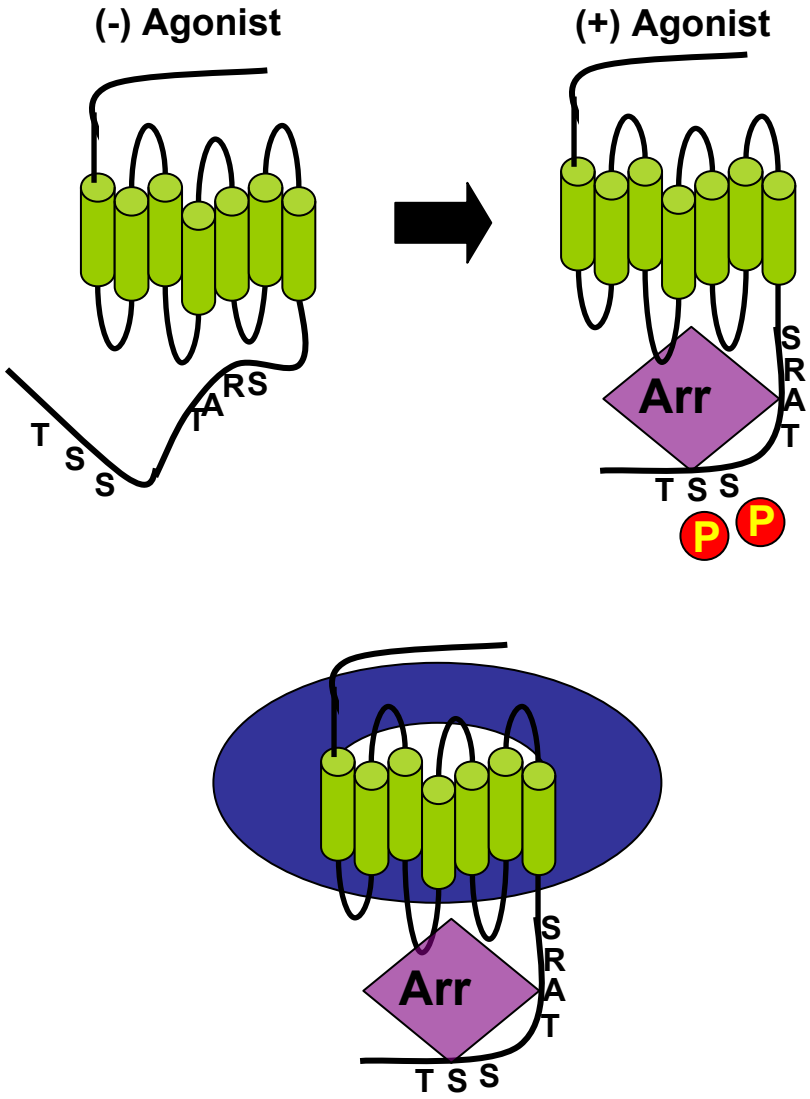


Fig. 3.9. Proposed model of $P2Y_1$ receptor internalization. In the absence of agonist, the C-terminal tail, through interactions of Ser336 with other parts of the receptor, structurally inhibits the binding of arrestin to the receptor. Upon ligand activation, Ser352 and Ser354 are phosphorylated, causing a conformational change and release of the tail from the body of the receptor. Activated arrestin is then able to bind to an intracellular loop of the receptor to facilitate internalization. See text for details

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Chapter 4

Conclusions and Future Directions

G protein-coupled receptors (GPCRs) are therapeutic targets for a broad range of human disease, including cardiovascular disease, gastrointestinal disease, and cancer. Thus, understanding how these receptors function in both normal and pathophyisological conditions, as well as how they are regulated, is critical in understanding the mechanisms of disease and how to treat them effectively.

The work presented in this dissertation involves two similar projects focused on signaling and regulation of GPCRs involved in cancer and/or cardiovascular physiology. The first project demonstrated the requirement for Protease-activated receptor-2 (PAR2) in coagulant protease-mediated signaling, migration, and invasion of breast carcinoma cells by utilizing siRNA techniques to specifically inhibit expression of either PAR1 or PAR2. The second project investigated the mechanisms by which another receptor important in the cardiovascular system, the $P2Y_1$ receptor, is internalized following agonist challenge. Receptor internalization is important in regulating GPCR signal termination, but the mechanisms involved in internalization of the $P2Y_1$ receptor had not been elucidated. These studies demonstrated that $P2Y_1$ internalization occurs through a novel mechanism by which a single amino acid, Ser336, within a conserved C-terminal motif, determines the rate and extent of agonist-promoted internalization of the $P2Y_1$ receptor.

4.1. Protease-activated receptors and coagulant proteases in cancer progression

In Chapter 2, we used siRNA to specifically deplete two cancer cell lines, MDA-MB-231 and BT549, of natively expressed PAR1 and PAR2, and showed that both PAR1 and PAR2 are critical mediators of cellular migration and invasion. We also showed that depleting cells of PAR2, but not PAR1, inhibits coagulant protease VIIa and Xa-induced migration of breast carcinoma cells.

In the tumor microenvironment, activation of the coagulation cascade is a hallmark of cancer and cell surface tissue factor (TF) is the major stimulant of the coagulation cascade. TF forms a catalytic enzyme complex with coagulant protease factors VIIa and Xa to trigger coagulation. Aberrant expression of PAR1 and PAR2, both of which can be activated by the TF-VIIa-Xa complex, has been correlated with the invasive and metastatic properties of tumor cells[1]. While we have been able to show that PAR2 is required for VIIa and Xa-induced migration of breast cancer cells *in vitro*, it is imperative to show that the same is true *in vivo* for potential therapeutic value.

Indeed, Versetteg et. al, 2008 addressed spontaneous mammary tumor development in $PAR1^{-/-}$ and $PAR2^{-/-}$ mice using the mouse model of polyoma middle T (PyMT) oncogene expression under the control of the mouse mammary tumor virus (MMTV) promoter to recapitulate important aspects of human breast cancer pathology. Their results demonstrate a critical role for PAR2, but not PAR1, in spontaneous breast cancer development and metastasis[2], supporting our findings. What was most interesting about this study, however, was that the progression from adenoma formation to adenocarcinoma was not prevented in $PAR2^{-/-}$ mice, but was only delayed, suggesting that PAR2 signaling does lead to tumor cell angiogenesis. It is uncertain whether the delayed angiogenic switch is due to deceased tumor growth, and while PAR2 signaling has been implicated in tumor cell proliferation and

apoptosis as well as migration [1,3-5], it is unlikey that PAR2 is a major contributor to tumor cell proliferation since tumor growth was normal once angiogenesis occurred. Thus, direct inhibition of PAR2 alone may not be sufficient for therapeutic treatment, but may be more effective when used in conjunction with other therapies by delaying the progression of tumor cell angiogenesis.

4.2. Regulation of $P2Y_1$ internalization

In Chapter 3, we showed that the Ser336 residue within an evolutionarily conserved motif, $S^{336}RAT^{339}$, regulates agonist-promoted internalization of the $P2Y_1$ receptor in MDCK (II) cells, a canine epithelial cell line. Our data are consistent with a model in which Ser336 interacts with unknown residues in the intracellular loops of the main body of the receptor in the basal state, and that this interaction puts the receptor in an arrestin-unreceptive state. Following agonist treatment, the C-terminal tail is phosphorylated at two Ser residues, Ser352 and Ser354, which weakens the interaction of the C-tail with the receptor and helps to convert the receptor into an arrestin-receptive state, allowing for interaction with arrestins to facilitate internalization. We also show that while arrestins are required for agonist-promoted internalization, an intact SRAT motif is not required for arrestin interaction. Interestingly, the $P2Y_1$ receptor is also unlike canonical G protein-coupled receptors in that it does not require agonist-promoted C-terminal tail phosphorylation in order to internalize provided that Ser336 has been mutated to Ala. Other GPCRs, such as δ-opioid and lutropin/choriogonadotropin receptors have been shown to activate arrestins independent of phosphorylation[6-7].

Based on studies conducted mainly with the β2-AR, the general dogma for GPCR internalization is that upon ligand activation, the C-termini of GPCRs are phosphorylated, which increases their affinity for arrestin. Once bound, arrestin promotes both desensitization and internalization. For several GPCRs, including the CXCR4 and luteinizing hormone/choriogonadotropin receptors, phosphorylation of the third intracellular loop is also a site of regulation for agonist-promoted internalization[8-9]. However, our studies with a mutant $P2Y_1$ receptor in which all Ser and Thr residues in intracellular domains ($P2Y_1$-Pan0P) were mutated to Ala demonstrated that the receptor internalized similarly as $P2Y_1$-0P, in which only the Ser/Thr residues in the C-terminal tail were mutated to Ala, in response to agonist challenge. These data thus ruled out the possibility of intracellular phosphorylation sites mediating arrestin binding to the $P2Y_1$ receptor.

Our laboratory previously demonstrated that $P2Y_1$ receptor internalization is differentially regulated based on which cells it is expressed. For example, the $P2Y_1$ receptor does not internalize following the addition of agonist in human embryonic kidney epithelial HEK293 cells, HeLa cervical carcinoma cells, or in 1321N1 astrocytoma cell lines. We do know that the lack of agonist-promoted $P2Y_1$ internalization in 1321N1 cells is not due to a defect in the endocytic machinery of the cell line, since $P2Y_2$ and $P2Y_4$ receptors internalize upon agonist internalization. We do know that in 1321N1 cells, the $P2Y_1$ receptor undergoes agonist-promoted phosphorylation (Fig. 3.5A), and recent preliminary data suggest that 1321N1 cells express both arrestin2 and arrestin3. Thus, the absence of agonist-promoted internalization of the $P2Y_1$ receptor in 1321N1 cells does not appear to be due to lack of phosphorylation or expression of arrestins. Thus, future investigation into the mechanisms involved in the different internalization properties observed in different cell lines may

provide valuable insight into the regulation of various processes regulated by $P2Y_1$ receptor signaling, including ion and water transport across epithelia, smooth muscle relaxation, and platelet activation. Future preliminary experiments may include screening the different cell lines for the expression (or lack thereof) of various endocytic adaptor proteins.

The work in Chapter 3 shows that arrestins are required for agonist-promoted $P2Y_1$ receptor internalization, but one aspect of the model remains untested: that the Ser336A mutation leads to an increase in both the rate and extent of arrestin binding upon addition of agonist. Experiments quantifying the receptor-arrestin interaction would allow a direct correlation between internalization and arrestin binding, and would help to test directly the model we have proposed. Bioluminescence Resonance Energy Transfer (BRET) is a technique that is capable of measuring both the magnitude and kinetics of arrestin binding; fusion of Renilla luciferase (Rluc) to the end of $P2Y_1$ wild type, -ARAT, and -0P receptors and co-expression of these receptors with arrestin3 fused to Yellow Fluorescent Protein (YFP) would, upon addition of luciferase substrate and receptor agonists, generate resonance transfer of light from luciferase to YFP provided that the two proteins interact. This approach will provide both kinetic data on how fast arrestins bind and to what level after addition of agonists, and these data would help to test our model that Ser-336 is a key gateway between receptor-unreceptive and –receptive states. Such experiments are currently in progress.

P2Y receptor signaling is important for platelet shape change and adhesion to mediate normal aggregation upon blood vessel injury. While clopidogrel, a $P2Y_{12}$ receptor-selective antagonist is commercially available and highly prescribed, no $P2Y_1$ receptor-selective therapies are currently available for therapeutic use. In fact, very little published information regarding $P2Y_1$ receptor regulation in platelets exists, despite its relevance as a target for

antiplatelet compounds. It has been recently shown that the $P2Y_1$ receptor rapidly desensitizes in platelets in response to the $P2Y_1$-selective agonist MRS 2365[10]. Other reports have suggested that the $P2Y_1$ receptor is rapidly phosphorylated and internalized in human platelets and astrocytoma cells[11], although the latter results conflict with our own data.

Studying the regulation of $P2Y_1$ receptor internalization in platelets is difficult due to the lack of a nucleus. Thus, we are unable express exogenous mutant receptors to study the mechanisms internalization in platelets directly. The MDCK cell line was chosen to study internalization based on two criteria: 1) the $P2Y_1$ receptor is natively expressed and 2) intact radioligand binding assays show that exogenously expressed receptors internalize at rates comparable to the native receptor. Further studies directly assessing the regulation of the $P2Y_1$ receptor in platelets is necessary in order to achieve a $P2Y_1$-specific antiplatelet compound.

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