The role of the $P2Y_2$ nucleotide receptor in inflammation: the mechanisms of $P2Y_2$ receptor-mediated activation of G proteins

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by

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The role of the $P2Y_2$ nucleotide receptor in inflammation: the mechanisms of $P2Y_2$ receptor-mediated activation of G proteins

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular calcium concentration
2-MeS-ATP	2-methyl-thio-adenosine 5'-triphosphate
Ab	antibody
ADP	adenosine 5'-diphosphate
AJ	adherens junction
ATCC	American Type Cell Collection
ATP	adenosine 5'-triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
DN	dominant-negative
EBM	endothelium basal medium
ECM	extracellular matrix
EDRF	endothelial-dependent relaxing factor
EGF	epithelial growth factor
eGFP	enhanced green fluorescence protein
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GEF	guanine nucleotide exchange factor
GPCR	G-protein-coupled receptor
GTPγS	guanosine 5'-*O*-(3-thiotriphosphate)
HA	hemagglutinin
HCAEC	human coronary artery endothelial cells
IB	immunoblotting

ICAM	intercellular cell adhesion molecule
IgG	immunoglobulin G
IP	immunoprecipitation
IP_3	inositol 1,4,5-triphosphate
JAM	junction adhesion protein
KO	knockout
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
MLC-2	myosin light chain-2
NFκB	nuclear factor κB
NDP	nucleoside diphosphate
NMP	nucleoside monophosphate
NO	nitric oxide
NOS	nitric oxide synthase
NTP	nucleoside triphosphate
PAK	p21-activated kinase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
pERK	phosphorylated extracellular signal-regulated kinase
PGI2	prostaglandin I_2
PH domain	pleckstrin homology domain
PI3K	phosphatidylinositol 3-kinase

PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin
PTy	phosphotyrosine
RBD	Rhotekin binding domain
RIPA	radio-immunoprecipitation assay
RGD	arginine-glycine-aspartic acid
RGE	arginine-glycine-glutamic acid
S.E.M.	standard error of mean
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
siRNA	short interfering RNA
SMC	smooth muscle cells
TBST	tris-buffered saline Tween-20
TJ	tight junction
TNF	tumor necrosis factor
UDP	uridine 5’-diphosphate
UTP	uridine 5’-triphosphate
VCAM	vascular cell adhesion molecule
VE-cad	vascular endothelial cadherin

VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2
WT	wild type

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Dr. Gary A. Weisman and Dr. Laurie Erb, Dissertation Supervisors

ABSTRACT

Adenine and uridine nucleotides (ATP, ADP, UTP and UDP) have been well-recognized for their role in modulating vascular inflammation and thrombosis. These nucleotides are released in the vascular system by red blood cells exposed to low blood oxygen as well as by aggregating platelets, migrating leukocytes and damaged vascular cells. Increasing evidence indicates that the extracellular ATP/UTP receptor, *i.e.*, the $P2Y_2$ receptor ($P2Y_2R$), mediates mitogenic and pro-inflammatory responses in the vasculature, including increased smooth muscle cell proliferation and migration, and the endothelium-dependent infiltration of monocytes and their transmigration into sites of infection, injury, or stress, responses associated with the *in vivo* up-regulation of the $P2Y_2$ receptor in vascular cells (*i.e.*, smooth muscle and endothelial cells).

This dissertation concerns the mechanisms whereby the $P2Y_2R$ mediates chemotaxis as well as the modulation of endothelial intercellular junctions. Since G proteins, such as heterotrimeric G_{12} and $G_{i/o}$ and the monomeric Rho family of GTPases, are responsible for regulating cellular actin dynamics and cytoskeletal changes that are central to chemotaxis and the stability of intercellular junctions, this dissertation focuses on the mechanisms underlying the $P2Y_2R$-mediated activation of G proteins.

The $P2Y_2R$ is a G protein-coupled receptor with an extracellular integrin binding domain (Arg-Gly-Asp or RGD) that enables this receptor to directly interact with $\alpha_v\beta_3/\beta_5$

integrins, cell adhesion molecules known to regulate chemotaxis. Mutation of the RGD sequence to Arg-Gly-Glu (RGE) prevented association between the $P2Y_2R$ and α_v integrins but did not prevent activation of G_q or G_q–mediated calcium mobilization by the $P2Y_2R$ agonist UTP. On the contrary, UTP-induced activation of G_{12} and G_{12}-mediated events, including RhoA activation, cofilin and myosin light chain-2 phosphorylation, stress fiber formation and chemotaxis were all inhibited by mutation of the RGD domain to RGE in the human $P2Y_2R$ expressed in human 1312N1 astrocytoma cells that are devoid of endogenous P2 nucleotide receptors. Similarly, UTP-induced activation of G_o and G_o-mediated events, including activation of Rac and vav2, a guanine nucleotide exchange factor for Rac, as well as chemotaxis towards UTP, were inhibited by the RGD to RGE mutation in the $P2Y_2R$. These responses were also inhibited by anti-$\alpha_v\beta_5$ integrin antibodies or α_v integrin-specific antisense oligonucleotides, further confirming that α_v integrin activity and expression are required for the $P2Y_2R$ to activate G_o and G_{12}, and subsequently, chemotaxis. Involvement of G_o and G_{12} in these UTP-triggered events was confirmed using the $G_{i/o}$ inhibitor, pertussis toxin or dominant negative $G\alpha_{12}$. Thus, the interaction between the $P2Y_2R$ and α_v integrin is required for the $P2Y_2R$ to activate heterotrimeric G_o and G_{12} proteins that control chemotaxis.

In migrating leukocytes, $P2Y_2Rs$ remain evenly distributed on the cell surface and are thought to provide directional sensing and amplification of chemotactic signals by sensing nucleotides released at the leading edge of the migrating cell. In chapter IV of this study, we analyzed the distribution of $P2Y_2Rs$ in quiescent endothelial cells. Activation of $P2Y_2Rs$ with the agonist UTP caused a rapid and transient clustering of GFP-tagged $P2Y_2Rs$ at the intercellular junctions of human coronary artery endothelial

cells (HCAEC). Co-immunoprecipitation experiments indicate that UTP causes a rapid and transient association of the $P2Y_2R$ and the vascular endothelial growth factor receptor-2 (VEGFR-2) with VE-cadherin, a transmembrane component of endothelial adherens junctions. Given that UTP infusion around stressed vascular tissue has been found to increase monocyte infiltration through the endothelium in collared rabbit carotid arteries, this dissertation addresses the mechanisms underlying monocyte infiltration by investigating $P2Y_2R$ interaction with endothelial intercellular junction proteins. The Rho family of GTPases has been shown to regulate endothelial permeability and leukocyte transmigration by controlling cytoplasmic actin dynamics. However, it is not well understood how a G protein-coupled receptor (GPCR) and junctional proteins coordinately regulate Rho GTPase activity. This dissertation reports that siRNA-mediated down-regulation of VE-cadherin or p120 catenin, a protein known to associate with VE-cadherin in adherens junctions, inhibited Rac activation induced by UTP. In addition, UTP promoted a prolonged interaction between p120 catenin and vav2, a response that correlates with the tyrosine phosphorylation of VE-cadherin and p120 catenin. UTP-induced responses, including the association of the $P2Y_2R$ with VE-cadherin, the association of p120 catenin with vav2, tyrosine phosphorylation of VE-cadherin and p120 catenin, and the activation of Rac, were inhibited by the Src inhibitor PP2 and the VEGFR-2 inhibitor SU1498, indicating that Src and VEGFR-2 activity are required for controlling $P2Y_2R$-initiated signaling to adherens junctions, consistent with the presence of SH3-binding motifs in the C-terminal domain of the $P2Y_2R$ known to regulate Src-dependent VEGFR-2 transactivation by the $P2Y_2R$.

Taken together, these data suggest that the $P2Y_2R$ requires direct interactions with α_v

integrin, growth factor receptors and VE-cadherin to activate G proteins involved in chemotaxis and modulate the activities of intercellular junction proteins. Although in different types of cells, the expression or distribution of $P2Y_2R$-associated proteins may vary, this dissertation provides novel findings indicating that to be fully functional, the $P2Y_2Rs$ must couple to a large complex containing multiple receptors and signaling proteins, each of which could be a potential target for the prevention and treatment of inflammatory diseases.

CHAPTER I

Introduction

A. Inflammation

Inflammation, a response triggered by trauma, toxin exposure, infection, ischemia and autoimmune injury, was recognized centuries ago. Today, chronic inflammation is considered to be a major factor in the pathophysiology of many diseases including rheumatoid arthritis, asthma, arteriosclerosis, diabetes and neurodegenerative diseases (Winsauer and de Martin, 2007).

Over the last decade, a view has emerged that an inflammatory reaction does not occur in response to foreign molecules but rather to self molecules released from damaged tissue (Heath and Carbone, 2003; Matzinger, 1994). The ideal characteristics of a molecule that causes inflammation is predicted to have the following features:

1. It should be easily and quickly generated, such as by release from a large intracellular pool into the extracellular environment.
2. It should be present extracellularly at very low concentrations under normal conditions to allow a high response upon release under pro-inflammatory conditions.
3. It should be easily transported or mobile in the pericellular environment.
4. It should be recognized by specific receptors on affected tissues.
5. It should be easily destroyed or masked to ensure a quick dampening of an inflammatory response.

Extracellular nucleotides fulfill all these features, thus making them good candidates as ‘triggering’ molecules in inflammation. There is increasing evidence supporting the importance of extracellular nucleotides and their receptors in the development of many

inflammatory diseases.

B. Endothelial-dependent mechanisms of leukocyte recruitment in inflammation

The endothelium is a monolayer of cells that covers the inner surface of the entire vascular system, providing a barrier that separates circulating blood from the tissue. The endothelium senses different chemical or physical stimuli and responds by producing a large variety of molecules that regulate blood fluidity. The endothelium regulates homeostasis by producing anti-coagulant and pro-coagulant factors, synthesizes and releases both vasodilating factors (such as nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2)) and vasoconstricting factors (such as endothelium-derived contracting factors (EDCFs)), thus modulating the relaxation and contraction of vascular smooth muscle. The endothelium also participates in leukocyte recruitment and activation, and smooth muscle cell migration and proliferation during the inflammatory process (Shimokawa, 1999; Esper et al., 2006). Loss of the capacity to maintain homeostasis (also called 'endothelial dysfunction') occurs under conditions of vascular injury or stress, such as hyperlipidemia, hypertension, smoking, diabetes, ischemia, and chronic bacterial infections. Endothelial dysfunction is accompanied by changes in the levels of cytokines, cell adhesion molecules, and other pro-inflammatory mediators, making local conditions favorable for the development of vascular inflammation (Winsauer and de Martin, 2007).

During an inflammatory event, leukocytes traffic from the blood into the underlying tissues to the sites of infection or injury. The process of leukocyte emigration involves several major steps: the recruitment of leukocytes from bone marrow into the circulation,

leukocyte rolling and attachment on the endothelium, leukocyte migration to the endothelial cell borders, transendothelial migration of leukocytes (diapedesis) in an ameboid-like fashion, and chemotaxis of leukocytes towards chemoattractants released by injured tissue (Ley, 1996).

Leukocyte recruitment requires specific interactions between endothelial cells and leukocytes to guide the migration of leukocytes from the blood to the sites of injury or infection. Leukocyte transendothelial migration is initiated by leukocyte rolling on the luminal surface of the endothelium, a process mediated by the low-affinity adhesion receptors termed selectins that weakly bind to glycoprotein ligands (McEver, 2002). Selectin-binding further activates β_2 integrins on leukocytes, as 'inside-out' signals, leading to firm leukocyte adhesion to the endothelium (Simon et al., 2000). In addition, the rolling leukocyte can be stimulated by chemokines or other chemotactic molecules, which activate firm adhesion on the endothelium. For example, monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-8 trigger the adhesion of monocytes through activation of the chemokine receptor, CCR2 (Gerszten et al., 1999). Leukocyte-endothelial adhesion is mediated by leukocyte surface integrins (β_1 and β_2 integrins) and their counter-receptors on endothelial cells, such as vascular endothelial cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1, -2 (ICAM-1, -2) (Imhof and Aurrand-Lions, 2004).

VCAM-1 preferentially recognizes $\alpha_4\beta_1$ integrin, and it also binds to $\alpha_4\beta_7$ integrin. VCAM-1 is considered to be the key molecule that mediates monocyte recruitment in early lesions and in vasculature predisposed to lesions (Cybulsky et al., 2001). Furthermore, VCAM-1 clustering generates endothelial cell 'outside-in' signals, such as

NADPH oxidase activation and endothelial cell actin reorganization, events thought to be important for VCAM-1-dependent lymphocyte migration, but not adhesion (Lorenzon et al., 1998; Matheny et al., 2000).

The final step in leukocyte extravasation is migration through the endothelial cell borders, a process called diapedesis. Although there is recent evidence suggesting that leukocytes can migrate through the body of an endothelial cell (transcellular pathway) (Carman and Springer, 2004), it is believed that transendothelial migration is largely due to leukocyte migration through interendothelial junctions (paracellular pathway). Diapedesis involves a rapid reorganization of the leukocyte cytoskeleton, creating pseudopod-like extensions into the endothelial intercellular junction that enable leukocyte passage through the junction by the use of mechanical force generated by the pseudopod (Luscinskas et al., 2002). Meanwhile, the endothelium communicates with leukocytes to facilitate diapedesis while maintaining the endothelial integrity (Huang et al., 1988). For example, neutrophil transmigration causes a rise in intracellular Ca^{2+} levels that is localized in endothelial cells close to the migrating neutrophil and neutrophil transmigration is inhibited by the treatment of cells with intracellular Ca^{2+} chelators (Huang et al., 1993; Su et al., 2000).

Endothelial junctions are composed of three morphologically distinct structures: tight junctions, adherens junctions and gap junctions. Molecular mechanisms regulating leukocyte transendothelial migration involve molecules present in tight junctions (*i.e.*, junctional adhesion molecules or JAMs, occludin and claudin) and adherens junctions (*i.e.*, VE-cadherin and catenins). Gap junctions are important for intercellular communication but are not thought to play a role in leukocyte transendothelial migration

(Luscinskas et al., 2002). In addition, platelet-endothelial cell adhesion molecule (PECAM)-1, a protein of the immunoglobulin (Ig) gene family, is localized to endothelial cell lateral junctions and has been shown to play a role in leukocyte transendothelial migration (Muller et al., 1993). PECAM-1 is expressed in both leukocytes and endothelial cells and is capable of both homophilic interaction and heterophilic interaction with $\alpha_v\beta_3$ (Piali et al., 1995). Studies show that extracellular domains 1 and 2 of leukocyte PECAM-1 are crucial for leukocyte transendothelial migration, whereas extracellular domain 6 is specifically important for interstitial migration (after transendothelial migration) (Liao et al., 1995).

JAM-1 is present on the surface of endothelial cells as well as leukocytes. *In vivo*, the up-regulation of JAM-1 expression is found in unstable atherosclerotic plaques and in atherosclerotic endothelium. *In vitro*, rather than up-regulation, JAM-1 redistributes from endothelial intercellular junctions to the apical surface upon co-stimulation with the cytokines tumor-necrosis factor-α (TNFα) and interferon-γ (IFNγ) (Weber et al., 2007).

Adherens junctions attract significant interest among research groups who study inflammation since they play an important role in leukocyte transendothelial migration. The adherens junction component, VE-cadherin, is a single transmembrane protein expressed exclusively in endothelial cells, with an extracellular domain responsible for Ca^{2+}-dependent homophilic interaction and a cytoplasmic tail that connects to the actin cytoskeleton via association with catenins. It has been found that treatment with antibodies to VE-cadherin leads to increased endothelial permeability *in vitro* as well as leukocyte transmigration *in vivo* (Gotsch et al., 1997; Hordijk et al., 1999). In fact, monocyte adhesion/transendothelial migration is associated with a transient decrease in

VE-cadherin localization at cell contact sites under flow conditions (Allport et al., 2000). Real time imaging of endothelial cells expressing GFP-tagged VE-cadherin during leukocyte transendothelial migration provides an insight into the kinetics of this dynamic process. During real time imaging experiments under flow conditions, monocytes crawl to intercellular junctions of endothelial cells and then migrate through gaps (4-6 μm) in VE-cadherin junctional distribution, and within 5 minutes the VE-cadherin redistributes and reseals the gaps. Interestingly, a certain percentage of leukocytes transmigrate through pre-existing gaps instead of *de novo* gaps formed as leukocytes adhere, consistent with previous studies (using postfixation staining techniques) indicating that leukocytes can migrate through pre-existing gaps at the border of three or more endothelial cells (Burns et al., 2000; Shaw et al., 2001). In addition, it has been found that rather than paracellular transendothelial migration, fMLP-induced neutrophil transendothelial migration occurs by a transcellular pathway (Feng et al., 1998). In both cases, transendothelial migration requires an interaction between leukocytes and endothelial cells, cytoskeletal rearrangements in both cell types and the transduction of intracellular signals that regulate this dynamic process.

After diapedesis, the leukocytes are directed by immobilized chemokines or by chemoattractant gradients to migrate across the endothelium and into the inflamed tissue. This directional migration is called chemotaxis, which will be discussed later in this chapter.

C. Role of extracellular nucleotides in inflammation

1. Extracellular nucleotides

Extracellular nucleotides are recognized as endogenous signaling molecules that function as hormones or neurotransmitters in a variety of mammalian cell types. Over the past few years, extracellular nucleotides have been characterized as an important factor modulating vascular inflammation and thrombosis (Di Virgilio and Solini, 2002). ATP, ADP, UTP and UDP are the major nucleotides that exert physiological effects through the activation of cell surface P2 nucleotide receptors that are known to regulate cardiac function, muscle relaxation and contraction, platelet aggregation, neurotransmission, inflammation, bone metabolism and cell growth (Dubyak and el-Moatassim, 1993; Hoebertz et al., 2003). Whereas intracellular concentrations of ATP are maintained at 3-10 mM (Bours et al., 2006), and reach 1 M in nerve vesicles (Burnstock, 2006a), the extracellular concentration of ATP is much lower. In human plasma, the physiological concentration of ATP is usually submicromolar (Ryan et al., 1991; Bours et al., 2006). Extracellular ATP can be released from secretory neuronal cells (Burnstock et al., 1978; Lew and White, 1987), aggregating platelets (Born and Kratzer, 1984), mast cells (Cockcroft and Gomperts, 1980), endothelial and smooth muscle cells (Pearson and Gordon, 1979), neutrophils (Chen et al., 2006), and from many cell types in response to mechanical or chemical stress including distention, osmotic shock, hypoxia, agonist stimulation, fluid shear stress and acute inflammation. (Bodin and Burnstock, 2001; Burnstock, 2006b). Several mechanisms of ATP release have been proposed but remain unclear. Besides the accepted theory of vesicular release from nerves and platelets, there is evidence of 'cytolysis' that follows cell membrane damage or cell death, and activation of ATP-binding cassette (ABC) transporters in non-neuronal cells (Bodin and Burnstock, 2001). Similar to ATP, UTP and other nucleotides can also be released from cells,

particularly under conditions of stress. However, the concentration of stored UTP is much lower than ATP. For example, UTP is stored in platelet granules at about 10% of ATP levels (Anderson and Parkinson, 1997). Extracellular nucleotides are rapidly metabolized by a ubiquitous family of membrane-bound enzymes (ecto-enzymes). A variety of ecto-ATPases and ecto-nucleotidases work together to hydrolyze NTP into NDP or N, NDP into NMP, and NMP into N (Anderson and Parkinson, 1997). Some of these metabolized products can also induce cellular responses, similar to ATP and UTP (Burnstock, 2002; Burnstock, 2007).

2. P2Y receptors and their physiological functions

Two types of purinoceptors, P1 and P2 receptors, mediate the effects of extracellular nucleotides and nucleosides. P1 receptors are mainly activated by adenosine or adenosine-like compounds. So far, four P1 receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) have been cloned (Burnstock, 2007). The main activating ligands for P2 receptors *in vivo* are extracellular ATP, ADP, UTP and UDP (Burnstock, 2007). P2 receptors are subdivided into two distinct categories, the metabotropic G protein-coupled (P2Y) receptors and the ionotropic ligand-gated ion channel (P2X) receptors. To date, 7 P2X and 8 P2Y receptor subtypes have been identified by molecular cloning and pharmacological characterization, leading to a greater understanding of the diversity of signal transduction pathways coupled to P2X and P2Y receptors in different systems. P2X receptors are ATP-gated ion channels that mediate rapid cell membrane depolarization and increase transmembrane transport of cations (*e.g.*, Na^+, K^+, and Ca^{2+}). P2Y receptors for purine and pyrimidine nucleotides belong to the superfamily of G protein-coupled receptors

(GPCRs) comprised of seven transmembrane domains. Currently, eight mammalian P2Y receptor subtypes have been cloned and characterized in heterologous expression systems: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors (see Table 1). Human P2Y receptors consist of 328 ($P2Y_6$) to 377 ($P2Y_2$) amino acids with glycosylation sites in the extracellular N-terminal domains. Although all P2Y receptors share a seven transmembrane domain tertiary structure, the amino acid sequences, pharmacological profiles and signal transduction pathways of these GCPRs vary significantly (see Table 1). Using rhodopsin as a template, models of the $P2Y_1$ and $P2Y_2$ receptor ligand binding sites have been produced that indicate the presence of several positively charged amino acids in highly conserved transmembrane regions 3, 6 and 7 that could act as counterions for the binding of negatively charged nucleotide ligands (Van Rhee et al., 1995). Consistent with this conclusion, site-directed mutagenesis of the $P2Y_2$ receptor to replace positively charged amino acid residues in transmembrane regions 6 and 7 with neutral amino acids produced receptors with dramatically altered agonist potencies as compared to the wild type $P2Y_2$ receptor (Erb et al., 1995). These positively charged amino acids are found in domains 6 and 7 of all P2Y receptors cloned to date.

Although the functions of some P2 receptor subtypes may be overlapping and therefore redundant under physiological conditions, as suggested by the phenotypes of P2Y receptor knockout mice (Cressman et al., 1999; Ghanem et al., 2005), there is increasing evidence that specific P2Y receptors in vascular endothelial smooth muscle cells and leukocytes are important mediators of thrombosis and vascular inflammation. For example, a $P2Y_1$ receptor-null mouse model was used to demonstrate that the lack of

$P2Y_1$ receptor expression leads to prolonged bleeding times and resistance to ADP-induced thromboembolism (Fabre et al., 1999; Leon et al., 1999), consistent with the role of this receptor in the regulation of platelet aggregation *in vitro* (Kunapuli et al., 2003). Other studies indicate that a frame-shift mutation in the $P2Y_{12}$ receptor gene may be responsible for a mild bleeding disorder (Hollopeter et al., 2001). Subsequently, the role of the $P2Y_{12}$ receptor in arterial thrombogenesis was confirmed in $P2Y_{12}$ receptor knockout mice that exhibit impaired platelet adhesion to von Willebrand factor (vWF) and decreased platelet activity, as indicated by lower fibrinogen-binding, delayed thrombus growth and unstable thrombi in injured arteries (Andre et al., 2003). ADP-induced activation of ERK1 is inhibited by the $P2Y_{12}$ and $P2Y_{13}$ receptor antagonist AR-C69931MX in human monocyte-derived dendritic cells, resulting in the inhibition of inflammatory cytokine production (Marteau et al., 2004).

Recently, $P2Y_6$ receptors have been found to act as mediators of phagocytosis in microglia (*i.e.*, brain immune cells), by sensing UDP signals (Koizumi et al., 2007). In addition, ATP released at sites of inflammation has been suggested to induce maturation of human monocyte-derived dendritic cells (MoDC) via $P2Y_{11}$ receptor-mediated activation of adenylyl cyclase and protein kinase A (Wilkin et al., 2001). $P2Y_{11}$ receptor activation also inhibits MoDC migration (Schnurr et al., 2003). Correlating with MoDC maturation, $P2Y_{11}$ receptor expression is down-regulated in mature MoDC, whereupon ATP becomes a less effective inhibitor of cell migration (Schnurr et al., 2003).

$P2Y_{14}$ receptors for UDP-glucose mediate bone marrow hematopoietic stem cell chemotaxis induced by UDP-sugars derived from the bone marrow microenvironment, suggesting a role for $P2Y_{14}$ receptor activity in responses to injury in which stem cells

provide the basic level of repair (Lee et al., 2003). UDP-glucose has been shown to increase $[Ca^{2+}]_i$ in immature human MoDC but not in mature dendritic cells, suggesting a role for this receptor in dendritic cell maturation (Skelton et al., 2003). The $P2Y_{14}$ receptor is also expressed in the rat brain where its mRNA can be up-regulated by immunologic challenge with lipopolysaccharide, suggesting an important role for $P2Y_{14}$ receptors in UDP-glucose-induced responses relating to neuroimmune function (Moore et al., 2003).

3. $P2Y_2$ receptor

$P2Y_2$ receptors have been cloned from human, rat, mouse and porcine cells or tissues (Lustig et al., 1993; Parr et al., 1994; Chen et al., 1996; Shen et al., 2004). ATP and UTP are equipotent agonists of the $P2Y_2$ receptor, whereas ADP and UDP are less effective (Lazarowski et al., 1995). $P2Y_2$ receptor mRNA is expressed in human skeletal muscle, heart, brain, spleen, lymphocytes, macrophages, bone marrow and lung, with lower levels expressed in liver, stomach and pancreas (Moore et al., 2001).

$P2Y_2$ receptors stimulate multiple and divergent intracellular signaling pathways that mediate a variety of physiological and pathological responses (Fig. 1). $P2Y_2$ receptors couple to both $G_{i/o}$ and G_q proteins to mediate the activation of phospholipase Cβ, leading to the production of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), second messengers for calcium release from intracellular storage sites and protein kinase C (PKC) activation, respectively (Weisman et al., 1998). Activation of PKC, in turn, promotes the synthesis and/or release of arachidonic acid (AA), prostaglandins, and nitric oxide (NO) (Xu et al., 2002, 2003a). In primary murine astrocytes, $P2Y_2$ receptors mediate activation

of PKC and ERK1/2, via both calcium-dependent and calcium-independent pathways, leading to activation of cytosolic phospholipases A_2 and the release of AA (Xu et al., 2002), the precursor of prostaglandins, eicosanoids, and leukotrienes (Balsinde et al., 2002). Studies have indicated that $P2Y_2$ receptor-mediated MAPK activation in C12 cells is dependent upon transactivation of the EGFR via a Src/adhesion focal tyrosine kinase (RAFTK)-dependent pathway (Soltoff et al., 1998). In addition, $P2Y_2$ receptor activation induces p38- and ERK1/2-dependent phosphorylation of the cAMP responsive element binding (CREB) protein and up-regulation of anti-apoptotic (*i.e.*, Bcl-2 and Bcl-xL) and other neuroprotective genes in human astrocytoma cells (Chorna et al., 2004). Other studies indicate that an integrin-binding domain (Arg-Gly-Asp) in the first extracellular loop of the $P2Y_2$ receptor mediates its association with $\alpha_v\beta_3/\beta_5$ integrins, enabling the coupling of the $P2Y_2$ receptor to G_o but not G_q proteins (Erb et al., 2001).

Functional analysis revealed that activation of $P2Y_2$ receptors in isolated UTP- or ATP-perfused rat hearts induced pronounced vasodilation of blood vessels (Godecke et al., 1996), consistent with earlier studies indicating that $P2Y_2$ receptor activation induces relaxation of smooth muscle through the endothelium-dependent release of NO and prostacyclin (Lustig et al., 1992; Pearson et al., 1992a, 1992b). In smooth muscle cells, $P2Y_2$ receptor expression is up-regulated by inflammatory agents, such as transforming growth factor (TGF)-β, basic fibroblast growth factor (bFGF), and even ATP (Hou et al., 1999). Up-regulation of $P2Y_2$ receptors occurs in response to balloon angioplasty (Seye et al., 1997) and causes intimal hyperplasia in collared rabbit carotid arteries due to the stimulation of smooth muscle cell proliferation and migration (Seye et al., 2002), suggesting an important role for $P2Y_2$ receptors in the initiation of atherosclerotic lesion

formation. Up-regulation of the $P2Y_2$ receptor also was observed by short-term culture (3 h to 6 days) of normal rat submandibular gland cells (Turner et al., 1997), and in activated mouse thymocytes (Koshiba et al., 1997). $P2Y_2$ receptor activation in astrocytes increases the synthesis of arachidonic acid and prostaglandin E2 as well as activation of type 2 cyclooxygenase (COX-2), which mediate inflammation and reactive astrogliosis associated with neurodegenerative diseases, including Alzheimer's disease (Brambilla et al., 1999; Brambilla and Abbracchio, 2001). In endothelial cells, $P2Y_2$ receptor activation causes up-regulation of vascular cell adhesion molecule-1 (VCAM-1) that mediates the adherence of monocytes to vascular endothelium (Seye et al., 2003), leading to their penetration into the blood vessel wall to promote arterial inflammation associated with cardiovascular disease (Seye et al., 2002). Recent studies have revealed that a Src homology-3 (SH3) binding domain in the C-terminal tail of the $P2Y_2$ receptor promotes the nucleotide-induced association of Src with the $P2Y_2$ receptor, leading to the transactivation of growth factor receptors such as the EGF and VEGF receptors (Liu et al., 2004). Deletion of the SH3 binding domain inhibits $P2Y_2$ receptor-mediated transactivation of the EGF and VEGF receptors and nucleotide-induced up-regulation of VCAM-1 (Liu et al., 2004; Seye et al., 2004), demonstrating a novel mechanism whereby $P2Y_2$ receptors can cause inflammatory responses in blood vessels.

Nucleotides are released during vascular injury from activated platelets and damaged cells (Schwiebert and Kishore, 2001; Oike et al., 2004). Released ATP and UTP can activate $P2Y_2$ receptors in human neutrophils to induce fibrinogen-dependent degranulation independent of arachidonic acid metabolites (Meshki et al., 2004). A recent study suggests that ATP is released from human neutrophils exposed to fMLP

(N-formyl-L-methionyl-L-leucyl-L-phenylalanine), a formylated tripeptide secreted by bacteria (Chen et al., 2006) and that $P2Y_2$ receptors are required for neutrophils to amplify the ATP-induced chemotactic signals that guide the cell to migrate since neutrophils from $P2Y_2^{-/-}$mice fail to undergo chemotaxis towards fMLP *in vitro* and *in vivo* (Chen et al., 2006). In addition, introduction of the $P2Y_2$R agonist UTP via a vascular collar placed around a rabbit carotid artery, causes a 4-fold increase in leukocyte migration into the intima (Seye et al., 2002). Since leukocyte infiltration and migration are key processes involved in atherosclerosis, these findings suggest that $P2Y_2$ receptors represent a novel target for reducing arterial inflammation associated with cardiovascular disease.

Activation of the $P2Y_2$ receptor also increases epithelial cell Cl^- secretion and inhibits Na^+ absorption, an effect that has been explored for its potential therapeutic application in the treatment of cystic fibrosis, a dehydrating disease characterized by defective Cl^- secretion due to genetic mutations in CFTR in airway epithelium (Clarke and Boucher, 1992; Parr et al., 1994; Kellerman et al., 2002). The role of the $P2Y_2$ receptor in regulating ion secretion in airway epithelial cells has been confirmed in $P2Y_2$ receptor knockout mice (Cressman et al., 1999). A selective $P2Y_2$ receptor agonist, INS27217, also has been shown to increase chloride and water secretion in tracheal epithelium, cilia beat frequency and mucin release from human airway epithelium, suggesting potential treatments for dry eye disease and cystic fibrosis (Yerxa et al., 2002). Activation of $P2Y_2$ and other P2 receptors in human keratinocytes has been postulated to play a role in the wound healing process (Burrell et al., 2003; Greig et al., 2003a, 2003b). $P2Y_2$ receptors also can promote α-secretase-dependent amyloid precursor protein processing in 1321N1

astrocytoma cells, suggesting a neuroprotective role (Camden et al., 2005). In contrast to these therapeutic effects, $P2Y_2$ receptors in osteoblasts function as negative modulators of bone remodeling by blocking bone formation by osteoblasts (Hoebertz et al., 2002). Thus, depending upon the tissue, $P2Y_2$ receptor expression and activation can promote deleterious (*e.g.*, endothelium, smooth muscle and bone) or beneficial (*e.g.*, epithelium and neuron) responses, which makes the $P2Y_2$ receptor a potential pharmacological target in atherosclerosis, inflammation, cystic fibrosis, osteoporosis, and neurodegenerative disorders.

D. Chemotaxis

Chemotaxis is the movement of a cell in response to a chemical gradient. In bacteria, chemotaxis is important for finding food (*e.g.*, glucose) by swimming towards the highest concentration of food molecules, or fleeing from poisons, such as phenol. In eukaryotes, the mechanism of chemotaxis is quite different from that in bacteria; however, sensing of chemical gradients by cell surface receptors is still a crucial step in this process. In the mammalian system, chemotaxis is required for many important processes, including embryonic development, angiogenesis, inflammatory responses and cancer cell metastasis (Li et al., 2005). In the process of inflammation, chemotaxis is a key step in leukocyte recruitment.

1. P2Y receptors and chemotaxis

P2Y receptors are able to induce chemotaxis in a variety of cell types, including smooth muscle cells (Pillois et al., 2002), epithelial cells (Klepeis et al., 2004),

endothelial cells (Kaczmarek et al., 2005), microglia (Honda et al., 2001), astrocytes (Wang et al., 2005), neutrophils (Chen et al., 2006), and bone marrow hematopoietic stem cells (Lee et al., 2003).

In rat aortic smooth muscle cells (rASMC), ATP, UTP and UDP, but not ADP, induce chemotaxis, suggesting involvement of $P2Y_2$ and $P2Y_6$ receptors (Pillois et al., 2002). UTP treatment causes a dose-dependent up-regulation of osteopontin, a ligand for $\alpha_v\beta_3$ integrins (Chaulet et al., 2001). UTP-induced ASMC migration is dependent on the expression of osteopotin via a pathway that involves Rho (a small GTPase responsible for cell morphological changes) and MAPK (Chaulet et al., 2001). Similarly, ATP and UTP, but not ADP, induce migration of human umbilical vein endothelial cells (HUVEC), a process associated with up-regulation of α_v integrins that requires activation of PI3K and focal adhesion kinase (Kaczmarek et al., 2005).

Actin cytoskeletal rearrangements have been seen in both smooth muscle cells and endothelial cells stimulated with extracellular nucleotides (Sauzeau et al., 2000; Kaczmarek et al., 2005). Studies indicate that $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are coupled to activation of RhoA (Sauzeau et al., 2000), although the $P2Y_1$ receptor is not thought to be involved in the migration of ASMC and endothelial cells since ADP does not have chemoattractant effects in these cells (Pillois et al., 2002; Kaczmarek et al., 2005). However, MRS-2179, a selective $P2Y_1$ receptor antagonist, reduced the rate of migration of wild type mouse neural progenitor cells, suggesting the involvement of $P2Y_1$ receptors in neural development. Furthermore, knockout of connexin 43, a tight junction protein implicated in the release of ATP from neutrophils (Eltzschig et al., 2006),

down-regulates the migratory ability of neural progenitor cells, an event that can be restored by exogenous expression of $P2Y_1$ receptors (Scemes et al., 2003).

Chen et al. showed that ATP is released from the protrusion region during neutrophil migration toward fMLP, a chemoattractant peptide secreted by certain pathogens (Chen et al., 2006). Due to the presence of ecto-nucleotidases, such as CD39 and CD73, in plasma membranes, ATP and ADP are converted into AMP, which is further hydrolyzed to adenosine. Chen et al. found that A3 adenosine receptors, but not $P2Y_2$ receptors, accumulate at the leading edge of migrating neutrophils exposed to a fMLP gradient and co-localized with F-actin-containing protrusions. A3 adenosine receptor-null cells perform normal directional sensing toward fMLP, but migrate at a much reduced speed than wild type cells, whereas $P2Y_2$ receptor-null cells have an impaired ability to sense the fMLP gradient. These findings suggest a model of coordinated purinergic signaling to achieve proper neutrophil chemotaxis during inflammation. Consistent with these data, CD39-null monocytes/macrophages fail to migrate into implanted Matrigel *in vivo* or towards nucleotides *in vitro*, indicating a possible role for nucleotides and nucleotide receptors in monocyte migration during inflammation (Goepfert et al., 2001).

2. Chemotaxis and GPCRs

Mammalian neutrophils and the amoeba *Dictyoselium* are two models frequently used to study the molecular mechanisms of chemotaxis. Chemotaxis requires a complicated coordination and integration of a series of biochemical signals. External signals for a cell to migrate include soluble chemoattractant, extracellular matrix proteins and mechanical stress. Cell surface receptors, most of which are heterotrimeric GPCRs, can sense a gradient of chemoattractants that has concentration differences as little as

1~2% (Zigmond, 1977; Tranquillo et al., 1988). Two characteristics contributing to chemotaxis are directional sensing and morphological polarization (Swanson and Taylor, 1982; Fisher et al., 1989; Parent and Devreotes, 1999). In response to chemokines, GPCRs activate GDP to GTP exchange on the Gα subunit and promote the dissociation between the Gα and Gβγ subunits, leading to the activation of downstream effectors. To date, four families of mammalian heterotrimeric G proteins have been identified according to their different α subunits: $G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{12/13}$ (Offermanns, 2003). Most of the mammalian chemotactic GPCRs are coupled to $G\alpha_{i/o}$, since pertussis toxin, a specific $G\alpha_{i/o}$ inhibitor prevents chemotaxis and many other responses to ligands of chemokine receptors (Gerard and Gerard, 1994), whereas some cells such as neutrophils utilize $G\alpha_{12/13}$, as well as $G\alpha_{i/o}$, to assemble divergent signaling complexes and to adopt an appropriate morphology in the presence of chemoattractants (Xu et al., 2003b).

Expression of two Gβγ subunit-sequestering proteins, a C-terminal fragment of the β-adrenoreceptor kinase or the α subunit of transducin (αt), inhibits G_i-mediated chemotaxis, suggesting an important role of the released Gβγ dimer in chemotaxis (Arai et al., 1997; Neptune and Bourne, 1997). In living cells during chemotaxis, Gβ subunits are mainly localized at the anterior end, whereas some chemoattractant receptors are evenly distributed along the surface of polarized cells (Servant et al., 1999; Jin et al., 2000). In fact, the agonist-induced release of Gβγ subunits from receptors leads to activation of phosphatidylinositol-3 kinase (PI3K) γ (Stephens et al., 1994). Studies with PI3Kγ knockout mice reveal an important role of PI3Kγ in chemoattractant-induced macrophage migration (Hirsch et al., 2000). The PI3K substrate PIP_3 and pleckstrin homology (PH) domain-containing proteins (such as Akt/PKB and adenylyl cyclase) are

found to be enriched at the leading edge of migrating cells following a chemoattractant gradient (Jin et al., 2000; Servant et al., 2000). Although inhibition of actin polymerization causes the lost of cell polarity and asymmetric distribution of Gβ subunits, it does not affect the recruitment of PH domain-containing proteins to the leading edge, suggesting that the polarized distribution of Gβ subunits depends on intrinsic signals whereas the appearance of PH domain binding sites indicates the direction of movement (Jin et al., 2000; Servant et al., 2000).

3. Rho GTPases

Rho GTPases, a family of small monomeric GTPases, are also crucial for controlling directed cell migration. The Rho family is divided into three subgroups: Rac, Rho and Cdc42. To date, twenty two mammalian genes encoding Rho GTPases have been described (Jaffe and Hall, 2005). Similar to other GTPases, Rho GTPases cycle between the inactive GDP-bound state and the active GTP-bound state. Three major groups of regulatory proteins control Rho GTPase activities: GEFs (guanine nucleotide exchange factors) that catalyze the exchange of GDP for GTP, GAPs (GTPase-activating proteins) that promote intrinsic GTPase activity to inactivate the protein, and GDIs (guanine nucleotide dissociation inhibitors) that block Rho GTPase self-activation (Jaffe and Hall, 2005). Different Rho GTPases have differential roles in regulating the morphology of a migrating cell. Generally, Rho-like family proteins are responsible for formation of stress fibers and focal adhesions, providing the cell with contractility; Rac-like family proteins promote the formation of membrane ruffles and lamellipodia; and Cdc42-like family proteins simulate the formation of filopodia (Burridge and Wennerberg, 2004). Actin

polymerization and actin/myosin contraction as well as filament elongation at the leading edge of cells are thought to provide the driving force for migration. Rho stimulates actin polymerization via the diaphanous-related formin (DRF), mDia1 in mammalian cells. mDia1 directly interacts with the profilin/actin complex and sends it to a filament end. Rho also induces assembly of contractile actin/myosin filaments via its target Rho-associated kinase (ROCK), leading to activation of LIM kinase (LIMK). LIMK promotes phosphorylation-induced inactivation of cofilin, which participates in actin filament disassembly by increasing actin monomer dissociation, leading to stabilization of the actin cytoskeleton. LIMK also inactivates myosin light chain (MLC) phosphatase by phosphorylation. This, in turn, increases MLC phosphorylation and stimulates the actin filament cross-linking activity of myosin II (Pollard and Borisy, 2003; Riento and Ridley, 2003; Jaffe and Hall, 2005).

Rho GTPases are also involved in directional sensing. For example, general inhibitor of Rho GTPases blocks the chemoattractant-induced translocation of PH domain-containing proteins, induces a round cell morphology and inhibits actin polymerization in response to a chemoattractant (Servant et al., 2000). Inhibition of Rac by overexpression of dominant-negative mutants in neutrophils blocks chemoattractant-induced actin polymerization, translocation of PH-domain containing proteins, and thus cell polarity, while inhibition of Cdc42 induces multiple leading edges instead of the normal formation of one leading front (Srinivasan et al., 2003). Both Rho and Rac are required for colony stimulating factor-1-induced macrophage migration, however, inhibition of Cdc42 prevents chemotaxis but keeps cell random migration intact, suggesting that Cdc42 is important for gradient sensing (Allen et al., 1998). It is likely

that different Rho GTPases are differentially regulated by different receptors/G proteins in separate spatial domains during chemotaxis. $G_{i/o}$ triggers the activation of Rac and mediates the formation of protrusions at the leading edge of a cell; $G_{12/13}$ promotes the activation of Rho and induces polarized actin reorganization at the rear of a cell to provide the rendering force (Xu et al., 2003b).

4. Regulation of chemotaxis by integrins and the extracellular matrix (ECM)

Cell motility requires the forward movement of the leading edge of a cell by extension, formation of adhesive contacts in these extensions and detachment of old adhesive contacts in the trailing edge (Ridley et al., 2003). Adhesion is mainly mediated by integrins, which are heterodimeric receptors consisting of α and β subunits with large extracellular domains and relatively short cytoplasmic domains. Integrins bind extracellular matrix (ECM) proteins and intracellular cytoskeletal proteins, thus linking signaling from the outside to the inside of cells (Hynes, 1992). Many of the integrins have been shown to play a role in cell migration. For example, $\alpha_v\beta_3$ integrins are found to play a key role in cell migration during angiogenesis (Brooks et al., 1994) and metastasis (Seftor et al., 1992); β_3 and β_2 integrins are found to be crucial for immune cell migration (Springer, 1995; Lacy-Hulbert et al., 2007).

The ECM, which consists of proteins that act as ligands for integrins, is able to regulate the speed and direction of chemotaxis by controlling integrin-ECM interactions and adhesion formation. At low levels of ECM, the cells can not form stable adhesions where integrins serve as contact sites to generate tractional forces that are transmitted from the interaction of myosin II and actin filaments. At high levels of ECM, the cell *can*

not break the integrin-ECM interactions at the rear of the cell and therefore can not move forward. Thus, integrin-mediated migration is dependent on the cell surface density of integrins, the density of ligands (ECM), and the affinity between integrin and ECM (Ridley et al., 2003; Li et al., 2005).

Although integrins lack any catalytic activities, they do transmit signals by direct or indirect interactions with other proteins. For example, the cytoplasmic domain of the α_4 integrin subunit interacts with a signaling adaptor protein, paxillin. This interaction is critical for effective $\alpha_4\beta_1$-dependent leukocyte migration (Rose, 2006). Talin, another cytoskeletal protein, binds to several β integrins and its head domain is responsible for activation of $\alpha_{IIb}\beta_3$ and $\alpha_L\beta_2$ integrins (Calderwood et al., 2002; Kim et al., 2003). Activated $\alpha_v\beta_{3/5}$ integrins, detected by the engineered antibody WOW-1, are found preferentially distributed to lamellipodia at the leading edge of a migrating cell (Kiosses et al., 2001). Pre-incubation with WOW-1 antibody blocks endothelial chemotaxis on fibrinogen (a ligand for $\alpha_v\beta_3$ integrins) towards bFGF (basic fibroblast growth factor), suggesting a role for high affinity integrins in chemotaxis (Kiosses et al., 2001). Integrin function is also regulated by post-translational modification. For example, phosphorylated α_4 integrins (on serine residues) are also found to accumulate in lamellipodia, and α_4 integrin phosphorylation and subsequent release of bound paxillin are thought to be required for $\alpha_4\beta_1$-dependent cell migration and lamellipodia stability (Goldfinger et al., 2003).

By participating in outside-in signaling, integrins can modulate the activities of Rho GTPases and other signaling molecules at focal adhesions to regulate cell migration. Cells plated on fibronectin exhibit a rapid activation of Rac and Cdc42 and spread by

forming filopodia and lamellipodia (Price et al., 1998). In contrast, Rho activity decreases initially when cells are plated on ECM proteins, followed by a transient peak of activation, which then returns to baseline (Ren et al., 1999), suggesting that Rho GTPase activity can be differentially modulated by integrin dynamics. Consistent with these observations, studies with fibroblasts indicate that ROCK inhibitor increases lysophosphatidic acid (LPA)-induced Rac activation (Tsuji et al., 2002). These signals are thought to be able to coordinate with each other during cell migration by a feedback loop, driving fluctuations in Rho and Rac activities to maintain cell polarity (Moissoglu and Schwartz, 2006).

Integrins can modulate other receptor functions by either direct association of the receptors or transactivation between receptors. For example, $\alpha_v\beta_3$ integrin is found to associate with activated platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor-2 (VEGFR-2), to play a synergistic role in growth factor-induced mitogenicity or cell migration (Schneller et al., 1997; Woodard et al., 1998; Soldi et al., 1999).

E. Cell-cell adherens junction proteins and Rho GTPases

Cell-cell adhesion is believed to support stability of normal tissue organizations in embryonic development. Major components of cell-cell adherens junctions include cadherins, a family of transmembrane proteins that form dimers with other cadherin molecules on adjacent cells, and catenins, a family of proteins that bind to cadherin's intracellular domain and serve as a connection between cadherins and actin cytoskelelton (Braga, 2002). Classic cadherins have a wide range of distribution. E-cadherin is found

mainly in epithelial cell junctions and P-cadherins are found in squamous epithelial cells, whereas fibroblasts express N-cadherin in cell-cell junctions and VE-cadherin expression is restricted to vascular endothelial cells (Angst et al., 2001). There is evidence that Rho GTPases regulate cadherin functions as inside-out signals and cadherins regulate Rho GTPase activities as outside-in signals. Rac activation induces increased E-cadherin and β-catenin distribution at cell-cell adhesion sites (Takaishi et al., 1997), whereas Rho has dual functions, either via ROCK to disrupt adherens junctions or via mDia to stabilize them (Sahai and Marshall, 2002). As an example of outside-in signaling, engagement of epithelial-like cadherins increases the activity of Rac and Cdc42, but inhibits Rho activation (Noren et al., 2001). Time-lapse microscopy shows that GFP-tagged Rac1 accumulates at the nascent contact membranes along the leading edges of cadherin-containing lamellipodia when migratory cells collide or in the presence of E-cadherin homopilic engagement (Ehrlich et al., 2002; Kovacs et al., 2002). Clustering of E-cadherin by mouse anti-cadherin antibody and anti-mouse IgG leads to activation of Rac1 in keratinocytes, an event dependent on epidermal growth factor receptor (EGFR) signaling (Betson et al., 2002). VE-cadherin, a unique adherens junction protein in endothelial cells, is found to be responsible for activation of Rac1, but not RhoA, probably due to the increased expression and membrane localization of the Rac1 GEF, Tiam 1, in VE-cadherin-expressing cells (Lampugnani et al., 2002). In contrast, N-cadherin engagement results in activation of RhoA, but not Rac1 or Cdc42 (Charrasse et al., 2002). Transfection with E-cadherin inhibits invasive carcinoma cell motility, and cell motility can again be induced by treatment with anti-E-cadherin antibodies, suggesting an inhibitory role for E-cadherin in cell motility (Frixen et al., 1991).

However, inappropriate expression of a non-epithelial cadherin such as N-cadherin or R-cadherin (retinal cadherin), which activate Rac and Cdc42, induces cell motility (Nieman et al., 1999; Johnson et al., 2004), suggesting that diverse cellular functions are probably regulated by cadherins.

How do cadherins regulate the activity of small Rho GTPase? One connection between cadherins and Rho GTPases is p120 catenin. It has been shown that cytosolic p120 catenin inhibits the activation of RhoA, but not Rac and Cdc42, probably via interacting with the Rho GTPase GEF, vav2 (Noren et al., 2000). In addition, $G\alpha_{12}$ family proteins have negative effects on E-cadherin-mediated inhibition of cell migration in a Rho-independent manner (Meigs et al., 2002), possibly because $G\alpha_{12/13}$ proteins interact with p120 catenin and overcome the inhibition of RhoA activity by p120 catenin (Krakstad et al., 2004). Alternatively, PI3K-dependent and EGFR-dependent intrinsic signaling might be involved in Rho GTPase regulation by cadherins (Betson et al., 2002; Kovacs et al., 2002).

F. Research goals and experimental approaches

There is increasing evidence that the $P2Y_2R$ plays an important role in the development of vascular inflammation. For example, UTP, the agonist for the $P2Y_2R$, increases neutrophil infiltration into the neointima (Seye et al., 2002). Furthermore, $P2Y_2R$ knockout mice are unable to recruit neutrophils into the sites of injury or bacterial infection (Chen et al., 2006). The central research goal of this dissertation is to explore the mechanisms of $P2Y_2R$-mediated inflammatory responses including leukocyte chemotaxis and endothelium-dependent leukocyte transendothelial migration. Since the

small Rho GTPases control these cellular processes via regulation of actin dynamics, one of the major aims of this dissertation is to examine the mechanisms involved in $P2Y_2R$-mediated activation of these Rho GTPases in different cells. Structure-function analysis reveals that the human $P2Y_2R$ contains an extracellular α_v integrin binding domain as well as a C-terminal SH3-binding domain. We, therefore, studied the role of these functional domains in $P2Y_2R$-mediated activation of Rho GTPases in 1312N1 human astrocytoma cells that do not express any endogenous P2 receptors by using cells that are stably transfected with wild type or mutant $P2Y_2Rs$. We also used pharmaceutical compounds or small interference RNA to block specific protein functions to further delineate the mechanisms of $P2Y_2R$-mediated activation of Rho GTPase in cells endogenously expressing the $P2Y_2R$ (*i.e.*, endothelial cells).

The experimental results of this dissertation are divided into the following chapters, according to their specific research aims:

1. Chapter II ─ determines the role of the $P2Y_2R/\alpha_v$ integrin interaction in $P2Y_2R$-mediated cell cytoskeletal rearrangements and delineates the signaling pathways involved in this process.
2. Chapter III ─ determines the role of the $P2Y_2R/\alpha_v$ integrin interaction in $P2Y_2R$-mediated cell chemotaxis and delineates the mechanisms involved.
3. Chapter IV ─ explores the crosstalk between the $P2Y_2R$ and adherens junction proteins in endothelial cells and examines the role of adherens junction proteins in $P2Y_2R$-mediated activation of Rho GTPases.

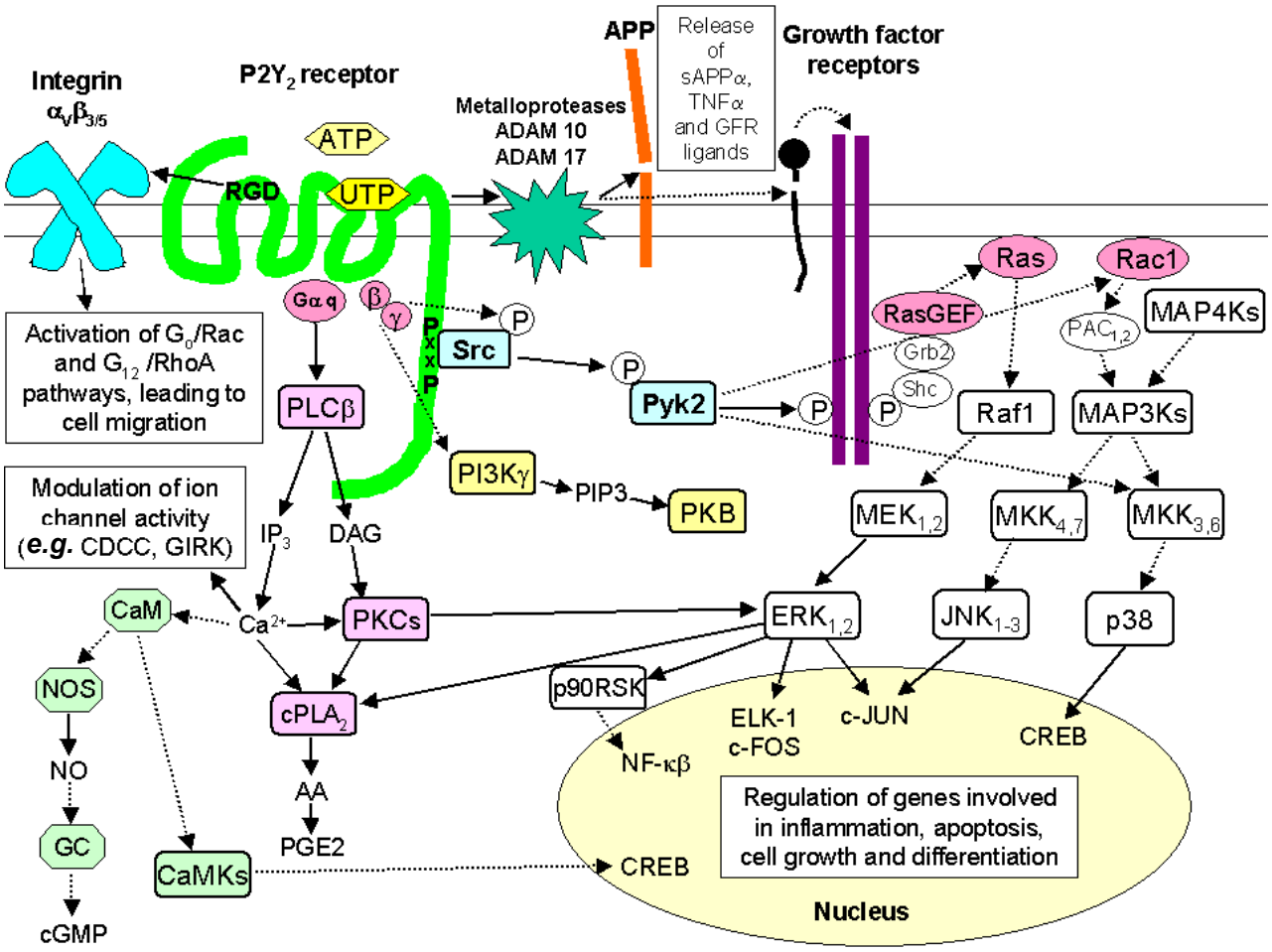
Table I-1. Cloned P2Y receptor subtypes and pharmacology

Lambrecht et al., 1992; Lustig et al., 1993; Parr et al., 1994; Chang et al., 1995; Communi et al., 1995; Lazarowski et al., 1995; Nguyen et al., 1995; Tokuyama et al., 1995; Ayyanathan et al., 1996; Boyer et al., 1996; Charlton et al., 1996; Chen et al., 1996; Communi et al.,1996; Communi et al., 1997; Robaye et al., 1997; Bogdanov et al., 1998; Palmer et al., 1998; Communi et al., 1999; Ingall et al., 1999; Chambers et al., 2000; Communi et al., 2001; Freeman et al., 2001; Hollopeter et al., 2001; Lazarowski et al., 2001; Zhang et al., 2001; Zhang et al., 2002; Abbracchio et al., 2003; Marteau et al., 2003; Fumagalli et al., 2004; Shen et al., 2004.

Table 1

Receptor subtypes	species cloned	agonists	antagonists	Transduction Mechanisms
$P2Y_1$	human, rat, mouse	2MeSADP > ADP > ATP	A3P5PS, PPADS, suramin	$G_{q/11,}$ PLC activation
$P2Y_2$	human, rat, mouse, porcine	UTP >= ATP > 5BrUTP	suramin	$G_{q/11}$, PLC activation; $G_{i/o,}$ PLC activation; G_{12}, Rho activation
$P2Y_4$	human, rat, mouse	UTP > ATP	PPADS	$G_{q/11}$ and $G_{i/o}$, PLC activation
$P2Y_6$	human, rat, mouse	UDP > UTP > ADP	PPADS, suramin	$G_{q/11}$, PLC activation
$P2Y_{11}$	human	ATPγS > BzATP > ATP	suramin	$G_{q/11}$ and $G_{s,}$ PLC activation
$P2Y_{12}$	human, rat, mouse	2MeSATP = 2MeSADP > ADP	suramin, AR-C6096, AR-C69931MX	$G_{i/o}$, inhibition of adenylate cyclase; possibly G_{12}
$P2Y_{13}$	human, rat, mouse	2MeSATP = 2MeSADP > ADP > ATP	AR-C69931MX, PPADS,suramin	$G_{i/o}$, inhibition of adenylate cyclase;
$P2Y_{14}$	human, rat, mouse	UDP-glucose > UDP-galactose	not defined	$G_{q/11,}$ PLC activation

Figure. I-1 Intracellular signaling pathways activated by the $P2Y_2$ receptor (Erb et al., 2006)**.** Responses to $P2Y_2$ receptor activation in different cell types may vary, possibly due to crosstalk occurring between different signaling pathways and to differential expression of cell-specific effector proteins. This diagram represents the divergent and comprehensive signaling events mediated by the $P2Y_2$ receptor. *Arrows with solid lines* show established responses mediated by the $P2Y_2$ receptor, whereas, *dashed lines* show established signaling pathways that have not yet been studied for $P2Y_2$ receptors. *AA* arachidonic acid, *ADAM* a disintegrin and metalloproteinase, *APP* amyloid precursor protein, *CaM* calmodulin, *CaMK* calmodulin-dependent protein kinase, *CDCC* calcium-dependent chloride channel, *cGMP* cyclic GMP, *$cPLA_2$* cytosolic phospholipase A2, *CREB* cAMP response element-binding protein, *DAG* diacylglycerol, *ELK-1* ETS-domain transcription factor, *ERK* extracellular signal-regulated protein kinase, *GC* guanylyl cyclase, *GIRK* G protein-activated inward rectifier, *GFR* growth factor receptor, *Grb2* growth factor receptor bound protein 2, *IP_3* inositol-1,4,5-trisphosphate, *JNK* c-Jun N-terminal kinase, *MAP4K* MAPK kinase kinase kinase, *MAP3K* MAPK kinase kinase, *MKK* MAPK kinase, *MEK* mitogen/extracellular signal-regulated protein kinase, NF-κB nuclear factor-κB, *NO* nitric oxide, *NOS* nitric oxide synthase, *p38* mitogen-activated protein-serine kinase p38, *p90RSK* p90 ribosomal S6 kinases, *PAK* p21-activated serine kinase, *PGE2* prostaglandin E2, *PI3K* phosphatidylinositol 3-kinase, *PIP_3* phosphatidyl-3,4,5-triphosphate, *PKB* protein kinase B, *PKC* protein kinase C, *PLCγ* phospholipase Cγ, *PXXP* proline-rich Src homology 3 domain, *Pyk2* proline-rich tyrosine kinase, *RGD* Arg-Gly-Asp integrin binding domain, *sAPPα* α-secretase-dependent amyloid precursor protein, *Rho, Rac, Ras, Raf* monomeric (small) G proteins, *Shc* Src-homology collagen protein, *TNF-α* tumor necrosis factor-α.


Integrin
$\alpha_V\beta_{3/5}$
P2Y$_2$ receptor
ATP
UTP
RGD
Metalloproteases
ADAM 10
ADAM 17
APP
Release of sAPPα, TNFα and GFR ligands
Growth factor receptors
Activation of G$_0$/Rac and G$_{12}$ /RhoA pathways, leading to cell migration
Gαq
β
γ
PXXP
P
Src
PLCβ
PI3Kγ
PIP3
PKB
Pyk2
Ras
Rac1
RasGEF
Grb2
Shc
PAC$_{1,2}$
MAP4Ks
Raf1
MAP3Ks
MEK$_{1,2}$
MKK$_{4,7}$
MKK$_{3,6}$
Modulation of ion channel activity (e.g. CDCC, GIRK)
IP$_3$
DAG
CaM
Ca^{2+}
PKCs
ERK$_{1,2}$
JNK$_{1-3}$
p38
NOS
NO
GC
cGMP
cPLA$_2$
AA
PGE2
CaMKs
p90RSK
NF-$\kappa\beta$
ELK-1
c-FOS
c-JUN
CREB
CREB
Regulation of genes involved in inflammation, apoptosis, cell growth and differentiation
Nucleus

CHAPTER II

The $P2Y_2$ nucleotide receptor requires interaction with α_V integrins to access and activate G_{12}

Abstract

The $P2Y_2$ nucleotide receptor ($P2Y_2R$) interacts with α_v integrins to activate G_o and induce chemotaxis in human 1321N1 astrocytoma cells. In this study, it was determined that the $P2Y_2R$ also requires interaction with α_v integrins to activate G_{12} and associated signaling pathways that control chemotaxis in 1321N1 cells. Mutation of the Arg-Gly-Asp (RGD) integrin-binding sequence in the first extracellular loop of the human $P2Y_2R$ to Arg-Gly-Glu (RGE), which prevents integrin interaction, did not inhibit G_q or ERK1/2 signaling by the $P2Y_2R$ agonist UTP but completely inhibited activation of G_{12} and G_{12}-mediated events, including Rho activation, cofilin and myosin light chain-2 phosphorylation, stress fiber formation and chemotaxis towards UTP. The involvement of G_{12} in all these events was verified by using a dominant negative $G\alpha_{12}$ construct. G_{12} activation by the $P2Y_2R$ also was inhibited by anti-$\alpha_v\beta_5$ integrin antibodies and α_v integrin antisense oligonucleotides, suggesting that α_v integrin activity and expression are required for the $P2Y_2R$ to activate G_{12}. Co-immunoprecipitation experiments confirmed that $G\alpha_{12}$ protein associates with the wild type $P2Y_2R$ and with α_v integrins but not with the RGE mutant $P2Y_2R$ or with α_3 integrins. Collectively, these results suggest that α_v integrin complexes provide the $P2Y_2R$ with access to G_{12}, thereby allowing activation of this heterotrimeric G protein that controls actin cytoskeletal rearrangements required for

chemotaxis.

Introduction

Chemotaxis is the movement of a cell towards a chemoattractant or away from a chemorepellant and is a fundamental feature of eukaryotic cells; it is important for many physiological and pathological processes, such as embryogenesis, neurogenesis, angiogenesis, wound healing, and homing of leukocytes to a site of infection. The ability of a cell to undergo chemotaxis requires the cell to assume a polarized morphology that is controlled by cell surface receptors that activate the Rho family of small GTPases including Cdc42, Rac, and Rho (Burridge and Wennerberg, 2004). Upon activation of a chemoattractant receptor, Cdc42 and Rac localize at the leading edge of a cell and control directional cell movement and the formation of a lamellipodium, respectively (Burridge and Wennerberg, 2004). Rho localizes at the rear and sides of a cell and controls the formation of contractile actin-myosin stress fibers (Xu et al., 2003b). Together, these GTPases promote cell migration towards a chemoattractant by mediating extension of the actin cytoskeleton at the front edge of the cell and retraction of the cytoskeleton at the rear.

A variety of compounds can act as chemoattractants by stimulating Rho family GTPases: these include growth factors that activate tyrosine kinase receptors, extracellular matrix proteins that activate integrins, and compounds that activate certain G protein-coupled receptors (GPCRs) (Burridge and Wennerberg, 2004). Recent studies have shown that GPCRs activate Rac and Rac-dependent lamellipodia formation through $G_{i/o}$, whereas the activity of Rho and Rho-dependent stress fiber formation is controlled

by $G_{12/13}$ (Hart et al., 1998; Kozasa et al., 1998; Xu et al., 2003b). Furthermore, studies have shown that the βγ subunits of $G_{i/o}$ are responsible for activation of Rac guanine nucleotide exchange factors (RacGEFs) that, in turn, activate Rac, whereas the α subunits of $G_{12/13}$ are responsible for activation of RhoGEFs (Neptune et al., 1999; Welch et al., 2002).

In this study, we examined the mechanism of Rho activation and Rho-dependent stress fiber formation mediated by the $P2Y_2$ nucleotide receptor ($P2Y_2R$), an integrin-associated GPCR activated by ATP or UTP that mediates stress fiber formation and chemotaxis in a variety of cell types (Satterwhite et al., 1999; Sauzeau et al., 2000; Bagchi et al., 2005; Kaczmarek et al., 2005; Wang et al., 2005) and plays an important role in monocyte (Seye et al., 2002) and neutrophil homing (Chen et al., 2006) to injured or infected tissues. Previously, we found that an RGD integrin-binding domain in the first extracellular loop of the $P2Y_2R$ enables the receptor to interact with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and this interaction is prevented by mutation of the RGD sequence to RGE (Erb et al., 2001). The RGD domain also was found to be necessary for coupling of the $P2Y_2R$ to G_o-mediated but not G_q-mediated calcium signaling (Erb et al., 2001) and a recent study by our group indicated that the $P2Y_2R$ requires interaction with α_v integrins to activate G_o and to initiate G_o-mediated events, including activation of Rac and the RacGEF vav2, up-regulation of vitronectin expression and increased cell migration (Bagchi et al., 2005). Here we report that α_v integrin interaction is required for the $P2Y_2R$ to access and activate G_{12}, leading to Rho activation and Rho-dependent stress fiber formation.

Results

$P2Y_2R$-mediated Rho activation and stress fiber formation require interaction with $\alpha_v\beta_5$ integrin

To determine whether integrin interaction with the $P2Y_2R$ is important for receptor signaling we mutated the Arg^{95}-Gly^{96}-Asp^{97} (RGD) sequence in the $P2Y_2R$ to Arg^{95}-Gly^{96}-Glu^{97} (RGE), a sequence that does not have high affinity for integrins (Erb et al., 2001), and compared signaling events mediated by the wild type and RGE mutant receptors that were expressed in human 1321N1 astrocytoma cells. These cells are devoid of endogenous G protein-coupled P2Y receptors (Parr et al., 1994) and, thus, provide a suitable null background for this study. Also, we incorporated a hemagglutinin (HA) epitope tag at the C-termini of the wild type and RGE mutant $P2Y_2Rs$ and used flow cytometry to verify that the cell surface expression levels of these receptor constructs were equivalent (Fig. II-1A). We found that mutation of the RGD sequence to RGE did not prevent ERK1/2 phosphorylation in response to the $P2Y_2R$ agonist UTP (Fig. II-1B) but completely prevented Rho activation (Fig. II-1B) and stress fiber formation (Fig. II-1C) induced by UTP at all concentrations tested (100 nM to 2 mM). A UTP dose-response curve indicated that the EC_{50} value for UTP-induced Rho activation by the wild type $P2Y_2R$ was ~1 μM (Supplemental Fig. II-S1), which is similar to the EC_{50} value of UTP for activation of other $P2Y_2R$-mediated responses (Erb et al., 2001). These results suggest that integrin interaction with the $P2Y_2R$, via the RGD integrin-binding domain, is necessary for the $P2Y_2R$ to activate Rho and cause stress fiber formation. Likewise, Rac activation and chemotaxis mediated by the $P2Y_2R$ in 1321N1 cell transfectants were found to require expression of the RGD integrin-binding

domain in the $P2Y_2R$ (Bagchi et al., 2005). As expected, untransfected 1321N1 cells that do not express P2Y receptors do not form stress fibers, undergo chemotaxis, or exhibit Rho or Rac activation in response to the $P2Y_2R$ agonists ATP or UTP (data not shown).

We have found that the RGE mutant $P2Y_2R$ can fully stimulate ERK1/2 phosphorylation (Fig. II-1B) and G_q-mediated calcium signaling (Erb et al., 2001), however, these responses require agonist concentrations that are 3 orders of magnitude higher than for the wild type receptor. This has raised questions as to whether the RGE mutation affects $P2Y_2R$ signaling by preventing integrin interaction or by affecting $P2Y_2R$ agonist binding affinity. For example, a recent study by Qi et al. confirmed that mutation of the RGD sequence to RGE in the $P2Y_2R$ decreases agonist potency for inositol phosphate formation by 1000-fold but mutation of the RGD sequence to AHN did not alter agonist potency, leading the authors to speculate that the RGD to RGE mutation was affecting agonist binding affinity (Qi et al., 2005). To verify that the loss of Rho signaling observed for the $P2Y_2R$ RGE mutant was due to decreased integrin binding and not to alteration in agonist binding affinity we constructed a $P2Y_2R$ mutant in which the entire RGD integrin-binding sequence was substituted with three alanines (AAA). We found that the AAA mutant $P2Y_2R$ had a similar UTP dose-response for ERK1/2 phosphorylation as the wild type receptor (Fig. II-2A) but was unable to activate Rho in response to UTP (Fig II-2B). Interestingly, 1321N1 cells expressing the AAA mutant did display a higher basal level of ERK1/2 phosphorylation and Rho activity as compared to cells expressing the wild type $P2Y_2R$ (Figs. II-2A and II-2B). The reason for the increased basal activity exhibited by the AAA mutant is unknown, nonetheless, these studies support the hypothesis that integrin interaction via the RGD domain of the

$P2Y_2R$ is important for controlling Rho activation by the $P2Y_2R$.

To further assess the role of $P2Y_2R$-integrin interaction in UTP-induced Rho activation and stress fiber formation mediated by the wild type $P2Y_2R$, we used function blocking antibodies directed against $\alpha_v\beta_5$, an integrin that interacts with the $P2Y_2R$, or α_3, an integrin that does not interact with the $P2Y_2R$ (Erb et al., 2001). Flow cytometry experiments indicate that 1321N1 cells express immunodetectable cell surface α_V, α_3 and β_5 integrin subunits, but not β_3 (Bagchi et al., 2005). Pretreatment of $P2Y_2R$-expressing cells with anti-$\alpha_v\beta_5$ integrin antibodies inhibited UTP-induced Rho activation (Fig. II-3A) and stress fiber formation (Fig. II-3B), whereas anti-α_3 integrin antibodies had no effect (Figs. II-3A and II-3B), suggesting that α_v integrin activity is important for $P2Y_2R$-mediated Rho activation and stress fiber formation. In contrast, anti-$\alpha_v\beta_5$ integrin antibodies did not inhibit Rho activation induced by fetal bovine serum (FBS) in 1321N1 cells (Supplemental Fig. II-S2), suggesting that α_v integrins are not involved in Rho activation by growth factors present in serum.

Integrin/$P2Y_2R$ interaction regulates Rho-dependent signaling

Activation of Rho leads to stress fiber formation by causing the phosphorylation of myosin light chain-2 (MLC-2) and cofilin, an actin depolymerizing protein that is inhibited when phosphorylated on Ser3 (Kimura et al., 1996; Kuhn et al., 2000). To verify that integrin/$P2Y_2R$ interaction is important for Rho signaling, we analyzed Rho-dependent signaling events in 1321N1 cells expressing the wild type $P2Y_2R$ or RGE mutant. UTP caused a dose-dependent increase in phosphorylation of MLC-2 (Fig. II-4A) and cofilin (Fig. II-4B) in cells expressing the wild type $P2Y_2R$ but not in cells

expressing the RGE mutant. Moreover, cofilin phosphorylation mediated by the wild type $P2Y_2R$ was inhibited by pretreatment of the cells with anti-$\alpha_v\beta_5$ antibodies but not anti-α_3 integrin antibodies (Fig. II-4C), further demonstrating that the $P2Y_2R$ interacts selectively with α_v integrins to activate Rho-mediated signaling events. Since cofilin phosphorylation can be regulated by proteins other than Rho, including Rac and testicular protein kinase 1 (Toshima et al., 2001; Burridge and Wennerberg, 2004), we used the ROCK-Rho pathway inhibitor Y-27632 to determine whether cofilin phosphorylation mediated by the $P2Y_2R$ occurs through the Rho signaling pathway. UTP-induced cofilin phosphorylation was completely inhibited when the cells were pretreated with Y-27632 (Fig. II-4D). Furthermore, pretreatment of the cells with the $G_{i/o}$ inhibitor pertussis toxin (PTX) did not inhibit UTP-induced cofilin phosphorylation (Fig. II-4D) but did inhibit UTP-induced Rac activation (Bagchi et al., 2005), suggesting that G_o-mediated activation of Rac is not involved in cofilin phosphorylation by the $P2Y_2R$.

Activation of $G\alpha_{12}$ by the $P2Y_2R$ requires interaction with α_v integrin

Rho activation and Rho-dependent stress fiber formation mediated by GPCRs are controlled by heterotrimeric G proteins in the $G_{12/13}$ family (Buhl et al., 1995; Xu et al., 2003b). Generally, GPCRs that stimulate stress fiber formation also couple to $G_{q/11}$ but regulate stress fiber assembly through activation of either G_{12} or G_{13} (Gohla et al., 1999). Here, we directly investigated whether the RGD integrin-binding domain of the $P2Y_2R$ is required for activation of specific G proteins (*i.e.*, G_{12} and G_q). Results indicated a 2.5 fold increase in [^{35}S]GTPγS binding to $G\alpha_{12}$ immunoprecipitated from UTP-treated membrane extracts of 1321N1 cells expressing the wild type $P2Y_2R$ as compared to

untreated controls, but extracts from cells expressing the RGE mutant receptor did not exhibit an increase in [^{35}S]GTPγS binding to $G\alpha_{12}$ in response to UTP (Fig. II-5A). In contrast, UTP induced a 2-3 fold increase in [^{35}S]GTPγS binding to $G\alpha_q$ upon activation of either the wild type or RGE mutant $P2Y_2R$ (Fig. II-5B). Activation of $G_{12/13}$ and $G_{q/11}$ proteins by the $P2Y_2R$ was also verified by analyzing serine/threonine phosphorylation of $G\alpha_{12/13}$ (Kozasa and Gilman, 1996) and tyrosine phosphorylation of $G\alpha_{q/11}$ (Umemori et al., 1997), as previously described. We found that UTP caused phosphorylation of both $G\alpha_{12}$ and $G\alpha_q$ in 1321N1 cells expressing the wild type $P2Y_2R$ (Fig. II-5C), whereas no phosphorylation of $G\alpha_{13}$ was detected in these cells (data not shown). UTP caused phosphorylation of $G\alpha_q$ but not $G\alpha_{12}$ in cells expressing the RGE mutant $P2Y_2R$ (Fig. II-5C), suggesting that α_v integrin interaction with the $P2Y_2R$ is required for UTP-induced activation of G_{12} but not G_q.

To further assess whether α_v integrins are involved in $P2Y_2R$-mediated activation of $G\alpha_{12}$, we tested the effects of inhibition of α_V activity or expression using anti-α_v integrin antibodies or α_v antisense oligonucleotides, respectively. We found that $G\alpha_{12}$ phosphorylation by UTP was inhibited by pretreatment with anti-α_v but not with anti-α_3 integrin antibodies in 1321N1 cells expressing the wild type $P2Y_2R$ (Fig. II-6A). Likewise, transfection of α_v antisense oligonucleotides in 1321N1 cells expressing the wild type $P2Y_2R$, which significantly suppressed α_v expression (Fig. II-6B), completely inhibited $G\alpha_{12}$ activation by UTP, as assessed by GTPγS binding (Fig. II-6B). Transfection of these cells with α_v sense oligonucleotides did not inhibit $G\alpha_{12}$ activation by UTP (Fig. II-6B). Together, these results suggest that α_V integrin expression and

activity are required for $P2Y_2R/G\alpha_{12}$ coupling.

$P2Y_2R$ accesses $G\alpha_{12}$ in a complex with α_v integrins

To determine whether the $P2Y_2R$ interacts with $G\alpha_{12}$ in a complex with α_V integrins, co-immunoprecipitation experiments were performed. Results indicated that endogenous α_v integrin co-immunoprecipitated with the HA-tagged wild type $P2Y_2R$ to a much greater extent than with the HA-tagged RGE mutant $P2Y_2R$ expressed in 1321N1 cells (Fig. II-7A). This association between α_v integrin and the wild type $P2Y_2R$ occurred with or without activation of the $P2Y_2R$, although 5 min stimulation with UTP did cause a slight but reproducible reduction in association between these proteins (Fig. 7A). We also found that endogenous $G\alpha_{12}$ co-immunoprecipitated with the wild type $P2Y_2R$ but not with the RGE mutant, whereas endogenous $G\alpha_q$ co-immunoprecipitated with both $P2Y_2R$ constructs (Fig. II-7A). Co-immunoprecipitation of both $G\alpha_q$ and $G\alpha_{12}$ with the wild type $P2Y_2R$ was inhibited after UTP treatment (Fig. II-7A), consistent with the concept that activation of GPCRs causes the release of receptor-coupled G protein subunits, thus triggering various downstream responses. Although we were unable to detect association between $G\alpha_{12}$ and α_v integrins when endogenous levels of these proteins were used, we did find that wild type $G\alpha_{12}$ overexpressed in 1321N1 cells co-immunoprecipitated with endogenous α_v integrins but not with endogenous α_3 integrins (Fig. II-7B), suggesting that G_{12} selectively associates with complexes containing α_v integrins. Interestingly, UTP treatment did not cause dissociation of $G\alpha_{12}$ and α_v integrins (Fig. II-7B) and fluorescence microscopy images indicated that UTP

treatment caused a slight redistribution of $G\alpha_{12}$ and $G\alpha_o$ onto membrane protrusions or lamellipodia in 1321N1 cells (Fig. II-8).

$G\alpha_{12}$ activity is required for $P2Y_2R$-mediated stress fiber formation and cell migration

To verify that G_{12} protein is responsible for $P2Y_2R$-mediated Rho activation and downstream signaling events, a dominant negative mutant of $G\alpha_{12}$ ($G\alpha_{12}DN$, Q231L/D299N) was used (Yang et al., 2005). Overexpression of $G\alpha_{12}DN$ in 1321N1 cells expressing the wild type $P2Y_2R$ completely inhibited UTP-induced Rho activation, cofilin phosphorylation, stress fiber formation and cell migration (Figs. II-9A, C and D), but did not inhibit UTP-induced ERK1/2 phosphorylation (Fig. II-9B), indicating that G_{12} is specifically required for $P2Y_2R$-mediated Rho activation and Rho-dependent signaling events leading to stress fiber formation and ultimately, cell migration.

Discussion

The G protein-coupled $P2Y_2R$ is known to activate several heterotrimeric G proteins, including G_o and G_q (Erb et al., 2001; Boarder et al., 1995). In this study, we show for the first time that the $P2Y_2R$ is also able to activate G_{12} and to initiate chemotactic signaling events downstream of G_{12}, including Rho activation, cofilin phosphorylation, stress fiber formation and directional cell migration (Figs. II-1, II-5, and II-9). Furthermore, we demonstrate here and in previous studies that an RGD integrin-binding domain in the first extracellular loop of the $P2Y_2R$ is necessary for the $P2Y_2R$ to activate G_o and G_{12}, but not G_q (Fig. II-5) (Bagchi et al., 2005; Erb et al., 2001), suggesting that integrin complexes provide the $P2Y_2R$ with access to select pools of

heterotrimeric G proteins. Since the $P2Y_2R$ is known to interact with α_V integrins (Erb et al., 2001), we performed a series of co-immunoprecipitation experiments to verify that this interaction is required for the $P2Y_2R$ to access G_{12} protein. These experiments showed that 1) $G\alpha_{12}$ co-immunoprecipitated with α_V integrins but not with α_3 integrins; 2) the $P2Y_2R$ co-immunoprecipitated with $G\alpha_q$, $G\alpha_{12}$ and α_v integrins; and 3) mutation of the $P2Y_2R$ integrin-binding domain (*i.e.*, substitution of RGD with RGE that does not bind integrins) did not affect the $P2Y_2R$'s ability to co-immunoprecipitate with $G\alpha_q$ but inhibited $P2Y_2R$ co-immunoprecipitation with $G\alpha_{12}$ and α_v integrins (Fig. II-7). Although many studies suggest that amino acids located in intracellular loop 2 and the NH_2- and COOH-terminal portions of intracellular loop 3 are the key elements responsible for GPCR selectivity of G protein recognition (Wess, 1997), the results presented here suggest that α_v integrin complexes are also important for establishing interaction between select G proteins and a GPCR. And, although it is well known that GPCRs require integrin activity to stimulate chemotaxis (Miettinen et al., 1998; Till et al., 2002), this is the first indication that a GPCR requires interaction with an integrin to provide access to specific heterotrimeric G proteins that regulate chemotaxis.

Similar to GPCR-mediated chemotaxis, there is some evidence that growth factor receptors and integrins use heterotrimeric G proteins to stimulate chemotaxis. Pertussis toxin (PTX), which specifically inactivates $G_{i/o}$ proteins by covalent modification of the α subunits, has been found to inhibit VEGF-induced monocyte migration (Barleon et al., 1996) as well as growth factor-induced Rac1 and Cdc42 activation by a chimeric EGF/VEGF receptor (Zeng et al., 2002). In addition, the latter study determined that overexpression of the $G\beta\gamma$ sequestering minigene, hβARK1, inhibited growth

factor-induced Rac1 and Cdc42 activation, suggesting that βγ subunits of PTX-sensitive $G_{i/o}$ proteins are involved in growth factor-induced activation of Rac and Cdc42 (Zeng et al., 2002). Vitronectin-induced chemotaxis of human melanoma cells mediated by the $\alpha_v\beta_3$ integrin is also inhibited by PTX (Aznavoorian et al., 1996), suggesting a role for $G_{i/o}$ proteins in this process. Although the mechanism of heterotrimeric G protein activation by growth factor receptors and integrins is unclear, it is possible that these chemoattractant receptors stimulate the release of ATP, thus triggering activation of G protein-coupled nucleotide and nucleoside receptors involved in chemotaxis. In support of this idea, studies have demonstrated that ATP is released from epithelial cells (McNamara et al., 2001; 2006) and migrating neutrophils (Chen et al., 2006) upon exposure to chemotactic bacterial proteins.

Recently, the general importance of the $P2Y_2R$ and the adenosine A3 receptor in mediating chemotaxis towards bacterial proteins was demonstrated in neutrophils (Chen et al., 2006). The authors showed that ATP is released at the leading edge of human neutrophils migrating towards the bacterial chemoattractant N-formyl-Met-Leu-Phe (fMLP) and is rapidly broken down to adenosine by ecto-ATPases on the cell surface. *In vivo* assessment of neutrophil infiltration into the peritoneal cavity of $P2Y_2R^{-/-}$ and A3 receptor$^{-/-}$ mice injected with a murine chemotactic protein (Trp-Lys-Tyr-Met-Val-Met-NH_2) or with *Staphylococcus aureus* bacteria indicated that both the $P2Y_2R$ and the adenosine A3 receptor are required for neutrophil recruitment. Furthermore, neutrophils lacking the adenosine A3 receptor migrated toward Trp-Lys-Tyr-Met-Val-Met-NH_2, but with diminished speed, whereas neutrophils lacking the $P2Y_2R$ showed a loss in sensing of the chemoattractant gradient. In agreement with

Chen et al.'s findings in neutrophils, we found that the $P2Y_2R$ remains evenly distributed in the plasma membrane of 1321N1 cells after receptor activation (not shown), which supports the conclusion that the $P2Y_2R$ controls chemotaxis by sensing and amplifying signals induced by chemoattractants.

Results presented in this study demonstrate that $G\alpha_{12}$ selectively associates with complexes containing α_v integrins (Fig. II-7). Although the mechanism of this interaction is unclear, it is possible that the cadherin family of cell-surface adhesion proteins may be involved since $G\alpha_{12}$ has been found to interact with the cytoplasmic tails of several cadherins (Meigs et al., 2001) and E-cadherin is known to associate with α_v integrins (von Schlippe et al., 2000). Another mechanism of $\alpha_v/G\alpha_{12}$ association may involve Tec tyrosine kinases. Members of the Tec family have been found to interact directly with $G\alpha_{12}$ and with focal adhesion kinase (Jiang et al., 1998; Chen et al., 2001), thus linking G_{12} to integrin complexes. Furthermore, $G\alpha_{12}$ can interact directly with leukemia-associated RhoGEF (LARG) and, upon phosphorylation of LARG by Tec, $G\alpha_{12}$ effectively stimulates the RhoGEF activity of LARG (Suzuki et al., 2003). Although we were unable to detect LARG expression in 1321N1 cells (data not shown), LARG belongs to a subfamily of RhoGEFs (including Lsc/p115 RhoGEF and PDZ-RhoGEF) that, unlike other RhoGEFs, contains a regulator of G protein signaling (RGS) domain that facilitates binding to $G\alpha_{12/13}$ (Fukuhara et al., 1999; Fukuhara et al., 2000; Reuther et al., 2001; Francis et al., 2006) and, in some instances, $G\alpha_q$ (Booden et al., 2002; Vogt et al., 2003). Members of this RhoGEF subfamily share the ability to specifically activate RhoA but not other Rho family GTPases, such as Rac1 and Cdc42 (Banerjee and Wedegaertner, 2004). Therefore, the link between this subfamily of RhoGEFs and the

$P2Y_2R$ warrants further investigation to better define how $P2Y_2Rs$ access G_{12} in α_v-containing complexes.

In summary, the present study indicates that the $P2Y_2R$ requires interaction with α_v integrins to access G_{12}, but not G_q, and to stimulate chemotactic signaling events mediated by G_{12}, including Rho activation, cofilin and MLC-2 phosphorylation and stress fiber formation. Since our previous work indicated that the $P2Y_2R$ also requires interaction with α_V integrins to activate G_o and G_o-mediated cell migration (Bagchi et al., 2005), these studies establish that α_v integrin complexes are required for the $P2Y_2R$ to access select heterotrimeric G proteins involved in chemotaxis.

Materials and Methods

Materials

Anti-human α_v (Q20), $\alpha_v\beta_5$ (P1F76) and α_3 (Ralph 3.2) monoclonal Abs, mouse IgG, polyclonal rabbit anti-human $G\alpha_{12}$, anti-human $G\alpha_{q/11}$, and anti-human ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-human cofilin, anti-phospho-cofilin, anti-phospho-MLC-2, and anti-phospho-ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA). The polyclonal rabbit anti-human actin antibodies were purchased from Cytoskeleton (Denver, CO). The mouse antiphosphoserine/threonine antibody and the anti-α_v integrin antibody for immunoblot analysis were purchased from BD Bioscience (San Jose, CA). The mouse anti-phosphotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-HA conjugated agarose beads and anti-HA antibody were purchased from Covance (Berkeley, CA). Oregon Green-conjugated phalloidin,

rhodamine-conjugated phalloidin and Texas Red-conjugated DNase I were purchased from Molecular Probes (Eugene, OR). The Rho-dependent kinase inhibitor Y27632 was purchased from Calbiochem (Indianapolis, IN). All other reagents including nucleotides were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Cell Culture and Transfection

Human 1321N1 astrocytoma cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 5% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in a humidified atmosphere of 5% $C0_2$ and 95% air. Cells were stably transfected with cDNA encoding either the wild type or RGE mutant $P2Y_2R$, as previously described (Erb et al., 2001). Both receptor constructs contained sequence encoding a hemagglutinin (HA) tag at the amino terminus of the $P2Y_2R$, as previously described (Erb et al., 2001). To make the AAA-$P2Y_2R$ mutant, the RGD sequence was also substituted with Ala-Ala-Ala using the QuikChange XL site-directed mutagenesis kit (Stratagene, CA), and the HA-tagged $P2Y_2R$ cDNAs were excised from pLXSN vectors by digesting with EcoRI and BamHI, and ligated into the pcDNA3.1(-) mammalian expression plasmid. The $G\alpha_{12}$ wild type and dominant-negative construct (Q231L/D299N) were obtained from Guthrie Cdna Resource Center (Sayre, PA). Human 1321N1 cells expressing the wild type $P2Y_2R$ were cultured to 80% confluence and transiently transfected with the $G\alpha_{12}$ constructs in the pcDNA3.1+ vector using the Lipofectamine 2000 reagent (Invitrogen, CA). The transfection efficiency for the $G\alpha_{12}$ constructs was determined to be ~60% using an indirect immunofluorescence assay. The day prior to experimental use of the cells, the

growth medium was replaced with serum-free medium. The α_v integrin was suppressed with α_v antisense oligonucleotides, as previously described (Bagchi et al., 2005).

Actin Stress Fiber Formation

Cells were plated on glass coverslips and treated as indicated at 37 °C in serum-free DMEM. Then, cells were washed in PBS, fixed for 10 min in 3.7% (v/v) formaldehyde, treated with 0.5% (v/v) Triton X-100, and rinsed in PBS. For staining of F-actin, cells were incubated with 5 μg/ml 488 Oregon Green-conjugated phalloidin for 45 min at room temperature and then washed with PBS. Texas Red-labeled DNaseI (5 μg/ml) was used to localize monomeric G-actin. In Figure II-8C, cells were incubated with rabbit anti-$G\alpha_{12}$ antibody, washed and then stained with rhodamine-conjugated phalloidin and Oregon Green-labeled goat anti-rabbit IgG for 45 mins. Coverslips were mounted on glass slides in ProLong antifade reagent (Molecular Probes) and examined using fluorescence microscopy (Nikon, Eclipse TE300) at room temperature. The objective was a Nikon Plan Fluor 40X/0.60 lens. Images were acquired and processed with Northern Eclipse 6.0 software via a QImaging camera (Qimaging, British Columbia, Canada). Single cells were selected by cropping the image.

Rho Activity Assay

A Rho activation assay kit (Upstate Biotechnology) was used to assess Rho activity according to the manufacturer's instructions. Briefly, cells were cultured in 100 mm tissue culture dishes in culture medium and starved with serum-free medium for 24 hours before stimulated with UTP for 5 min at 37 °C. Cells then were washed three times

with ice-cold PBS, suspended in Lysis Buffer containing 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% (v/v) Igepal CA-630, 50 mM $MgCl_2$, 5 mM EDTA and 10% (v/v) glycerol, and the lysates were transferred to 1.5 ml tubes. Forty microliters of Rhotekin Rho binding domain (RBD)-agarose that only recognizes GTP-bound Rho were added to 500 µl of lysate for 45 min at 4 °C. The beads were pelleted by centrifugation (30 sec; 14,000 x g; 4 °C) and washed three times with Lysis Buffer. Finally, the beads were resuspended in 40 ml of 2X Laemmli's sample buffer (120 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) sucrose, 1 mM EDTA, 50 mM dithiothreitol, and 0.003% (w/v) bromophenol blue) and Western blot analysis (see below) was performed with 1:1000 anti-Rho antibody (Upstate Biotechnology).

[^{35}S]GTPγS Binding Assay

Membranes (80 µg of protein) from 1321N1 astrocytoma cell transfectants expressing the wild type or RGE mutant $P2Y_2R$ or the pLXSN vector were isolated, as previously described (Tian et al., 1994), and incubated in assay buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 µM guanosine 5'-diphosphate, 1X protease inhibitor cocktail (Roche), and 50 nCi of [^{35}S]GTPγS; 1250 Ci/mmol; Perkin Elmer, CA) containing the indicated concentration of UTP. Samples were incubated for 20 min at 30 °C followed by addition of 0.5 ml of ice-cold buffer containing 50 mM Tris-Cl (pH 7.4), 100 mM NaCl, and 5 mM $MgCl_2$. The samples were centrifuged at 100,000 x g for 15 min at 4 °C and the resulting pellets were resuspended in 500 µl of solubilization buffer (100 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1.25% (v/v) NP40, 0.2% (w/v) SDS, and 1X protease inhibitor cocktail). Extracts were

incubated overnight with 1:1000 anti-$G\alpha_{12}$ or anti-$G\alpha_q$ antibody at 4 °C. The extract was then incubated with 50 μl of a 50% (v/v) protein G-agarose suspension, and the immune complexes were collected by centrifugation and washed three times in wash buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, and 5 mM $MgCl_2$). [^{35}S]GTPγS binding in the immunoprecipitates was quantified by liquid scintillation counting.

Immunoblot Analysis and Immunoprecipitation

Immunoblotting (IB) was performed as previously described (Liu et al., 2004). After the IB procedure, the membranes were stripped and reprobed with anti-actin or anti-ERK1/2 antibody to assess protein loading. Lysates from 1321N1 cell transfectants expressing the HA-tagged wild type or RGE mutant $P2Y_2R$, or the pLXSN vector were used for immunoprecipitation (IP) with anti-HA conjugated agarose beads, as previously described (Liu et al., 2004). IP was also performed with cell lysates and anti-$\alpha_v\beta_5$ or anti-α_3 antibody or normal goat IgG (negative control). The immune complexes were precipitated by protein G-conjugated beads and analyzed by IB with anti-G_{12} antibody. Phosphorylation of G proteins was detected by IP of G_{12} or tyrosine-phosphorylated proteins using anti-G_{12} or anti-phosphotyrosine antibody, respectively. The immunoprecipitated samples were analyzed by IB with anti-phosphoserine/threonine or anti-G_q antibody, respectively.

Cell Migration Assay

Cell migration assays were performed with 8-μm pore size Transwells (Costar), as described (Bagchi et al., 2005). In brief, the cells were cultured at 37 °C for 24 h in

DMEM supplemented with 5% (v/v) fetal bovine serum, suspended by trypsinization, washed, and resuspended in 100 μl of serum-free DMEM (5×10^4 cells) and placed in the upper chamber of the Transwells. The lower chamber was filled with 600 μl of serum-free medium with or without 100 μM UTP. The cells were allowed to migrate for 16 h at 37 °C. Cells migrating to the lower side of the membrane were fixed with cold methanol and stained with Accustain. Cells were counted in 10 microscopic fields at 20X magnification.

Figure II-1. $P2Y_2R$-mediated Rho activation and stress fiber formation require $P2Y_2R$ interaction with α_v integrins. (A) Cell surface expression of HA-tagged wild type (WT) or RGE mutant (RGE) $P2Y_2Rs$ in 1321N1 cells was determined by flow cytometry. Cells stained with only secondary antibody were used as a negative control. (B) Cells expressing the WT or RGE mutant $P2Y_2R$ were treated with or without 1 mM UTP for 5 min prior to measuring ERK1/2 phosphorylation and Rho activity. Results shown are representative of 3 experiments. (C) Cells expressing the WT or RGE mutant $P2Y_2R$ were treated with or without 1 mM UTP or 20% FBS (positive control) for the indicated time and stress fibers were visualized by staining filamentous actin (shown in green) with Oregon-Green labeled phalloidin. Texas Red-labeled DNase I was used to label monomeric actin (shown in red). More than 100 cells were examined in three separate experiments, and representative images are shown. Bar = 50 μm.

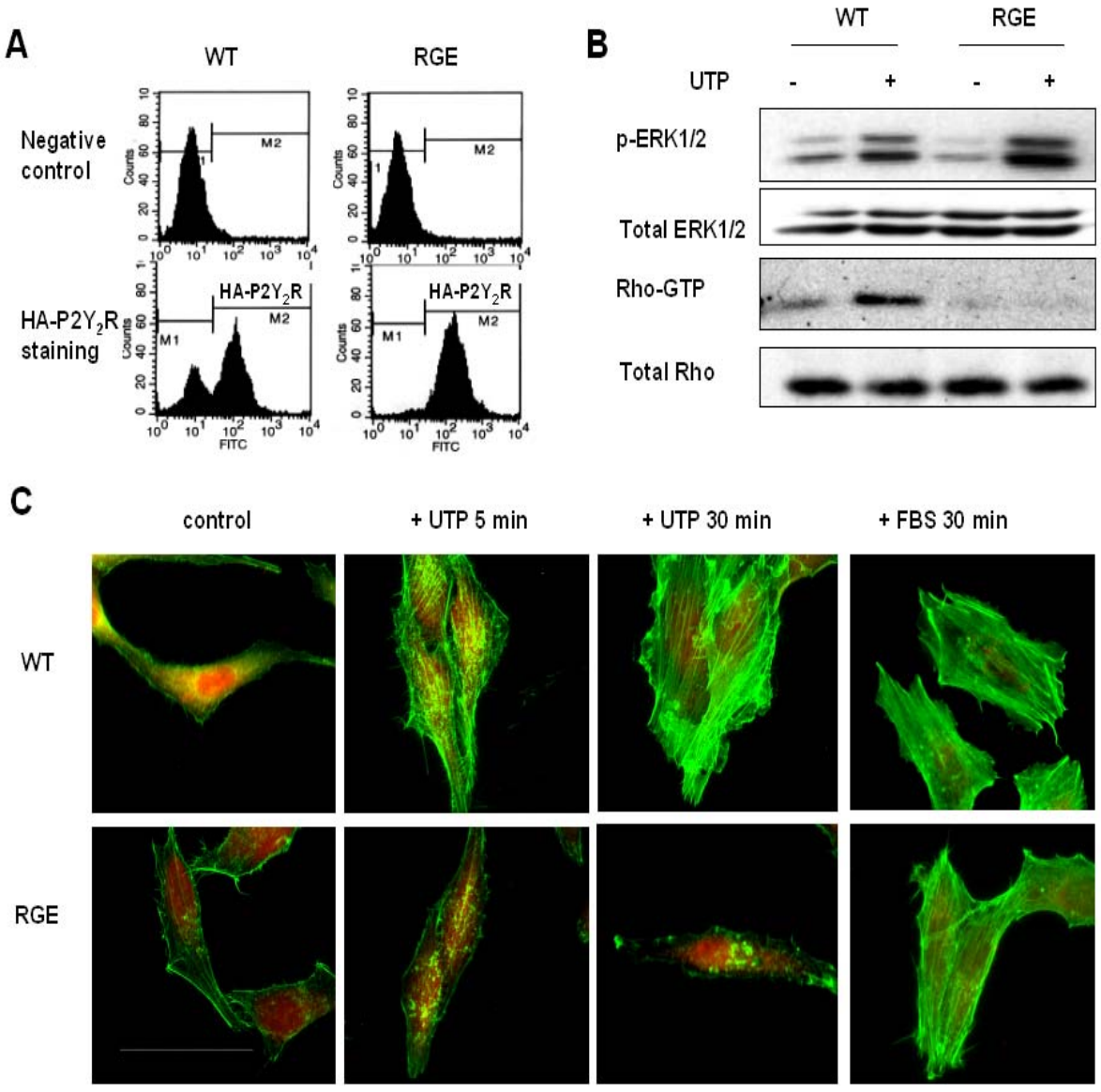

A
WT
RGE
Negative control
HA-P2Y2R staining
M1
M2
HA-P2Y2R
Counts
FITC
B
WT
RGE
UTP
- + - +
p-ERK1/2
Total ERK1/2
Rho-GTP
Total Rho
C
control
+ UTP 5 min
+ UTP 30 min
+ FBS 30 min
WT
RGE

Figure II-2. ERK1/2 and Rho activation mediated by WT and AAA mutant $P2Y_2$Rs. Human 1321N1 cells were transiently transfected with either WT (RGD) or AAA mutant $P2Y_2$R cDNA in pcDNA3.1(-). Transfected cells were starved overnight and stimulated with the indicated concentration of UTP for 5 min prior to measuring ERK1/2 phosphorylation (A) or Rho activity (B). (A) UTP-induced phosphorylation of ERK1/2 was expressed as fold increase over basal levels. Data points represent the means ± S.E.M. of results from three experiments. (B) The Rho-GTP band density was normalized to total Rho and relative intensities are shown below each band as means ± S.E.M. of results from four experiments.

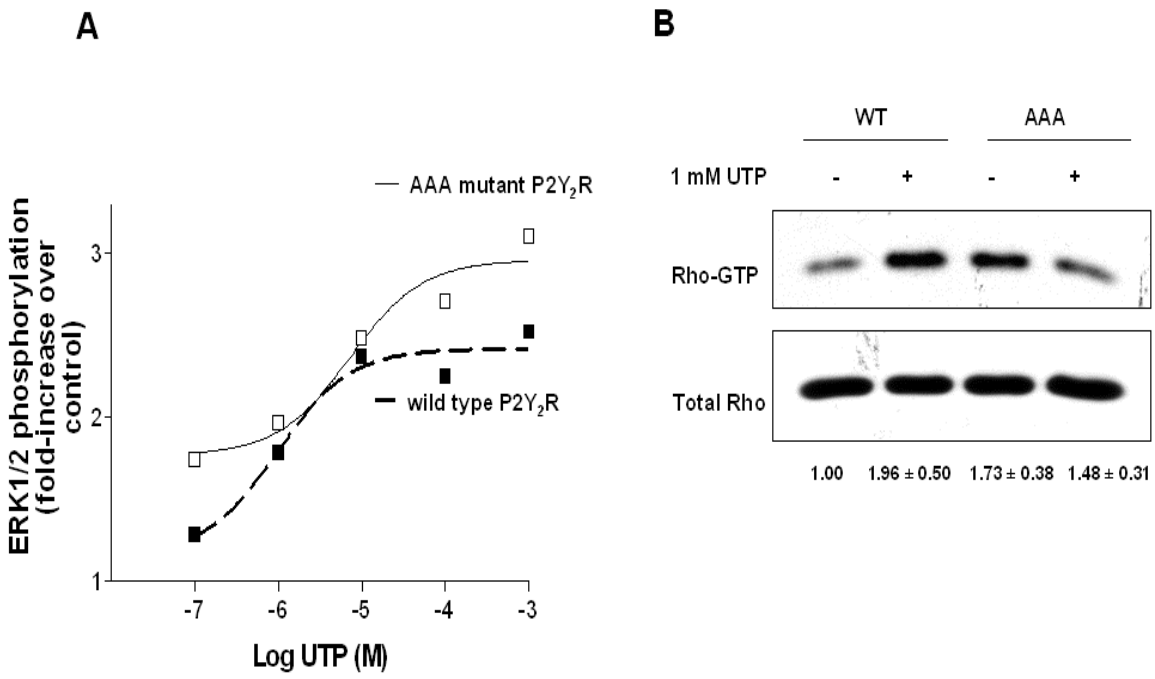

A
B
AAA mutant P2Y2R
wild type P2Y2R
ERK1/2 phosphorylation (fold-increase over control)
Log UTP (M)
WT
AAA
1 mM UTP
Rho-GTP
Total Rho
1.00
1.96 ± 0.50
1.73 ± 0.38
1.48 ± 0.31

Figure II-3. $P2Y_2R$-mediated Rho activation and stress fiber formation require α_v integrin activity. Human 1321N1 cells expressing the WT $P2Y_2R$ were incubated overnight at 37 °C in serum-free medium with 10 μg/ml anti-$\alpha_v\beta_5$ or anti-α_3 antibody. Cells were then treated with or without 100 μM UTP for 5 min prior to analysis of Rho activity (A) or 30 min prior to analysis of stress fiber formation (B). (A) Representative blots from three experiments are shown. (B) More than 100 cells were examined in three separate experiments, and representative images are shown. Bar = 50 μm.

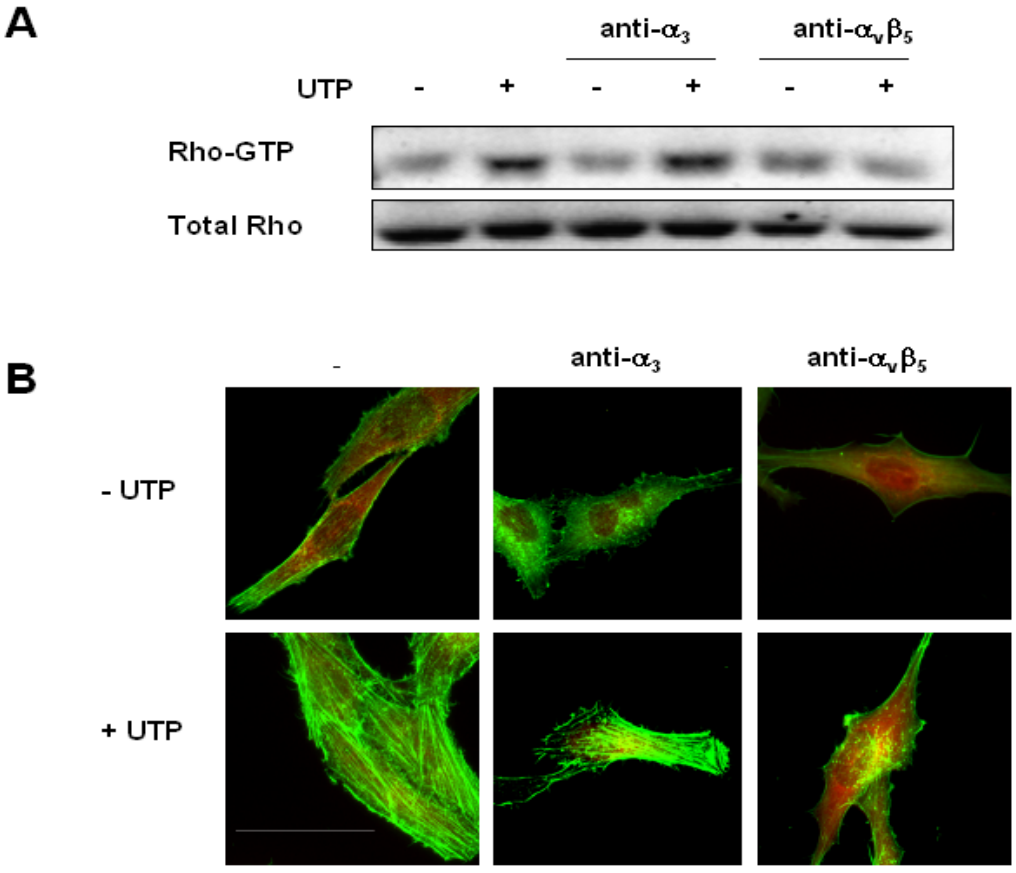

A
anti-α3
anti-αvβ5
UTP
-
+
-
+
-
+
Rho-GTP
Total Rho
B
-
anti-α3
anti-αvβ5
- UTP
+ UTP

Figure II-4. $P2Y_2R/\alpha_v$ integrin interaction regulates Rho-mediated signaling. (A and B) Human 1321N1 cells expressing the WT or RGE mutant $P2Y_2R$ were incubated with the indicated concentration of UTP for 5 min at 37 °C. Cell lysates were prepared and analyzed by immunoblotting with (A) anti-phospho-myosin light chain 2 (p-MLC-2) or (B) anti-phospho-cofilin antibodies. (C) Cells expressing the WT $P2Y_2R$ were incubated overnight with or without 10 μg/ml anti-$\alpha_v\beta_5$ or anti-α_3 antibody, then stimulated with 100 μM UTP for 5 min at 37 °C. Cell lysates were prepared and analyzed by immunoblotting with anti-phospho-cofilin antibodies. Relative intensities are shown below each band as means ± S.E.M. of results from three experiments. (D) Cells expressing the WT $P2Y_2R$ were pretreated at 37 °C in serum-free medium with the Rho-dependent kinase inhibitor Y-27632 (10 μM) for 1 h or 200 ng/ml *Bordetella pertussis* toxin (PTX) overnight, then stimulated with 100 μM UTP for 5 min at 37 °C. Cell lysates were analyzed by immunoblotting with anti-phospho-cofilin antibodies. Protein loading in each lane was evaluated by stripping the membrane of antibodies and re-probing with anti-actin antibodies. Blots representative of three-five experiments are shown.

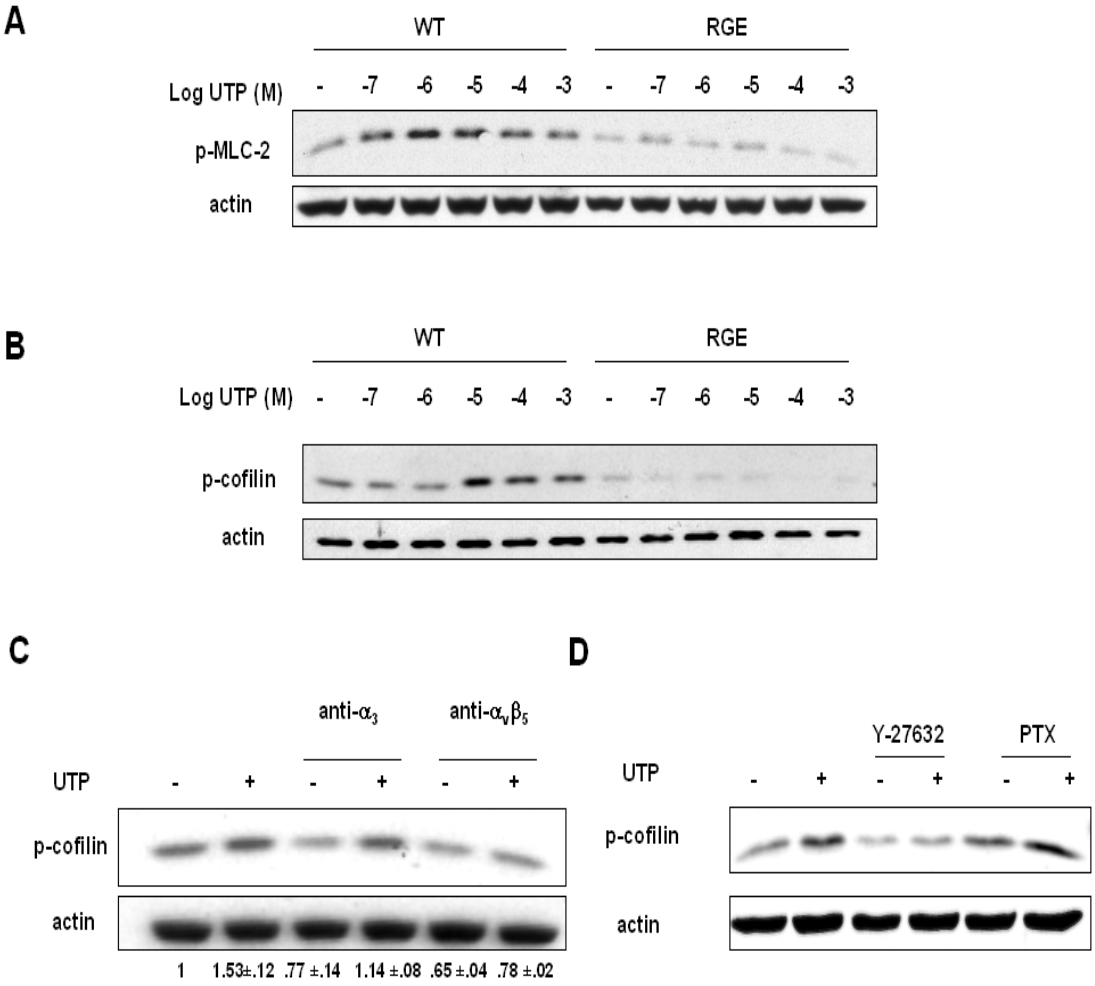

A
WT
RGE
Log UTP (M)
- -7 -6 -5 -4 -3 - -7 -6 -5 -4 -3
p-MLC-2
actin
B
WT
RGE
Log UTP (M)
- -7 -6 -5 -4 -3 - -7 -6 -5 -4 -3
p-cofilin
actin
C
anti-α3
anti-αvβ5
UTP
- + - + - +
p-cofilin
actin
1 1.53±.12 .77 ±.14 1.14 ±.08 .65 ±.04 .78 ±.02
D
Y-27632
PTX
UTP
- + - + - +
p-cofilin
actin

Figure II-5. $P2Y_2R/\alpha_v$ integrin interaction is required for G_{12} coupling. (A and B) Membrane preparations from 1321N1 cells expressing the WT or RGE mutant $P2Y_2R$ or pLXSN vector-transfected cells (negative control) were used in [^{35}S]GTPγS binding assays in the presence or absence of 1 mM UTP (see "Materials and Methods"). After termination of the assay, samples were immunoprecipitated with antiserum against (A) $G\alpha_{12}$ or (B) $G\alpha_{q/11}$ and radioactivity in the immunoprecipitates was calculated. Data are the means ± S.E.M. of results from three separate experiments and are shown as fold increase over untreated cells. (C) Human 1321N1 cells expressing the WT or RGE mutant $P2Y_2R$ were incubated with 1 mM UTP for 2 min at 37 °C. G_{12} activation was detected by immunoprecipitation (IP) of $G\alpha_{12}$ with anti-$G\alpha_{12}$ antibody and immunoblotting (IB) of $G\alpha_{12}$ with anti-phosphoserine/threonine antibody. $G_{q/11}$ activation was detected by IP with anti-phosphotyrosine antibody and IB with anti-$G\alpha_{q/11}$ antibody. Blots representative of three experiments are shown.

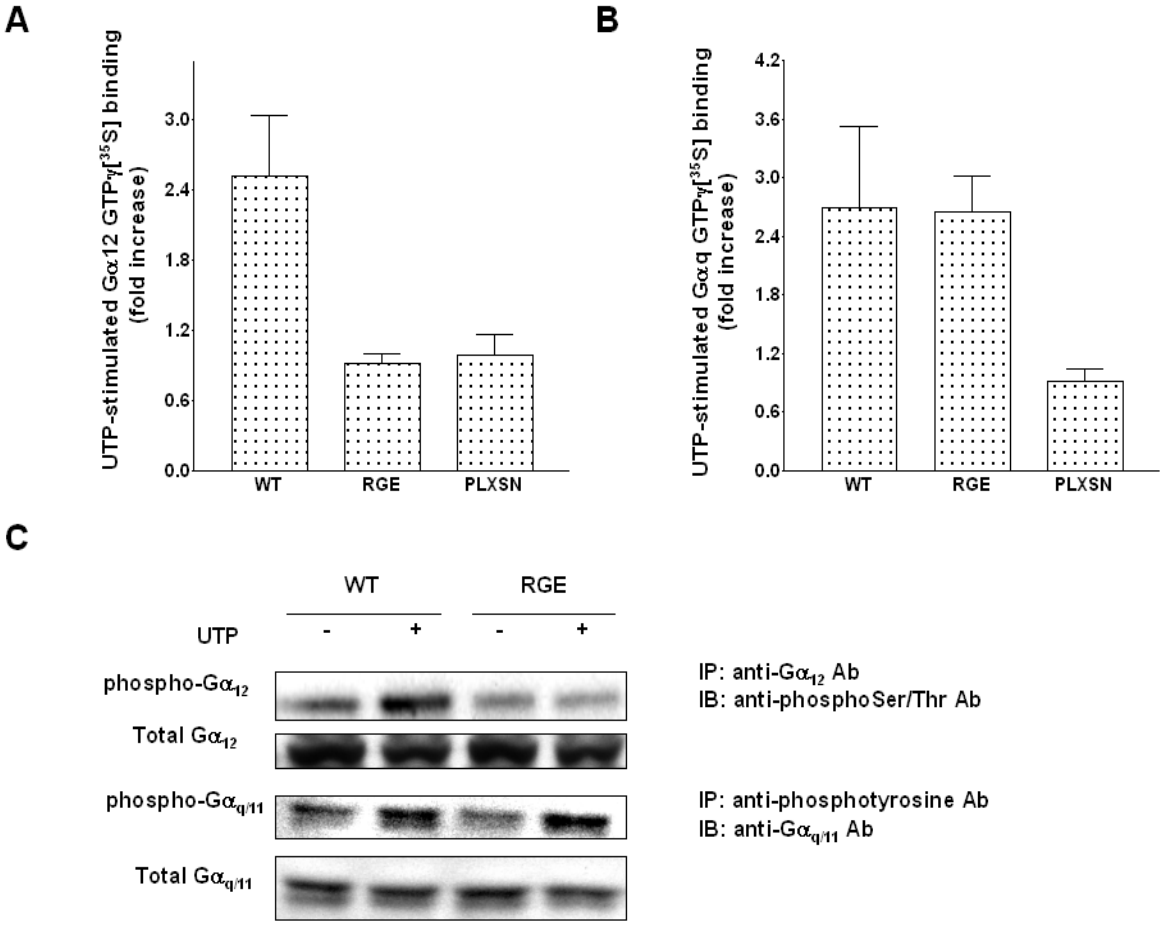
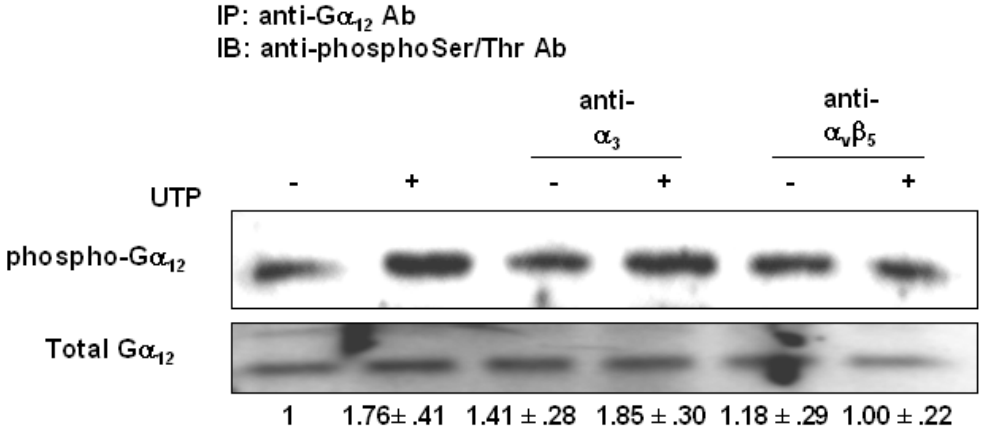

A
UTP-stimulated Gα12 GTPγ[35S] binding (fold increase)
3.0
2.4
1.8
1.2
0.6
0.0
WT
RGE
PLXSN
B
UTP-stimulated Gαq GTPγ[35S] binding (fold increase)
4.2
3.6
3.0
2.4
1.8
1.2
0.6
0.0
WT
RGE
PLXSN
C
WT
RGE
UTP
-
+
-
+
phospho-Gα12
Total Gα12
phospho-Gαq/11
Total Gαq/11
IP: anti-Gα12 Ab
IB: anti-phosphoSer/Thr Ab
IP: anti-phosphotyrosine Ab
IB: anti-Gαq/11 Ab

Figure II-6. $P2Y_2R$-mediated G_{12} activation requires α_v integrin expression and activity. (A) Cells expressing the WT $P2Y_2R$ were incubated overnight at 37 °C in serum-free medium with 10 μg/ml anti-$\alpha_v\beta_5$ or anti-α_3 antibody, then stimulated with 100 μM UTP for 2 min at 37 °C. $G\alpha_{12}$ phosphorylation was detected, as described in Fig. 5. Relative intensities are shown below each band as means ± S.E.M. of results from four experiments. (B) Cells expressing the WT $P2Y_2R$ were transfected with 5 μg of α_v antisense (AS) or sense (S) oligonucleotides, as described (Bagchi et al., 2005). Lipofectamine transfected cells served as a negative control. Expression of α_v integrin in these cells was determined by immunoblot analysis and a representative blot is shown. UTP-induced binding of [^{35}S]GTPγS to $G\alpha_{12}$ was determined, as described in Fig. 5. Data are the means ± S.E.M. of results from three separate experiments and are shown as fold increase over untreated cells.

A


IP: anti-Gα12 Ab
IB: anti-phosphoSer/Thr Ab
anti-α3
anti-αvβ5
UTP
- + - + - +
phospho-Gα12
Total Gα12
1 1.76± .41 1.41 ± .28 1.85 ± .30 1.18 ± .29 1.00 ± .22

B

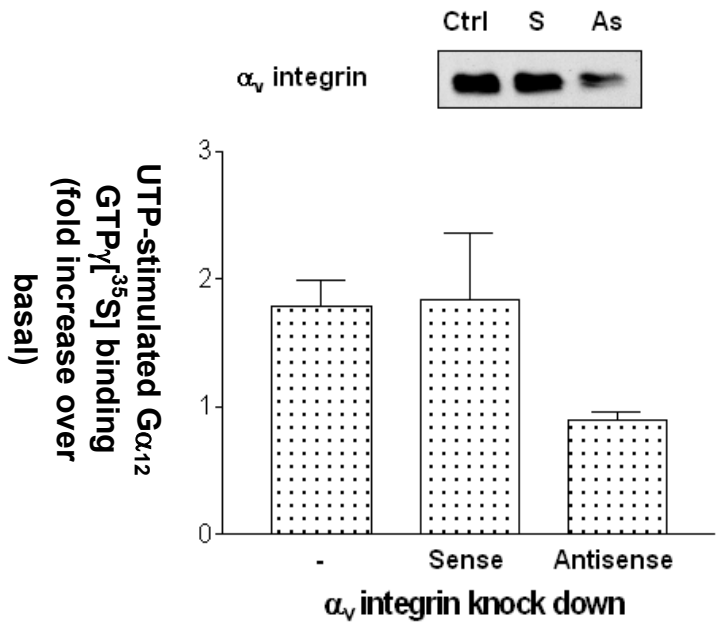

Ctrl S As
αv integrin
UTP-stimulated Gα12 GTPγ[35S] binding (fold increase over basal)
3
2
1
0
-
Sense
Antisense
αv integrin knock down

Figure II-7. The $P2Y_2R$ accesses G_{12} in a complex with α_v integrins. (A) RGD-dependent association of the $P2Y_2R$ with α_v integrins and $G\alpha_{12}$. Cells expressing the HA-tagged WT or RGE mutant $P2Y_2R$ and endogenous $G_{q/11}$, G_{12} and α_v integrins were treated with or without 1 mM UTP for 5 min at 37 °C and lysates were subjected to IP with mouse anti-HA antibody conjugated to agarose beads and IB with anti-$G\alpha_{12}$, anti-$G\alpha_{q/11}$, anti-α_v integrin, or anti-HA antibody. Cells transfected with the pLXSN vector served as a negative control. (B) $G\alpha_{12}$ associates with α_v integrins. Human 1321N1 cells expressing cDNAs for the WT $P2Y_2R$ and $G\alpha_{12}$ were treated with or without 100 μM UTP for 5 min at 37 °C and lysates were subjected to IP with IgG, anti-α_3 integrin, or anti-α_v integrin antibody followed by IB with anti-$G\alpha_{12}$, anti-α_3 integrin or anti-α_v integrin antibody. Blots representative of three experiments are shown.

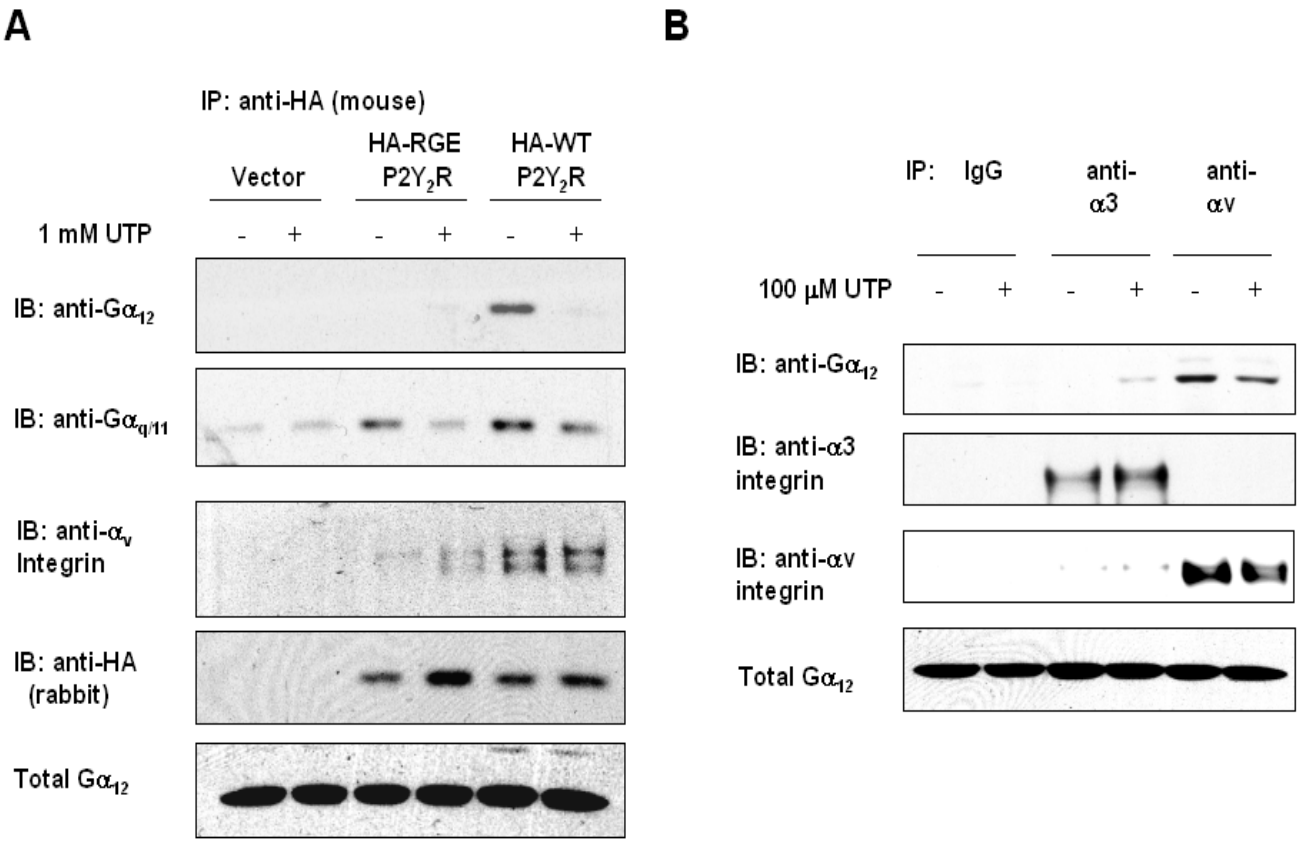

A
IP: anti-HA (mouse)
Vector
HA-RGE P2Y2R
HA-WT P2Y2R
1 mM UTP
- + - + - +
IB: anti-Gα12
IB: anti-Gαq/11
IB: anti-αv Integrin
IB: anti-HA (rabbit)
Total Gα12
B
IP: IgG
anti-α3
anti-αv
100 μM UTP
- + - + - +
IB: anti-Gα12
IB: anti-α3 integrin
IB: anti-αv integrin
Total Gα12

Figure II-8. Co-immunostaining of $G\alpha_{12}$ and $G\alpha_o$ in cells exposed to a UTP gradient. Human 1321N1 cells expressing wild type $P2Y_2Rs$ were plated on chambered coverslides and incubated overnight in 300 μl serum-free medium at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. UTP (1 μl of a 100 mM solution) was added at the bottom left and after 30 min at 37 °C, cells were washed, fixed, permeabilized and stained with rabbit anti-$G\alpha_{12}$ and mouse anti-$G\alpha_o$ antibodies. Texas Red-conjugated anti-rabbit IgG and Alexa Fluor 488 conjugated anti-mouse IgG were then used to localize $G\alpha_{12}$ and $G\alpha_o$, respectively. The slides were examined by fluorescence microscopy, as described in "Materials and Methods", and images representative of three experiments are shown. Arrows indicate membrane protrusions indicative of lamellipodia formation.

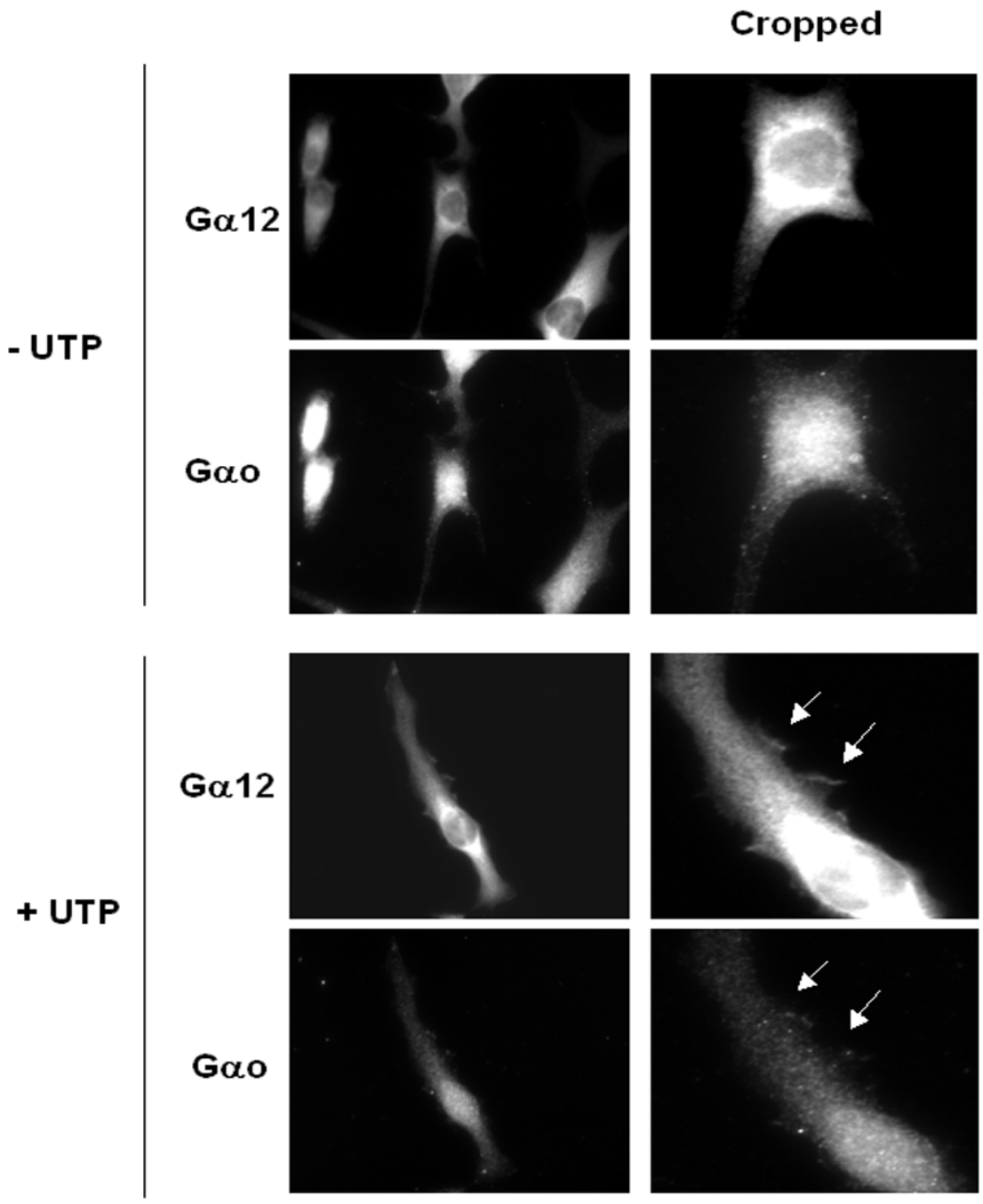
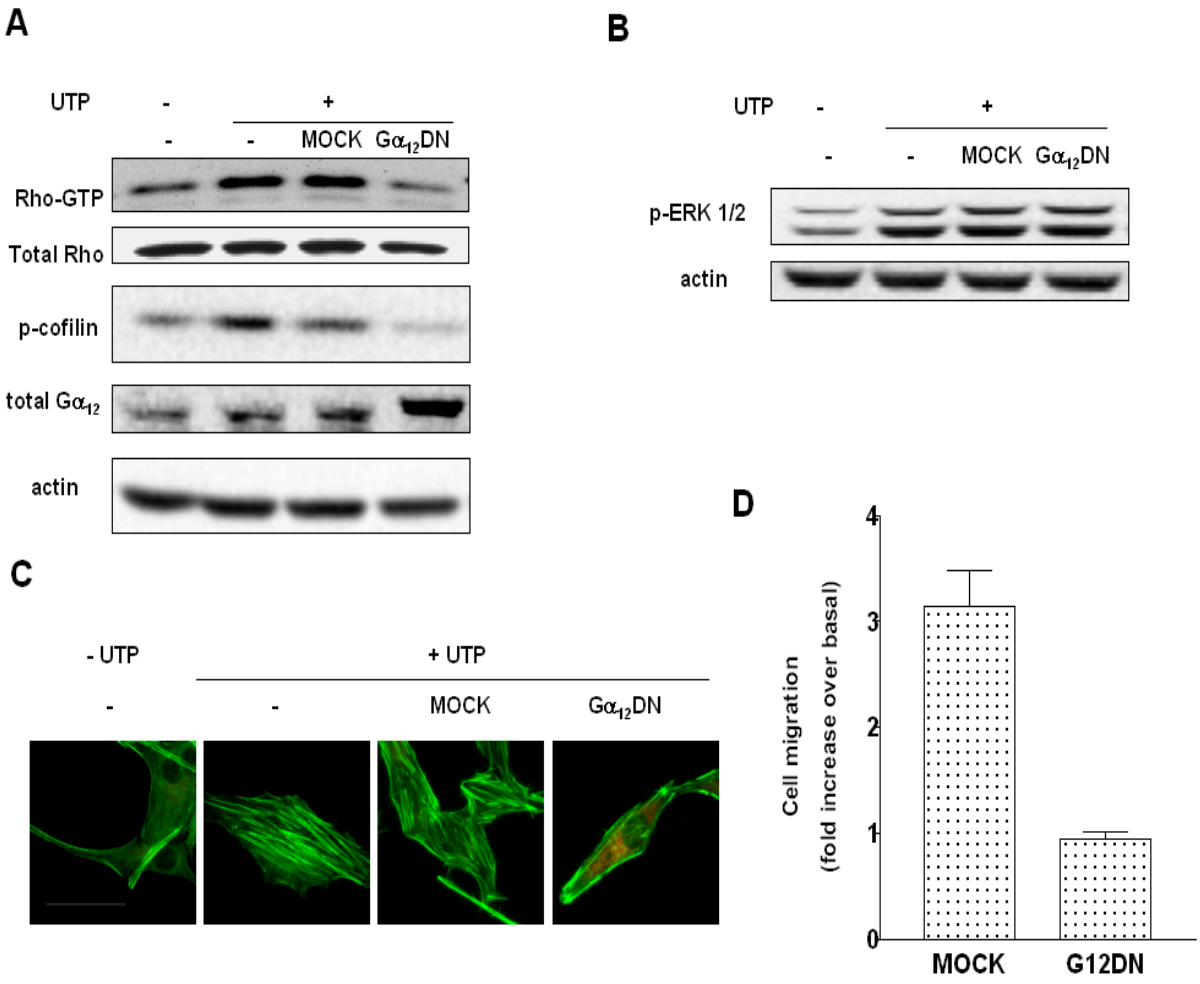
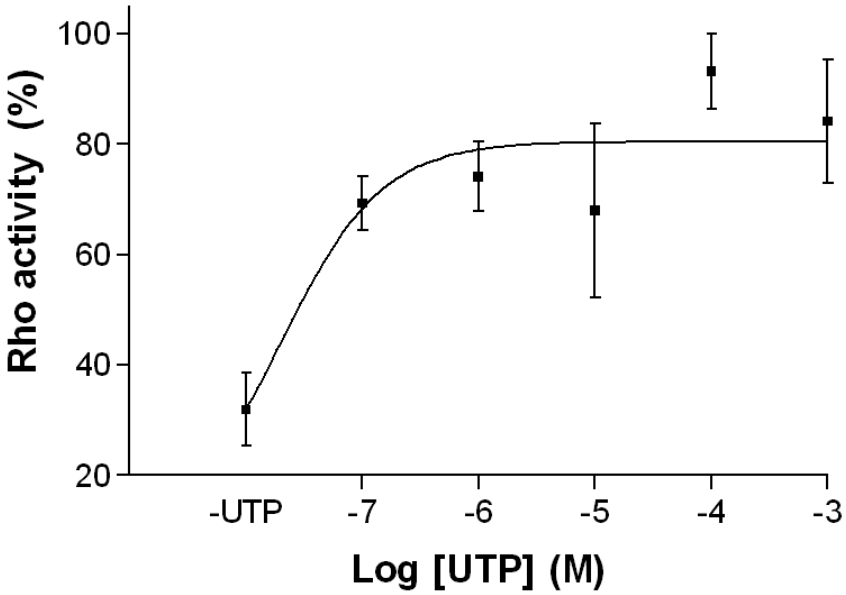

Cropped
- UTP
Gα12
Gαo
+ UTP
Gα12
Gαo

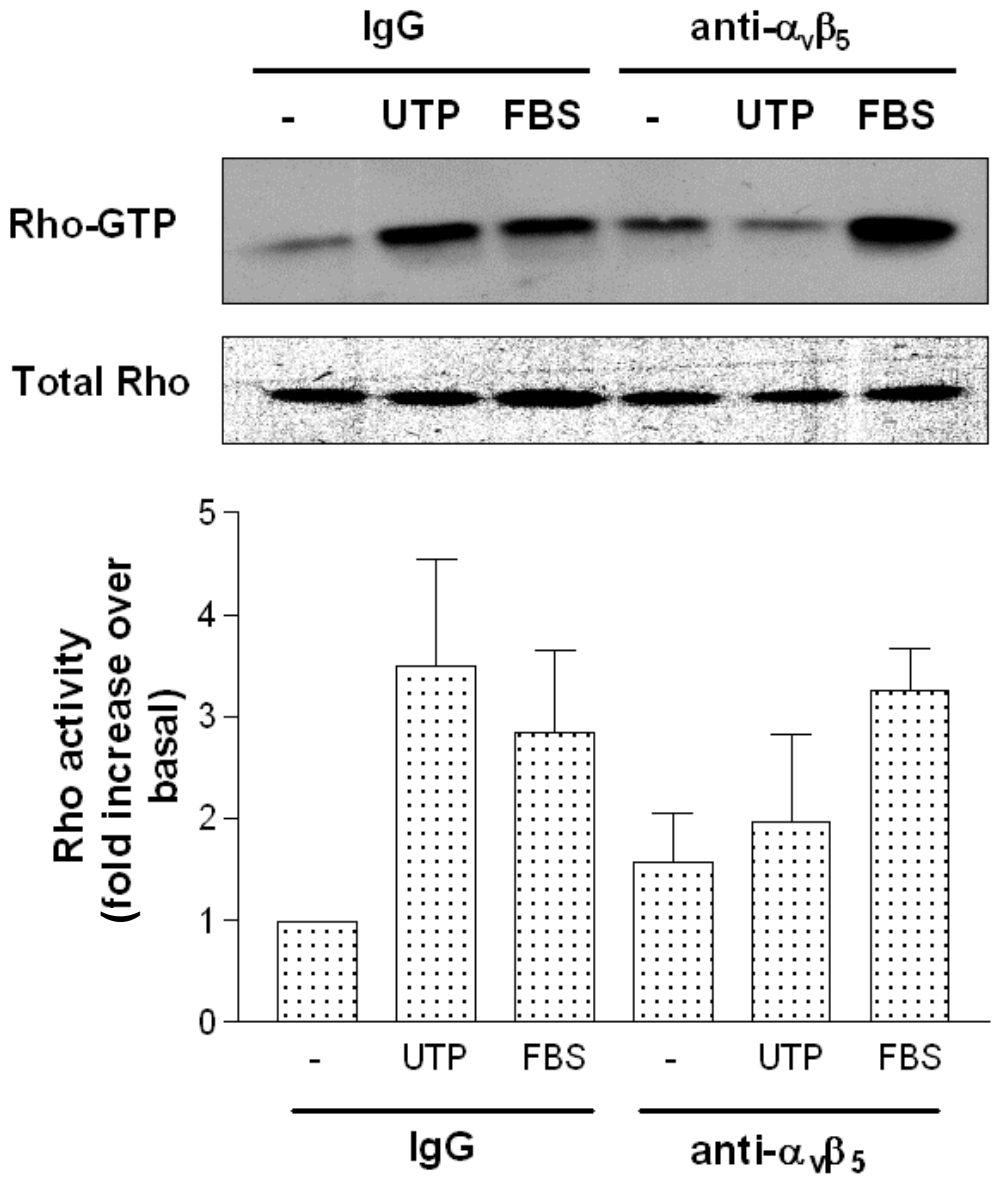
Figure II-9. Effect of dominant-negative $G\alpha_{12}$ ($G\alpha_{12}$ DN) on signaling events mediated by the $P2Y_2R$. (A-D) Human 1321N1 cells expressing the WT $P2Y_2R$ were cultured to 80% confluence and transiently transfected with the $G\alpha_{12}$DN mutant in the pcDNA3.1+ vector. MOCK: vector transfected negative control. Transfected cells were incubated with 100 μM UTP for 5 min (A and B) or 30 min (C) at 37 °C and assayed for (A) Rho activity and cofilin phosphorylation, (B) ERK1/2 phosphorylation and (C) stress fiber formation, as described in "Materials and Methods". Total $G\alpha_{12}$ was detected with anti-$G\alpha_{12}$ antibody, as shown in (A). Antibody-labeled $G\alpha_{12}$ in (C) is shown in red and phalloidin-labeled filamentous actin is shown in green. (D) Cells (5×10^4) transfected with the indicated constructs were seeded into the upper chamber of Transwells. Lower chambers contained serum-free medium with or without 100 μM UTP. Cell migration was evaluated 16 h after UTP stimulation at 37 °C and expressed as the fold increase in the number of cells that moved across the Transwell membranes in response to UTP as compared to untreated controls. (A and B) Blots representative of three-five experiments are shown. (C) Images representative of three separate experiments are shown. Bar = 40 μm. (D) Data shown are the means ± S.E.M. of results from five experiments.


A
UTP
-
+
-
-
MOCK
Gα12DN
Rho-GTP
Total Rho
p-cofilin
total Gα12
actin
B
UTP
-
+
-
-
MOCK
Gα12DN
p-ERK 1/2
actin
C
- UTP
+ UTP
-
-
MOCK
Gα12DN
D
Cell migration
(fold increase over basal)
0
1
2
3
4
MOCK
G12DN

Supplemental Figure II-S1. UTP induces a dose-dependent activation of Rho in 1321N1 cells expressing $P2Y_2$ receptors. Human 1321N1 cells expressing the WT $P2Y_2R$ were treated with the indicated concentration of UTP for 5 min at 37 °C. Rho activity was then measured, as described in "Materials and Methods". UTP-induced Rho activation is expressed as a percentage of maximum response levels. Data points represent the means ± S.E.M. of results from three experiments.


Rho activity (%)
100
80
60
40
20
-UTP
-7
-6
-5
-4
-3
Log [UTP] (M)

Supplemental Figure II-S2. Effects of anti-$\alpha_v\beta_5$ integrin antibody on UTP- and FBS-stimulated Rho activation. Human 1321N1 cells expressing the WT $P2Y_2R$ were incubated overnight at 37 °C in serum-free medium with 10 μg/ml of anti-IgG or anti-$\alpha_v\beta_5$ antibody. Cells were then treated with or without 100 μM UTP or 10% (v/v) fetal bovine serum (FBS) for 5 min at 37 °C prior to analysis of Rho activity. Representative blots and relative intensities as means ± S.E.M. of results from three experiments are shown.


IgG
anti-αvβ5
- UTP FBS - UTP FBS
Rho-GTP
Total Rho
Rho activity
(fold increase over
basal)
0
1
2
3
4
5
- UTP FBS - UTP FBS
IgG
anti-αvβ5

CHAPTER III

The $P2Y_2$ nucleotide receptor interacts with α_v integrins to activate G_o and induce cell migration

Abstract

Extracellular ATP and UTP induce chemotaxis, or directed cell migration, by stimulating the G protein-coupled $P2Y_2$ nucleotide receptor ($P2Y_2R$). Previously, we found that an arginine-glycine-aspartic acid (RGD) integrin-binding domain in the $P2Y_2R$ enables this receptor to interact selectively with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, an interaction that is prevented by mutation of the RGD sequence to arginine-glycine-glutamic acid (RGE) (Erb et al., 2001). This RGD domain also was found to be necessary for coupling the $P2Y_2R$ to G_o- but not G_q-mediated intracellular calcium mobilization, leading us to investigate the role of $P2Y_2R$ interaction with integrins in nucleotide-induced chemotaxis. Here we show that mutation of the RGD sequence to RGE in the human $P2Y_2R$ expressed in 1321N1 astrocytoma cells completely prevented UTP-induced chemotaxis as well as activation of G_o, Rac, and vav2, a guanine nucleotide exchange factor for Rac. $P2Y_2R$-mediated chemotaxis, Rac and vav2 activation were inhibited by pretreatment of the cells with anti-$\alpha_v\beta_5$ integrin antibodies or the $G_{i/o}$ inhibitor, pertussis toxin. Thus, the RGD-dependent interaction between the $P2Y_2R$ and α_v integrins is necessary for the $P2Y_2R$ to activate G_o and to initiate G_o-mediated signaling events leading to chemotaxis.

Introduction

Chemotaxis is the movement of a cell in response to a chemical gradient and is required for many physiological events, including embryonic development, immune system function, and wound healing (Cox and Huttenlocher, 1998; Sanchez-Madrid and del Pozo, 1999; Van Haastert and Devreotes, 2004). The process of chemotaxis is also important for understanding diseases that result from abnormal cell migration, such as chronic inflammation, atherosclerosis, and cancer metastasis. To migrate, cells must establish dynamic and highly regulated adhesive interactions with the extracellular matrix, which are mediated by integrin receptors (Cox and Huttenlocher, 1998). For example, recent studies have shown that α_v integrins play an important role in controlling cell adhesion, spreading, and motility in several cell types, including human vascular smooth muscle cells and pancreatic beta cells (Kaido et al., 2004; Stawowy et al., 2004). Upon activation, many types of integrin receptors cluster together and recruit a host of cytoskeletal and cytoplasmic proteins into specialized adhesive structures called focal adhesions. These focal adhesion complexes not only serve as a physical link between the extracellular and intracellular matrix but also are important sites of signal transduction for integrins and many other types of receptors that mediate cell migration (Martin et al., 2002). Chemotaxis also requires a cell to assume a polarized morphology that is controlled by cell surface receptors that activate the Rho family of GTPases, including Cdc42, Rac, and Rho (Etienne-Manneville and Hall, 2002; Burridge and Wennerberg, 2004). Upon activation of a chemoattractant receptor, Cdc42 and Rac localize at the leading edge of a cell and control directional movement and the formation of lamellipodia containing highly branched actin filaments, respectively (Burridge and Wennerberg, 2004). Rho localizes at the rear and sides of a cell and controls the

formation of contractile actin-myosin stress fibers (Xu et al., 2003b). Together, these GTPases promote cell migration towards a chemoattractant by mediating extension of the actin cytoskeleton at the front edge of the cell and retraction of the cytoskeleton at the rear edge of the cell.

Recent studies have shown that G protein-coupled receptors (GPCRs) regulate Rac and Rac-dependent lamellipodia formation by activating the $G_{i/o}$ family of heterotrimeric G proteins, whereas activation of Rho and Rho-dependent stress fiber formation are controlled by the $G_{12/13}$ family (Xu et al., 2003b). Furthermore, studies have shown that the $\beta\gamma$ subunits of $G_{i/o}$ are responsible for activation of Rac guanine nucleotide exchange factors (RacGEFs) that, in turn, activate Rac, whereas the α subunits of $G_{12/13}$ are responsible for activation of RhoGEFs (Neptune and Bourne, 1997; Burridge and Wennerberg, 2004).

The $P2Y_2$ nucleotide receptor ($P2Y_2R$), a GPCR activated by extracellular ATP or UTP, is an important regulator of monocyte chemotaxis (Seye et al., 2002). Although the $P2Y_2R$ is normally expressed in monocytes, neutrophils, and other immune cells (Jin et al., 1998), recent studies have shown that the $P2Y_2R$ is up-regulated in epithelial and vascular tissue in response to injury or stress (Seye et al., 1997). Upon activation, the $P2Y_2R$ mediates a variety of biological functions, including mitogenesis, angiogenesis, vasodilation, chemotaxis, and inflammation (Satterwhite et al., 1999; Chaulet et al., 2001). Furthermore, recent work from our group has demonstrated that arterial stress in rabbit carotid arteries induces up-regulation of the $P2Y_2R$ in vascular endothelium and smooth muscle and that *in vivo* activation of the $P2Y_2R$ by UTP leads to a 7-fold increase in intimal thickening and a 4-fold increase in leukocyte infiltration into the neointima

(Seye et al., 2002). Thus, the $P2Y_2R$ represents an exciting target for wound healing as well as chronic inflammatory diseases.

Previously, we reported that the $P2Y_2R$ contains a consensus RGD integrin-binding domain in the first extracellular loop that interacts with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Erb et al., 2001). Furthermore, we found that the RGD domain in the $P2Y_2R$ is required for G_o- but not G_q-mediated calcium signaling (Erb et al., 2001), leading us to speculate that α_v integrin interaction with the $P2Y_2R$ is important for nucleotide-induced chemotaxis. The purpose of the present study was to evaluate the role of α_v integrin interaction with the $P2Y_2R$ in nucleotide-induced chemotaxis and to identify upstream signaling events involved in $P2Y_2R$-mediated chemotaxis.

Results

An RGD integrin-binding domain in the $P2Y_2R$ is required for UTP-induced cell migration

We previously reported that interaction between the $P2Y_2R$ and α_v integrins is prevented by mutation of the $P2Y_2R$ RGD sequence to RGE (Erb et al., 2001). To determine whether the RGD integrin-binding domain in the $P2Y_2R$ is important for $P2Y_2R$-mediated chemotaxis, a modified Boyden chamber assay was used. The results showed that UTP induced a concentration-dependent increase in the migration of cells transfected with the WT $P2Y_2R$ in contrast to cells transfected with the RGE mutant $P2Y_2R$ or vector alone (Fig. III-1). Also, we found that $P2Y_2R$-transfected cells did not migrate significantly when UTP was placed in the upper chamber instead of the lower chamber or when equal concentrations of UTP were placed in both chambers (data not

shown), indicating that UTP acts as a chemoattractant in cells expressing the WT $P2Y_2R$. The cell transfectants expressed similar surface levels of WT and RGE mutant $P2Y_2Rs$ and α_v integrin, suggesting that variations in the magnitude of UTP-induced migration were not due to differences in cell-surface protein levels (Bagchi et al., 2005). Thus, the RGD integrin-binding domain of the $P2Y_2R$ is apparently required for UTP-induced chemotaxis.

The $P2Y_2R$ RGD domain is required for UTP-induced activation of vav2 and Rac

The Rac subfamily of small GTPases stimulates the formation of lamellipodia and is necessary for cell migration (Allen et al., 1997; Hall, 1998). Furthermore, the efficiency of Rac signaling is dependent on RacGEFs, such as vav2 (Das et al., 2000), an ubiquitously expressed member of the vav family of RhoGEFs that is necessary for lamellipodia formation and activation of Cdc42, Rac1 and RhoA (Schuebel et al., 1998; Abe et al., 2000; Liu and Burridge, 2000; Marignani and Carpenter, 2001). Taking into consideration the importance of Rac and vav2 in cell motility, we determined whether Rac and vav2 are activated by UTP and the relationship of these activities to RGD-dependent $P2Y_2R$ interaction with α_v integrins. We found that even at very high UTP concentrations (*e.g.*, 1 mM), vav2 and Rac were only activated in cells expressing the WT $P2Y_2R$ but not in cells expressing the RGE mutant $P2Y_2R$ (Fig. III-2), suggesting that the $P2Y_2R$ requires interaction with α_v integrins to activate Rac and vav2. Furthermore, transient transfection of 1321N1 cells expressing the WT $P2Y_2R$ with a dominant negative mutant of vav2 (vav2 DN) abolished UTP-induced Rac1 activation (Bagchi et al., 2005), consistent with the role of vav2-dependent Rac1 activation in

lamellipodia formation and cell spreading in other cell lines (Marignani and Carpenter, 2001).

Functional activity of α_v integrins is essential for UTP-induced cell migration, vav2 and Rac activation

To further evaluate the role of α_v integrins in $P2Y_2R$-mediated cell migration, we tested the effect of anti-integrin Abs on UTP-induced migration and upstream signaling events in 1321N1 cells transfected with the WT $P2Y_2R$. The increase in migration of $P2Y_2R$-transfected cells in response to UTP was inhibited by anti-α_v and anti-$\alpha_v\beta_5$ Abs in a dose-dependent manner, whereas negative control Ab (anti-IgG) did not inhibit UTP-induced cell migration (Bagchi et al., 2005). UTP-induced chemokinesis of $P2Y_2R$-transfected cells was also inhibited by anti-$\alpha_v\beta_5$ Ab at a concentration of 50 μg/ml (Bagchi et al., 2005), indicating that α_v integrins are required for UTP-induced cell migration. Likewise, Rac and vav2 activation in response to UTP were significantly inhibited by treatment with anti-$\alpha_v\beta_5$ Ab, but not with Ab against another integrin subtype (α_3) that is also expressed in 1321N1 cells (Fig. III-3). Note: fluorescence-activated cell sorter analysis indicated that $\alpha_v\beta_5$ and α_3 integrins, but not $\alpha_v\beta_3$, are expressed in 1321N1 cells (data not shown).

Involvement of G_o signaling in $P2Y_2R$-mediated cell migration

Pertussis toxin (PTX), which inhibits $G_{i/o}$ signaling, has been shown to block chemotaxis mediated by chemokine receptors (Haque et al., 2004). Previous work from our

laboratory has shown that PTX partially inhibits intracellular Ca^{2+} mobilization mediated by the WT $P2Y_2R$, but does not block Ca^{2+} mobilization mediated by the RGE mutant $P2Y_2R$, suggesting that the RGD integrin-binding domain is required for the $P2Y_2R$ to activate G_o (Erb et al., 2001). Here, we directly investigated whether the RGD domain in the $P2Y_2R$ is required for activation of G_o. Accordingly, membrane extracts were prepared from 1321N1 cells expressing the WT or RGE mutant $P2Y_2R$ and stimulated with 1 mM UTP in the presence of [^{35}S]GTPγS. Immunoprecipitation of $G\alpha_o$ or $G\alpha_q$ with anti-$G\alpha_o$ or anti-$G\alpha_{q/11}$ Abs indicated that UTP induced a 2-fold increase in [^{35}S]GTPγS binding to $G\alpha_o$ in cells expressing the WT $P2Y_2R$, whereas no increase in [^{35}S]GTPγS binding to $G\alpha_o$ was seen in cells expressing the RGE mutant receptor (Bagchi et al., 2005). In contrast, activation of both the WT and RGE mutant $P2Y_2R$ by UTP induced a 2-fold increase in [^{35}S]GTPγS binding to $G\alpha_q$ (Bagchi et al., 2005). These results directly show that the RGD integrin-binding domain in the $P2Y_2R$ is required for activation of G_o, but not G_q, and suggest that integrin interaction with the $P2Y_2R$ is important for access to specific heterotrimeric G proteins involved in cell migration.

Furthermore, we found that PTX inhibited UTP-induced cell migration by 70-80% in 1321N1 cells expressing the WT $P2Y_2R$ (Bagchi et al., 2005), indicating that G_o activation is involved in $P2Y_2R$-mediated cell migration. PTX also completely inhibited UTP-induced vav2 and Rac activation (Fig. III-4) and vitronectin up-regulation (Bagchi et al., 2005) in 1321N1 cells expressing the WT $P2Y_2R$, indicating that all of these responses are dependent on G_o activity.

$P2Y_2R$-mediated Akt phosphorylation does not require integrin interaction

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is known to regulate actin reorganization and cell migration by some, but not all, chemokine receptors (Firtel and Chung, 2000; Cronshaw et al., 2004), and a recent study has shown that extracellular ATP and UTP can increase migration of human umbilical vein endothelial cells (HUVEC) in a PI3K-dependent manner (Kaczmarek et al., 2005). Therefore, we investigated whether the PI3K/Akt pathway is involved in chemotaxis mediated by the $P2Y_2R$ expressed in 1321N1 cells. We found that the PI3K inhibitor LY294002 inhibited Akt phosphorylation induced by UTP but failed to significantly inhibit UTP-induced migration of 1321N1 cells expressing the WT $P2Y_2R$ (data not shown). We also found that both the WT and RGE mutant $P2Y_2R$ could activate Akt but the RGE mutant $P2Y_2R$ required ~1000-fold higher concentrations of UTP, as compared with the WT $P2Y_2R$ (Fig. III-5), which is similar to the agonist potency of the RGE mutant $P2Y_2R$ in the stimulation of intracellular Ca^{2+} mobilization, ERK1/2 phosphorylation (Erb et al., 2001) and inositol phosphate accumulation (Qi et al., 2005). Thus, at high UTP concentrations, both the WT and the RGE mutant $P2Y_2Rs$ are equally efficacious in the activation of Akt, ERK1/2, intracellular Ca^{2+} mobilization, and inositol phosphate formation. And, although untested, it was recently suggested that the RGE mutation in the $P2Y_2R$ may be affecting these pathways by decreasing the agonist binding affinity of the $P2Y_2R$ (Qi et al., 2005). We next used PTX to evaluate the role of G_o in $P2Y_2R$-mediated Akt phosphorylation. PTX caused a 50% inhibition of Akt phosphorylation induced by UTP in 1321N1 cells expressing the WT $P2Y_2R$, whereas Akt phosphorylation was insensitive to PTX treatment in cells expressing the RGE mutant receptor (Bagchi et al., 2005). Therefore, we conclude that $P2Y_2R$-mediated Akt phosphorylation in 1321N1 cells occurs by both G_o- and

G_q-dependent pathways, although UTP-induced cell migration is largely G_o-dependent. Moreover, the involvement of the PI3K/Akt pathway in $P2Y_2R$-mediated migration appears to be cell type specific and does not play a significant role in $P2Y_2R$-mediated chemotaxis in 1321N1 cells.

Discussion

Previously, we found that an integrin-binding sequence (RGD) in the G protein-coupled $P2Y_2R$, promotes interaction with $\alpha_v\beta_3/\beta_5$ integrins and is required for nucleotide-induced activation of G_o- but not G_q-mediated intracellular calcium mobilization (Erb et al., 2001). In the present study, we have shown that the $P2Y_2R$ agonist UTP can induce migration of human 1321N1 cells expressing the WT $P2Y_2R$ but not a $P2Y_2R$ in which the RGD sequence was mutated to RGE (Fig. III-1), a sequence change that prevents integrin interaction (Erb et al., 2001). Our results strongly suggest that interaction of the $P2Y_2R$ with α_v integrins is essential for UTP-induced cell migration and this report is the first demonstration that a GPCR can interact with an integrin to facilitate cell migration. In addition, two other lines of evidence demonstrate the involvement of α_v integrins in $P2Y_2R$-mediated cell migration. First, selective inhibition of α_v integrin expression by antisense oligonucleotide inhibited UTP-induced cell migration of 1321N1 cells expressing the WT $P2Y_2R$ (Bagchi et al., 2005). Second, anti-α_v and anti-$\alpha_v\beta_5$ Abs inhibited UTP-induced migration of WT $P2Y_2R$ transfected cells (Bagchi et al., 2005). These observations are consistent with other reports indicating that integrin activity is required for both GPCR-mediated (Chernyavsky et al., 2004; Idzko et al., 2004) and

growth factor receptor-mediated cell migration (Meyer et al., 2004; Clemmons and Maile, 2005).

During cell migration, integrins attach to and detach from their extracellular matrix ligands to provide the necessary traction for cell crawling (Zhelev and Alteraifi, 2002). It also has been demonstrated that in migrating neutrophils, the active form of the $\alpha_v\beta_3$ integrin is concentrated at the leading edge of the cell (Lawson and Maxfield, 1995; Kindzelskii et al., 1996; Sanchez-Madrid and del Pozo, 1999) and that this integrin cycles between an active (*i.e.*, extracellular matrix-bound form that is tyrosine phosphorylated on the β_3 subunit) and an inactive conformation in a calcium- and calcineurin-dependent manner (Lawson and Maxfield, 1995). In the chemotaxis assays performed in our study, the Transwell membranes were not coated with extracellular matrix proteins, and thus we presumed that activation of the $P2Y_2R$ induced the synthesis and secretion of extracellular matrix proteins needed to facilitate cell migration. We demonstrated that UTP increased the expression of the extracellular matrix proteins vitronectin and osteopontin (Bagchi et al., 2005) in 1321N1 cells expressing the WT but not the RGE mutant $P2Y_2R$, suggesting that integrin interaction also is required for up-regulation of extracellular matrix proteins by the $P2Y_2R$. Furthermore, anti-vitronectin Abs inhibited UTP-induced migration of 1321N1 cells expressing the WT receptor (Bagchi et al., 2005). From these observations, it seems likely that $P2Y_2R$-mediated cell migration is enhanced by the synthesis and secretion of extracellular matrix ligands for $\alpha_v\beta_3/\beta_5$ integrins. Many extracellular matrix proteins like osteopontin and vitronectin contain RGD domains that interact with $\alpha_v\beta_3/\beta_5$ integrins (Chaulet et al., 2001). Therefore, it also seems likely that the $P2Y_2R$ and extracellular matrix proteins compete for binding to $\alpha_v\beta_3/\beta_5$ integrins and this competition

may facilitate the cyclic attachment/detachment of the cell to the extracellular matrix that is necessary for cell migration. In support of this idea, we observed that 1321N1 cells expressing the WT $P2Y_2R$ adhered to vitronectin-coated dishes at a slower rate than untransfected cells or cells expressing the RGE mutant (data not shown). Further investigation, however, is required to examine this hypothesis.

Recent advances in the understanding of the mechanism of F-actin polymerization have shown that small GTPases like Cdc42, RhoA, and Rac are major regulators of actin dynamics (Hall, 1998; Nobes and Hall, 1999) and play a critical role in cell motility. For example, activated Cdc42 stimulates the formation of actin rich filopodia, activated RhoA induces the formation of stress fibers and focal adhesions, and activated Rac stimulates the formation of lamellipodia and membrane ruffles (Hall, 1998). Rac is also required to recruit the active form of $\alpha_v\beta_3$ integrin to the lamellipodia, which promotes cell migration (Kiosses et al., 2001). In our experiments, UTP stimulated Rac activation in 1321N1 cells expressing the WT $P2Y_2R$, but not in cells expressing the RGE mutant $P2Y_2R$ (Fig. III-2). Dominant-negative Rac inhibited UTP-induced migration of cells expressing the WT $P2Y_2R$ (Bagchi et al., 2005), providing direct evidence for the involvement of Rac in $P2Y_2R$-mediated cell migration. The small GTPases cycle between active GTP-bound and inactive GDP-bound states depending on their regulation by guanine nucleotide exchange factors (GEFs). One GEF for Rac is vav2, which is activated by tyrosine kinase-dependent phosphorylation (Bustelo, 2000), and UTP has been found to cause a strong time-dependent increase in the tyrosine phosphorylation of vav2 in human coronary artery endothelial cells (Liu et al., 2004). In the present study, vav2 phosphorylation was stimulated by UTP in 1321N1 cells expressing the WT $P2Y_2R$, but not in cells expressing

the RGE mutant $P2Y_2R$ (Fig. III-2), suggesting that vav2 phosphorylation, like Rac activation, was dependent on $P2Y_2R$ interaction with α_v integrins. Furthermore, vav2 DN completely inhibited UTP-induced Rac activation (Bagchi et al., 2005), thus establishing that vav2 plays a crucial role in $P2Y_2R$-mediated Rac activation and chemotaxis. The requirement for $P2Y_2R$ and α_v integrin interaction in the activation of vav2 and Rac was demonstrated in two other ways. First, selective inhibition of α_v integrin expression with α_v antisense oligonucleotide inhibited the UTP-induced activation of vav2 and Rac (Bagchi et al., 2005). Second, UTP-induced phosphorylation of vav2 and Rac were significantly inhibited by an overnight treatment of WT $P2Y_2R$ transfected cells with anti-$\alpha_v\beta_5$ Ab (Fig. III-3). Thus, our results strongly suggest that vav2 and Rac activation are required for UTP-induced cell migration, responses that are dependent upon interaction between the $P2Y_2R$ and α_v integrins.

Heterotrimeric G proteins in the $G_{i/o}$ family were recently reported to be responsible for GPCR-mediated cell migration by activating RacGEFs, which, in turn, activate Rac (Burridge and Wennerberg, 2004). In this study, we utilized the [^{35}S]GTPγS binding assay to provide direct evidence that the RGD integrin-binding domain in the $P2Y_2R$ is required for this receptor to activate G_o but not G_q (Bagchi et al., 2005). We also found that the $G_{i/o}$ inhibitor PTX decreased UTP-induced migration of 1321N1 cells expressing the WT $P2Y_2R$ by 70-80% (Bagchi et al., 2005) and completely inhibited $P2Y_2R$-mediated vav2 and Rac activation (Fig. III-4). Taken together, these results suggest that α_v integrin interaction with the $P2Y_2R$ is required to activate G_o and initiate G_o-mediated vav2 and Rac activation and vitronectin up-regulation, leading to increased cell migration.

These results provide novel insight into the mechanism of chemotactic signaling by a GPCR and suggest that α_v integrin-based complexes can be important for allowing access to specific heterotrimeric G proteins involved in cell migration. Since α_v integrins have been shown to play a critical role in cell migration and angiogenesis in vascular endothelial cells (Kiosses et al., 2001; Ziche et al., 2004), therapeutic strategies that disrupt $P2Y_2R$ interaction with α_v integrins could be useful in the treatment of diseases involving inflammation and angiogenesis, such as atherosclerosis, diabetes, Alzheimer's disease, and cancer.

Materials and Methods

Cell Culture and Transfection

Human 1321N1 astrocytoma cells were stably transfected with cDNA encoding a hemagglutinin (HA)-tagged wild-type (WT) or RGE mutant $P2Y_2R$ using the pLXSN retroviral vector, as previously described (Erb et al., 1995). The cells were cultured in DMEM containing 5% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml Geneticin (G418, GIBCO BRL) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cell surface expression of the HA-tagged WT or RGE mutant $P2Y_2R$ was determined by flow cytometry, as previously described (Garrad et al., 1998). Surface expression of endogenous α_v integrin in 1321N1 cells expressing the WT or RGE mutant $P2Y_2R$ was quantitated by flow cytometry, using a 1:100 dilution of mouse monoclonal anti-human α_v antibody (P2W7, Santa Cruz Biotechnology) as the primary antibody and a 1:100 dilution of goat anti-mouse IgG-FITC (Santa Cruz Biotechnology) as the secondary antibody. Cells were washed with PBS, fixed and

analyzed on an EPICS 753 flow cytometer (Coulter Corp., Hialeah, FL).

Migration Assay

Cell migration assays were performed with 3 μm pore size Transwells (Costar), as described (Chaulet et al., 2001). In brief, cells were suspended by trypsinization, washed, and resuspended in 100 μl of serum-free DMEM ($5x10^4$ cells) and placed in the upper chamber of the Transwells. The lower chamber was filled with 500 μl of serum-free DMEM supplemented with varying concentrations of UTP (Amersham), as indicated. The cells were allowed to migrate for 16 h at 37 °C. Cells migrating to the lower side of the polycarbonate membrane were fixed with ice-cold methanol and stained with Accustain (Sigma). Cells were counted in 10 microscopic fields at 20X magnification.

Immunoblot Analysis

Immunoblot analysis was done as previously described (Erb et al., 2001). For signal normalization, membranes were probed with a 1:1000 dilution of goat anti-human actin Ab (Cytoskeleton Inc.).

Rac activity and vav2 Phosphorylation

Human 1321N1 cells transfected with the WT or RGE mutant $P2Y_2R$ were plated on uncoated 100 mm tissue culture dishes, serum-starved for 24 h and stimulated with UTP for 5 min at 37 °C. Rac-GTP was precipitated from cell extracts with Rac/Cdc42 Assay Reagent (PAK-1 PBD-agarose), comprised of the P-21 binding domain of human PAK-1 bound to glutathione agarose (Upstate Biotechnology), following the manufacturer's

instructions. SDS-PAGE was performed with the precipitated protein. Rac activity was detected by immunoblotting with a 1:1000 dilution of mouse anti-human Rac (clone 23A8, Upstate Biotechnology) as the primary Ab and a 1:2000 dilution of HRP-conjugated goat anti-mouse IgG as the secondary Ab (Santa Cruz Biotechnology).

vav2 phosphorylation was analyzed by immunoprecipitation of vav2 with 2 μg of mouse anti-human phosphotyrosine Ab (Upstate Biotechnology), followed by immunoblotting of the precipitated protein with a 1:500 dilution of rabbit anti-human vav2 (Zymed Laboratories Inc.) as the primary Ab and a 1:2000 dilution of HRP–conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) as the secondary Ab. For normalization of the signal, aliquots of total cell lysates were subjected to SDS-PAGE and immunoblotted with a 1:1000 dilution of mouse anti-human Rac or rabbit anti-human vav2 as the primary Abs, which bind to Rac or vav2, respectively, independent of their phosphorylation state.

Figure III-1. The RGD integrin-binding domain in the $P2Y_2R$ is required for UTP-induced migration of 1321N1 cells. Chemotaxis of 1321N1 cells ($5x10^4$) transfected with the WT or RGE mutant $P2Y_2R$ or the empty vector (pLXSN) seeded in the upper chamber of Transwells. Lower chambers contained the indicated concentration of UTP or serum-free medium (control). Chemotaxis expressed as the number of cells that moved across the membrane was evaluated 16 h after UTP stimulation.

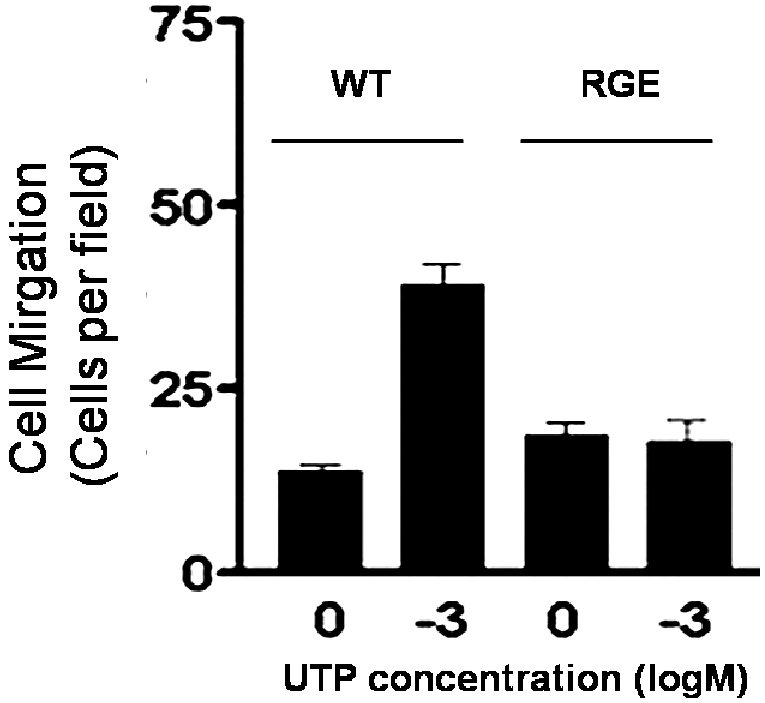

75
50
25
0
WT
RGE
Cell Mirgation
(Cells per field)
0
-3
0
-3
UTP concentration (logM)

Figure III-2. The RGD domain of the $P2Y_2R$ is required for vav2 and Rac activation. Human 1321N1 cells expressing the WT or RGE mutant $P2Y_2R$ were serum-starved overnight and treated with or without 1 mM UTP for 5 min at 37 °C prior to determining vav2 tyrosine phosphorylation and Rac GTPase activity, as described in "Materials and Methods". The data shown are representative of results from 3-4 experiments.

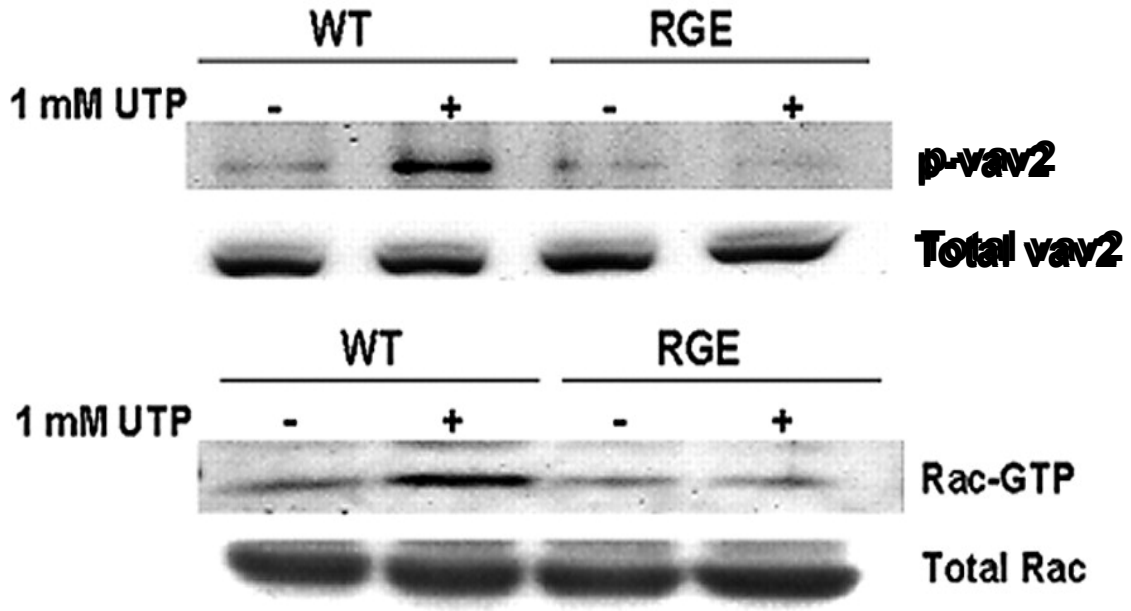

WT
RGE
1 mM UTP
-
+
-
+
p-vav2
Total vav2
WT
RGE
1 mM UTP
-
+
-
+
Rac-GTP
Total Rac

Figure III-3. $P2Y_2R$-mediated vav2 and Rac activation requires α_v integrin activity. Human 1321N1 cells expressing the WT $P2Y_2R$ were incubated overnight with 10 μg/ml anti-α_3 or anti-$\alpha_v\beta_5$ Abs, then stimulated with 100 μM UTP for 5 min at 37 °C prior to determining vav2 phosphorylation and Rac GTPase activity, as described in the "Materials and Methods". The data shown are representative of results from 3-4 experiments.

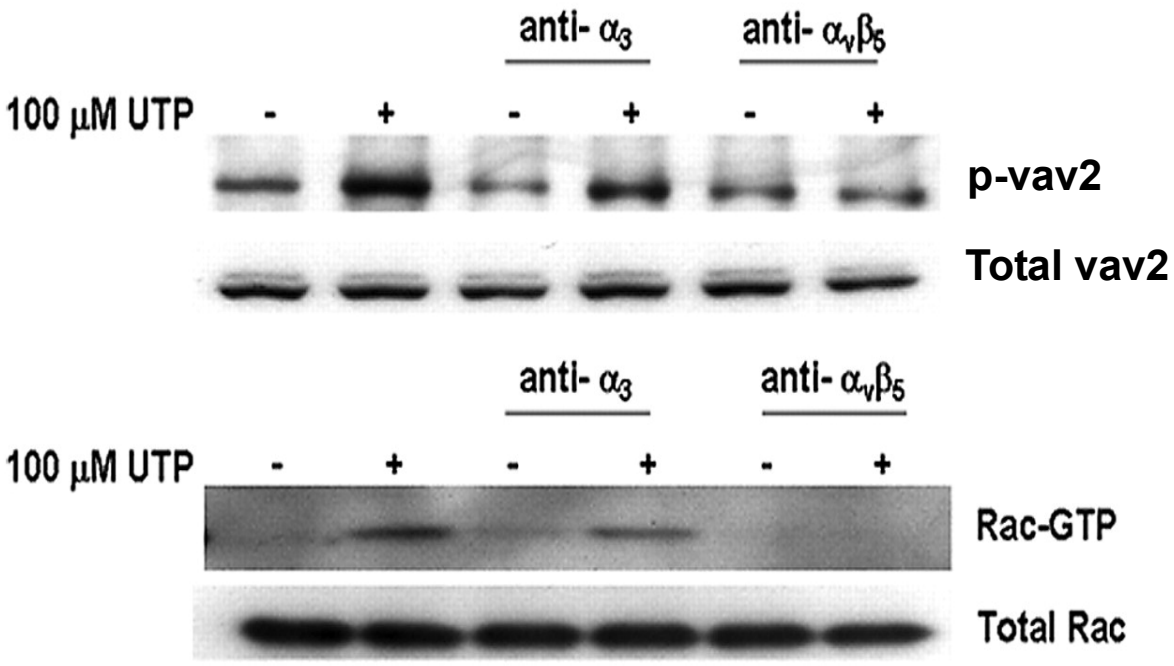

anti- α3
anti- αvβ5
100 μM UTP
- + - + - +
p-vav2
Total vav2
anti- α3
anti- αvβ5
100 μM UTP
- + - + - +
Rac-GTP
Total Rac

Figure III-4. Role of G_o in $P2Y_2R$-mediated vav2 and Rac activation. Human 1321N1 cells expressing the WT $P2Y_2R$ were pretreated with PTX for 16 h in serum-free medium then stimulated with 100 μM UTP for 5 min at 37 °C prior to determining vav2 phosphorylation and Rac GTPase activity, as described in the "Materials and Methods". The data shown are representative of results from 3-4 experiments.

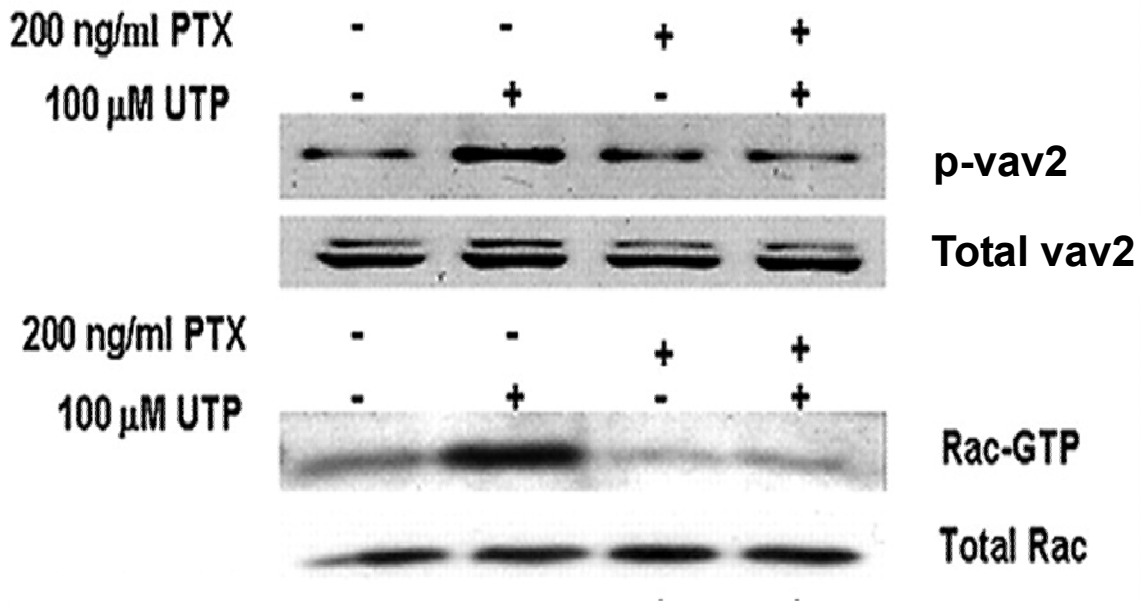
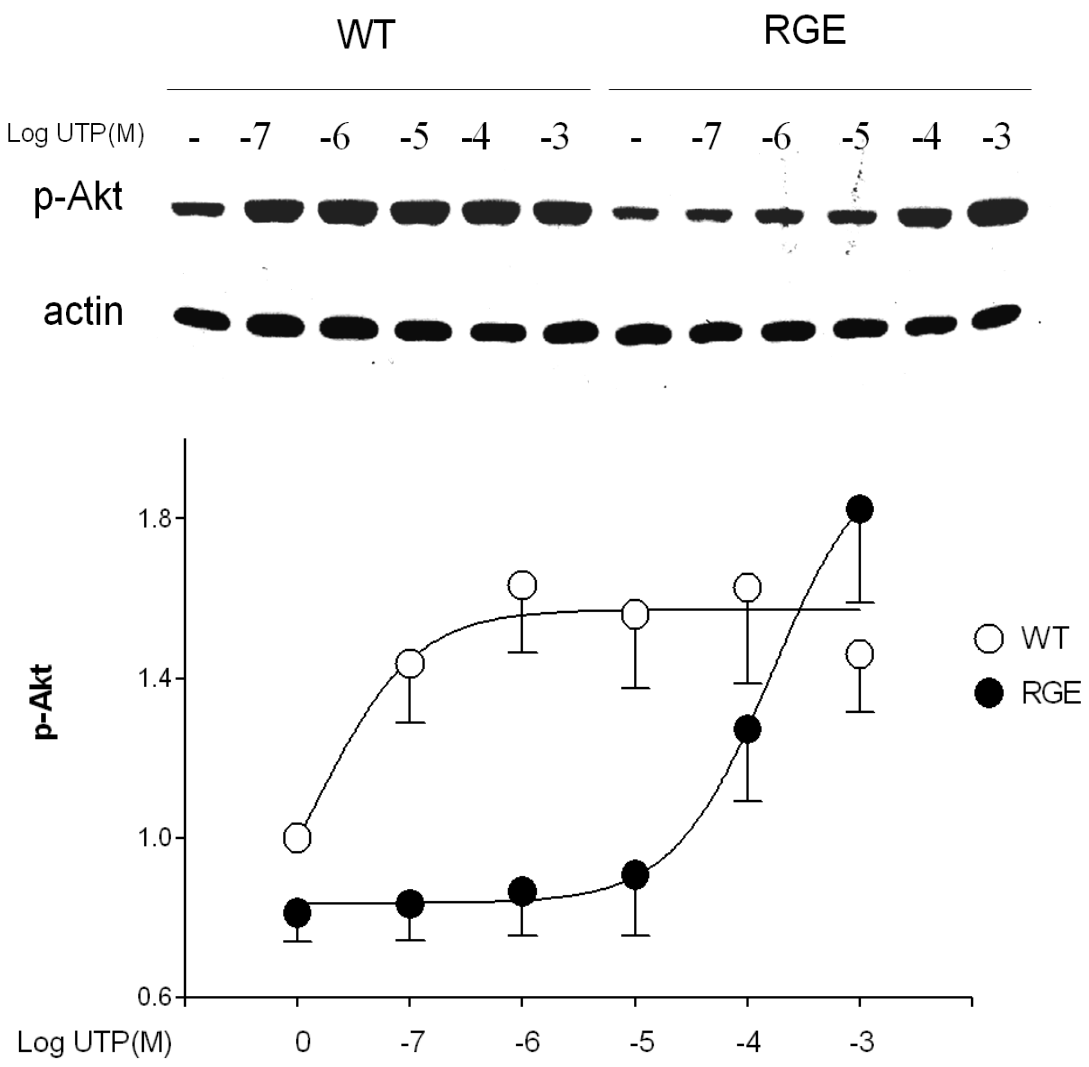

200 ng/ml PTX
100 μM UTP
p-vav2
Total vav2
200 ng/ml PTX
100 μM UTP
Rac-GTP
Total Rac

Figure III-5. Role of the $P2Y_2R$ RGD domain in UTP-induced Akt phosphorylation. Human 1321N1 cells expressing the WT or RGE mutant $P2Y_2R$ were stimulated with the indicated concentration of UTP for 10 min at 37 °C prior to analysis of Akt phosphorylation using anti-phospho-Akt Ab. The phospho-Akt band density was normalized to total actin and shown in the lower panel. Data shown are representative of results from 3 experiments.


WT
RGE
Log UTP(M)
-
-7
-6
-5
-4
-3
-
-7
-6
-5
-4
-3
p-Akt
actin
p-Akt
1.8
1.4
1.0
0.6
Log UTP(M)
0
-7
-6
-5
-4
-3
WT
RGE

CHAPTER IV

Activated $P2Y_2$ receptors transiently relocate to endothelial adherens junctions to regulate Rac activity

Abstract

$P2Y_2$ nucleotide receptors ($P2Y_2Rs$) are involved in several stages of an inflammatory response, including up-regulation of endothelial VCAM-1, a vascular cell adhesion molecule that promotes tight binding of leukocytes to endothelium, and the extravasation and migration of leukocytes into infected or injured tissue (Seye et al., 2004; Chen et al., 2006). In migrating neutrophils, $P2Y_2Rs$ are evenly distributed on the cell surface and are thought to sense nucleotides released at the leading edge of a cell to amplify bacterial chemotactic signals (Chen et al., 2006). In this study, we analyzed the distribution of $P2Y_2Rs$ in quiescent and nucleotide-activated endothelial cells. Activation of $P2Y_2Rs$ in human coronary artery endothelial cells (HCAEC) with the agonist UTP caused a rapid and transient clustering of GFP-tagged $P2Y_2Rs$ at the intercellular junctional zones. Co-immunoprecipitation experiments indicated that UTP also caused a rapid and transient association of the $P2Y_2R$ and VEGFR-2 (vascular endothelial growth factor receptor-2) with VE-cadherin, a transmembrane component of endothelial adherens junctions. Knockdown of VE-cadherin expression with siRNA in HCAEC led to a loss of UTP-induced Rac activation and tyrosine phosphorylation of p120 catenin, a cytoplasmic protein known to interact with VE-cadherin. Activation of the $P2Y_2R$ by UTP also was found to cause a prolonged interaction between p120 catenin and vav2 (a guanine nucleotide exchange factor for Rac) that correlated with the kinetics

of UTP-induced tyrosine phosphorylation of p120 catenin and VE-cadherin. Inhibitors of VEGFR-2 or Src significantly diminished UTP-induced Rac activation, tyrosine phosphorylation of p120 catenin and VE-cadherin, and association of the $P2Y_2R$ with VE-cadherin and p120 catenin with vav2. These findings suggest that the $P2Y_2R$ uses Src and VEGFR-2 to mediate association of the $P2Y_2R$ with adherens junctions and to activate Rac in endothelial cells.

Introduction

Transendothelial migration of leukocytes in blood vessels is controlled by the formation and dissociation of endothelial cell adhesion structures comprised of adherens junctions, tight junctions and gap junctions (Mehta and Malik, 2006). It is well known that endothelial barrier function is regulated by the stability of these junctional structures and the contractility provided by the actomyosin cytoskeleton. Rho family GTPases control both the stability of intercellular junctions and actin cytoskeletal organization (Wojciak-Stothard and Ridley, 2002). For example, endothelial Rho GTPase and Rho kinase promote the sealing of intercellular junctions by controlling phosphorylation of myosin light chain (Saito et al., 2002). In contrast, alternative roles for other Rho family GTPases are suggested by studies demonstrating that dominant negative Rac enhances thrombin-induced permeability of macromolecules, whereas dominant negative Cdc42 has no effects (Wojciak-Stothard et al., 2001). VEGF, thrombin, TNFα and histamine also can increase endothelial permeability, due to modulation of protein distribution in adherens junctions and the activities of Rho GTPases (Rabiet et al., 1996; Esser et al., 1998; Kevil et al., 1998; Vouret-Craviari et al., 1998; Wojciak-Stothard et al., 1998;

Andriopoulou et al., 1999; Wong et al., 1999; Soga et al., 2001).

Extracellular nucleotides have been shown to play a role in regulating blood vessel permeability and leukocyte infiltration. For example, extracellular ATP reduces the permeability of endothelial cell monolayers to macromolecules, most likely via activation of P2 receptors (Noll et al., 1999), whereas 2-MeSATP, an agonist of the $P2Y_1$ receptor, increases endothelial monolayer permeability (Tanaka et al., 2004). A recent study has shown that the $P2Y_2R$ activation by ATP released at the leading edge of migrating neutrophils can amplify bacterial chemotactic signals that recruit neutrophils to the site of an infection (Chen et al., 2006). It also has been shown that local infusion of the $P2Y_2R$ agonist UTP into a silicone collar placed around a rabbit carotid artery promotes intimal hyperplasia and increases monocyte infiltration into the intimal tissue (Seye et al., 2002). Other studies indicate that the $P2Y_2R$, by virtue of a RGD integrin-binding motif in its extracellular domain, mediates the activation of small Rho GTPases (Bagchi et al., 2005; Liao et al., 2007) and, by virtue of SH3-binding motifs in its intracellular domain, promotes the Src-dependent activation of VEGFR-2 that up-regulates the expression of the leukocyte binding protein VCAM-1 on endothelial cells (Liu et al., 2004; Seye et al., 2004). Since the $P2Y_2R$ regulates both leukocyte adhesion to endothelium and Rho GTPase activities (Wheelock and Johnson, 2003; Seye et al., 2004; Bagchi et al., 2005; Liao et al., 2007), we speculated that the $P2Y_2R$ may modulate the permeability of endothelium to leukocytes by affecting the stability of adherens junctions.

Among the proteins in endothelial cell junctions, VE-cadherin has been well recognized for its role in regulating recruitment and endothelial permeability of leukocytes (Breviario et al., 1995; Gotsch et al., 1997; Matsuyoshi et al., 1997; Corada

et al., 1999). VE-cadherin is exclusively expressed in vascular endothelial cells (Dejana et al., 1999) and deletion of VE-cadherin in mice causes severe defects in vascular development and embryonic death (Carmeliet et al., 1999; Gory-Faure et al., 1999). Down-regulation of VE-cadherin has been associated with vascular tumor growth (Zanetta et al., 2005), whereas treatment of endothelial cells with VE-cadherin neutralizing antibody increases VEGF-induced VEGFR-2 activity (Rahimi and Kazlauskas, 1999). Compared to endothelial cells expressing VE-cadherin, VE-cadherin-null endothelial cells have thinner actin stress fibers, decreased vinculin-positive focal contacts, and attenuated activity of Rac, a small GTPase involved in actin reorganization and cell migration (Lampugnani et al., 2002). The N-terminal extracellular domain of VE-cadherin mediates Ca^{2+}-dependent homophilic adhesion while the cytoplasmic domain interacts with various intracellular binding partners, including α-, β-, and p120 catenins, which may provide a linkage to actin cytoskeleton (Vincent et al., 2004). Modulation of cell-cell contacts that regulate cell adhesion and cell motility likely requires interactions between cadherins and catenins. It has been shown that p120 catenin regulates actin cytoskeletal organization and cell motility by activation of Rho GTPase (Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001). In addition, VE-cadherin may associate with VEGFR-2 and some intracellular signaling molecules such as Shc and Csk (Zanetti et al., 2002; Baumeister et al., 2005), and protein phosphatases such as VE-PTP (vascular endothelial protein tyrosine phosphatase) (Nawroth et al., 2002). These interactions may be important for regulating cell-cell contacts, cell adhesion, and growth factor signaling (Dejana et al., 1999).

In the present study, we investigated the distribution of $P2Y_2Rs$ in human coronary

artery endothelial cells (HCAEC) and found that stimulation of the receptor with UTP caused clustering of $P2Y_2Rs$ at intercellular junctions, a process that was dependent on Src and VEGFR-2 activity. We also demonstrate that UTP causes transient association of the $P2Y_2R$ and VEGFR-2 with VE-cadherin. This led us to investigate the role of VE-cadherin in $P2Y_2R$-mediated signaling in endothelial cells. These studies suggest that Rac activation by the $P2Y_2R$ in endothelial cells involves tyrosine phosphorylation of VE-cadherin and p120 catenin and association of p120 catenin with vav2, a Rac guanine nucleotide exchange factor (GEF).

Results

UTP causes clustering of the $P2Y_2R$ in intercellular junctions

It has been shown that VE-cadherin inhibits the internalization of VEGFR-2 in response to VEGF, and thus VEGF-induced signaling (Lampugnani et al., 2006), possibly via interaction between VE-cadherin and VEGFR-2 (Zanetti et al., 2002). Consistent with this finding, we found that overexpression of VE-cadherin in 1321N1 astrocytoma cells expressing the wild type HA-$hP2Y_2R$ inhibited UTP-induced internalization of the HA-$hP2Y_2R$ (Supplemental Fig. IV-S1). To further investigate whether the $P2Y_2R$ redistributes in endothelial cells in response to UTP, we transfected HCAEC with cDNA encoding the GFP-tagged $hP2Y_2R$ and examined the localization of GFP-$hP2Y_2Rs$ in the absence and presence of UTP. Thirty-six hours after transfection, most GFP-$hP2Y_2Rs$ were localized to an intracellular compartment (not shown), possibly early endosomes, whereas 84 h after transfection, the GFP-$hP2Y_2R$ appeared at the peripheral cell membrane (Fig. IV-1). In ~50% of the transfected cells, a 5 min stimulation with UTP

caused clustering of GFP-hP2Y$_2$R in peripheral membranes between adjacent cells, and co-localization with the adherens junction protein VE-cadherin (Fig. IV-1), suggesting that VE-cadherin recruits the activated GFP-hP2Y$_2$R to junctional zones of endothelial cells.

UTP induces transient association between VE-cadherin and the P2Y$_2$R

Since the P2Y$_2$R was translocated to intercellular adherens junctions upon activation (Fig. IV-1), we examined whether the P2Y$_2$R was recruited by interacting with an adherens junction protein. Accordingly, HCAEC were transfected with cDNA encoding the HA-hP2Y$_2$R and immunoprecipitation with anti-HA matrix was performed. An interaction between the HA-hP2Y$_2$R and VE-cadherin in HCAEC was observed within 5 min of exposure to 100 μM UTP, but was not detected 10 min later (Fig. IV-2A).

The P2Y$_2$R mediates tyrosine phosphorylation of VE-cadherin

VEGF (vascular endothelial growth factor) can stimulate the tyrosine phosphorylation of adherens junction proteins, including VE-cadherin, β-catenin, plakoglobin, and p120 catenin (Esser et al., 1998). It has been found that activation of the G protein-coupled P2Y$_2$R induces the Src-dependent transactivation of growth factor receptors, including the EGFR, PDGFR (Liu et al., 2004) and VEGFR-2 (Seye et al., 2004). Therefore, we hypothesized that activation of the P2Y$_2$R would increase the tyrosine phosphorylation of VE-cadherin. Indeed, we found that the P2Y$_2$R agonist UTP induced sustained tyrosine phosphorylation of VE-cadherin in HCAEC, as detected by immunoprecipitation of cell lysates with anti-phosphotyrosine antibody (Fig. IV-3A) or

anti-VE-cadherin (data not shown). To confirm that UTP-induced tyrosine phosphorylation is mediated by the $P2Y_2R$, we suppressed expression of the endogenous $P2Y_2R$ in HCAEC with specific $P2Y_2R$ siRNA duplexes. Results indicated that down-regulation of $P2Y_2R$ mRNA expression inhibited UTP-induced tyrosine phosphorylation of VE-cadherin in HCAEC (Figs. 3B and 3C), demonstrating the involvement of the $P2Y_2R$. In addition, expression of HA-h$P2Y_2R$ and VE-cadherin in P2 receptor-deficient 1321N1 astrocytoma cells enabled UTP to induce tyrosine phosphorylation of VE-cadherin (data not shown), further supporting the ability of $P2Y_2R$ activation to mediate VE-cadherin phosphorylation.

Src-dependent VEGFR-2 transactivation plays a role in $P2Y_2R$-mediated tyrosine phosphorylation of VE-cadherin

Since VEGFR-2 and VE-cadherin interaction is required for VEGF-induced tyrosine phosphorylation of VE-cadherin (Zanetti et al., 2002) and since $P2Y_2R$ activation promotes the Src-dependent transactivation of VEGFR-2 (Seye et al., 2004), we hypothesized that $P2Y_2R$-mediated tyrosine phosphorylation of VE-cadherin in HCAEC is dependent on Src-dependent VEGFR-2 transactivation. $P2Y_2R$ activation by UTP caused a transient interaction between VE-cadherin and VEGFR-2 that decreases within 5-15 min of UTP addition (Fig. IV-2B), similar to the kinetics of VE-cadherin and $P2Y_2R$ association in response to UTP (Fig. IV-2A). UTP-induced tyrosine phosphorylation of VE-cadherin was sustained for more than 30 min (Fig. IV-3A and data not shown), possibly due to auto-phosphorylation of the VEGFR-2. To further investigate the role of Src and VEGFR-2 in $P2Y_2R$-mediated tyrosine phosphorylation of VE-cadherin, we

treated HCAEC with PP2 (1 μM) or SU1498 (10 μM), inhibitors of Src or VEGFR-2 activity, respectively. PP2 and SU1498 inhibited both UTP-induced tyrosine phosphorylation of VE-cadherin (Fig. IV-4), and the UTP-induced interaction between the HA-hP2Y$_2$R and VE-cadherin (Fig. IV-2C), consistent with a role for Src-dependent VEGFR-2 transactivation in mediating signal transduction between the P2Y$_2$R and VE-cadherin. Furthermore, a mutant hP2Y$_2$R (Del) that lacks the C-terminal SH3-binding domains required for Src-dependent VEGFR-2 transactivation (Seye et al., 2004) did not induce tyrosine phosphorylation of VE-cadherin or interaction of VE-cadherin with the Del-hP2Y$_2$R in 1321N1 cells treated with UTP (Supplemental Fig. IV-2), demonstrating that the binding of Src to SH3-binding domains in the P2Y$_2$R is required for VEGFR-2 transactivation and the stimulation of VE-cadherin phosphorylation.

Both VE-cadherin and VEGFR-2 are required for UTP-induced Rac activation

Recent studies have demonstrated an important role for VE-cadherin-containing adherens junctions in regulating cell proliferation (Grazia Lampugnani et al., 2003) and actin cytoskeletal organization (Lampugnani et al., 2002). To investigate the role of VE-cadherin in P2Y$_2$R-mediated signal transduction, VE-cadherin-specific siRNA was used to down-regulate the expression of VE-cadherin in HCAEC (Fig. IV-5A). It was reported that the presence of VE-cadherin decreased MAPK phosphorylation in response to VEGF (Grazia Lampugnani et al., 2003). However, we found that P2Y$_2$R-mediated MAPK (*i.e.*, ERK1/2) activation in HCAEC was not affected by the presence of VE-cadherin (Fig. IV-5A). In contrast, down-regulation of VE-cadherin led to an

inhibition of UTP-induced activation of Rac (Fig. IV-5B). Furthermore, UTP-induced Rac activation in HCAEC was inhibited by PP2 or SU1498 (Fig. IV-5C), suggesting roles for Src and VEGFR-2 in the $P2Y_2R$-mediated activation of Rac. Consistent with a previous report (Lampugnani et al., 2002), we also found that knockdown of VE-cadherin expression in HCAEC caused an increase in basal Rho activity, which, however, was not further stimulated by UTP (Supplemental Fig. IV-3). Since both UTP-induced phosphorylation of VE-cadherin and activation of Rac are regulated by VEGFR-2, we conclude that VEGFR-2 activity is required for VE-cadherin-dependent activation of Rac mediated by the $P2Y_2R$.

The $P2Y_2R$ regulates p120 catenin phosphorylation via VEGFR-2 and VE-cadherin

Other adherens junction proteins that interact with VE-cadherin provide a linkage with actin cytoskeleton as well as intracellular signaling pathways (Vincent et al., 2004). Among these adherens junction proteins, p120 catenin binds to the cytoplasmic domain of cadherins in the juxtamembrane region and regulates the activity of small Rho GTPases, thereby modulating actin cytoskeletal organization and cell motility (Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001). Tyrosine phosphorylation of p120 catenin is dependent upon an interaction between p120 and VE-cadherin, thereby regulating endothelial barrier function (Iyer et al., 2004). We found that in HCAEC, UTP also caused tyrosine phosphorylation of p120 catenin, which was inhibited by transfection of cells wih $P2Y_2R$-specific siRNA, indicating that UTP-induced phosphorylation of p120 catenin is mediated by the $P2Y_2R$ (Fig. IV-6A). $P2Y_2R$-mediated tyrosine phosphorylation of p120 catenin is also prevented by treatment

of HCAEC with PP2 or SU1498 (Figs. IV-6B and 6C), indicating a role for Src-dependent VEGFR-2 transactivation in UTP-induced p120 catenin phosphorylation. Furthermore, down-regulation of VE-cadherin with VE-cadherin-specific siRNA inhibited UTP-induced tyrosine phosphorylation of p120 catenin (Fig. IV-6D), indicating that VE-cadherin is required for modulation of p120 catenin phosphorylation in response to $P2Y_2R$ activation.

p120 catenin regulates $P2Y_2R$-mediated activation of Rac

To examine the role of p120 catenin in VE-cadherin-dependent activation of Rac mediated by the $P2Y_2R$, we transfected HCAEC with p120 catenin-specific siRNA to down-regulate expression of p120 catenin. UTP-induced activation of Rac was inhibited by p120 catenin-specific siRNA transfection (Fig. IV-7), further supporting the idea that adherens junction proteins are necessary for the $P2Y_2R$ to modulate Rho GTPase activity. In addition, p120 catenin-specific siRNA also increased the basal activity of Rho in HCAEC, similar to VE-cadherin-specific siRNA, although no further activation by UTP was seen (data not shown). Previously, we found that vav2, a Rac guanine nucleotide exchange factor (GEF), regulated $P2Y_2R$-mediated Rac activation, by demonstrating that expression of dominant negative vav2 inhibits Rac activity in UTP-treated 1321N1 cells expressing the $P2Y_2R$ (Bagchi et al., 2005). It has been postulated that vav2 binding to p120 is required for $P2Y_2R$-mediated Rac activation (Noren et al., 2000). We found that UTP increased the interaction between p120 catenin and vav2 in HCAEC (Fig. IV-8A), supporting the conclusion that vav2 interaction with p120 catenin is necessary for $P2Y_2R$-mediated activation of Rac. Furthermore, the UTP-induced interaction of vav2

and p120 catenin was inhibited by SU1498 or PP2 (Fig. IV-8B), consistent with a role for Src-dependent VEGFR-2 activation in $P2Y_2R$-mediated Rac activity.

Discussion

In contrast to the uniform distribution of $P2Y_2Rs$ in migrating neutrophils (Chen et al., 2006), we found that stimulation of human coronary artery endothelial cells (HCAEC) with the $P2Y_2R$ agonist UTP promotes a rapid and transient re-distribution of GFP-tagged $P2Y_2Rs$ into intercellular junctions (Fig. IV-1). This correlated with the rapid and transient association of the $P2Y_2R$ with VE-cadherin (Fig. IV-2A), an endothelial cell-specific component of intercellular adherens junctions that is important for the regulation of endothelial barrier functions.

Association of the $P2Y_2R$ with VE-cadherin induced by UTP was prevented by pretreatment of the cells with PP2 or SU1498, compounds that inhibit Src and VEGFR-2 activity, respectively (Fig. IV-2C), suggesting that Src and VEFGR-2 mediate $P2Y_2R$ signaling in endothelial adherens junctions. In support of this finding, UTP also caused a rapid and transient association of VEGFR-2 with VE-cadherin (Fig. IV-2B) and the Src- and VEGFR-2-dependent tyrosine phosphorylation of VE-adhering (Fig. IV-4) and the VE-cadherin-associated protein, p120 catenin (Figs. IV-6B and 6C). VEGFR-2 has also been found to associate with VE-cadherin in response to shear stress in vascular endothelial cells (Shay-Salit et al., 2002). Since nucleotides are known to be released in response to mechanical stress (Born and Kratzer, 1984), this suggests that the $P2Y_2R$ and other nucleotide receptors act as shear stress receptors in the vasculature.

VEGF and the GPCR ligands thrombin, histamine and sphingosine-1-phosphate

(S1P) have been shown to regulate changes in endothelial permeability via modulation of VE-cadherin function (Rabiet et al., 1996; Andriopoulou et al., 1999; Sanchez et al., 2007). Although we did observe an increase in endothelial permeability to FITC-labeled dextran after UTP treatment, the increase in permeability occurred only after 12 h incubation with UTP and may be related to the increase in VEGF expression caused by UTP treatment (data not shown), whereas VEGF-, thrombin-, histamine- and S1P-induced permeability changes occurred within the first hour of treatment (Rabiet et al., 1996; Andriopoulou et al., 1999; Sanchez et al., 2007). This suggests that the function of the $P2Y_2R$ in endothelial cells is not to regulate vascular permeability to macromolecules. Based on results presented in this study, we hypothesize that one function of endothelial $P2Y_2Rs$ may be to escort leukocytes to paracellular junctions.

Previous studies in endothelial cells indicate that UTP treatment causes a transient association of the $P2Y_2R$ with VEGFR-2 (Liu et al., 2004; Seye et al., 2004). This association is accompanied by tyrosine phosphorylation of VEGFR-2 and up-regulation of the leukocyte binding protein VCAM-1, a process that is dependent on VEGFR-2 expression and kinase activity (Liu et al., 2004; Seye et al., 2004). In addition, the mechanism of this transactivation process has been shown to involve binding of Src to SH3-binding domains in the $P2Y_2R$ cytoplasmic tail, which promotes the Src-dependent association of $P2Y_2R$ with several growth factor receptors, including EGFR, PDGFR and VEGFR-2 (Liu et al., 2004; Seye et al., 2004). In this study, we used selective inhibitors of VEGFR-2 (SU1498) and Src (PP2) and found that, in HCAEC, VEGFR-2 and Src kinase activity are required for UTP-induced association of the $P2Y_2R$ with VE-cadherin as well as tyrosine phosphorylation of VE-cadherin and the VE-cadherin-associated

protein p120 catenin, and the association of the Rac GTP exchange factor vav2 with p120 catenin leading to Rac activation (Fig. IV-9). Furthermore, we found that down-regulation of VE-cadherin expression with specific siRNA abolished UTP-induced phosphorylation of p120 catenin and Rac activation and that down-regulation of p120 catenin inhibited UTP-induced Rac activation in HCAEC. Together, these results demonstrate that in HCAEC, $P2Y_2R$-mediated activation of Rac requires Src-dependent transactivation of VEGFR-2, which is responsible for proper assembly of proteins involved in Rac activation at adherens junctions.

Although the ultimate function of Rac activation by the $P2Y_2R$ in quiescent endothelial cells is unclear, it is known that Rac participates in cytoskeletal rearrangements important for endothelial permeability (Wojciak-Stothard et al., 2001) and transendothelial migration of leukocytes (van Wetering et al., 2003) and there is evidence indicating that the $P2Y_2R$ is involved in leukocyte migration and extravasation during inflammation. For example, introduction of the $P2Y_2R$ agonist UTP via a vascular collar placed around a rabbit carotid artery, caused a 4-fold increase in monocyte migration into the neointima (Seye et al., 2002). Also, $P2Y_2R^{-/-}$ mice are defective in the recruitment of neutrophils towards a site of bacterial infection *in vivo* (Chen et al., 2006). The present study demonstrates that VE-cadherin is required for Rac activation mediated by the $P2Y_2R$ in endothelial cells, which likely regulates actin cytoskeletal rearrangements important for transendothelial migration of leukocytes.

VE-cadherin interacts homophilically between neighboring endothelial cells. This interaction is reduced during angiogenesis and leukocyte extravasation and it is well established that VE-cadherin serine and tyrosine phosphorylation regulates VE-cadherin

endocytosis and adherens junction integrity in response to VEGFR-2 activation by VEGF (Gavard and Gutkind, 2006; Mukherjee et al., 2006). We found that UTP treatment caused tyrosine phosphorylation of VE-cadherin and the VE-cadherin-associated protein, p120 catenin (Figs. IV-3 and 6), but did not significantly alter the distribution of VE-cadherin in endothelial cells after 5 min (Fig. IV-1) or 30 min (data not shown), suggesting that the $P2Y_2R$ does not regulate VE-cadherin endocytosis.

Besides VEGFR-2, integrins such as $\alpha_v\beta_3/\beta_5$ may also play a role in regulating $P2Y_2R$/VE-cadherin interactions. It has been shown that fibronectin binding to integrins disrupts VE-cadherin-containing adherens junctions in a Src-dependent manner (Wang et al., 2006). The $P2Y_2R$ contains a RGD domain that promotes interactions with $\alpha_v\beta_3/\beta_5$ integrins (Erb et al., 2001) and when mutated to RGE, the RGE-$P2Y_2R$ mutant did not exhibit UTP-induced phosphorylation of VE-cadherin(date not shown), suggesting that $P2Y_2R$/integrin interactions play a role in signaling to VE-cadherin.

The role of VE-cadherin in controlling cell proliferation has been well recognized, and requires interactions between VE-cadherin and the cell cytoskeleton, Src kinases, and catenins (Ferber et al., 2002; Nelson and Chen, 2003; Baumeister et al., 2005). VE-cadherin also regulates cell proliferation by preventing VEGF-induced internalization of VEGFR-2 that is required for activation of the mitogen-activated protein kinases ERK1/2 (Lampugnani et al., 2006). We found that overexpression of VE-cadherin in 1321N1 cells stably expressing the HA-tagged $P2Y_2R$ inhibits UTP-induced internalization of the $P2Y_2R$ (Supplemental Fig. IV-S1). Unlike Rac activation, however, UTP-induced ERK1/2 activation was unaffected by either down-regulation (Fig. IV-5) or overexpression of VE-cadherin (data not shown). Since the G_q-coupled $P2Y_2R$ has been

reported to couple to other G proteins, including G_o and G_{12} that regulate $P2Y_2R$-mediated activation of Rho GTPases in an integrin-dependent manner (Bagchi et al., 2005; Liao et al., 2007), it is likely that specific G proteins also regulate $P2Y_2R$/VE-cadherin interactions.

Since UTP-induced tyrosine phosphorylation of p120 catenin is dependent on the presence of VE-cadherin (Fig. IV-6D), we conclude that p120 catenin acts downstream of VE-cadherin to regulate Rac activity. Furthermore, we found that the UTP-induced interaction between p120 catenin and the Rac GEF, vav2, occurs in a VEGFR-2-dependent manner (Fig. IV-8), thereby elucidating a novel pathway by which the $P2Y_2R$ regulates Rac activity.

In summary, results presented here indicate that in endothelial cells, activation of the G protein-coupled $P2Y_2R$ induces the Src- and VEGFR-2-dependent activation of VE-cadherin in adherens junctions, which leads to the p120 catenin- and vav2-dependent activation of Rac (Fig. IV-9). Since the $P2Y_2R$ can associate with VE-cadherin (Fig. IV-2) as well as VEGFR-2 (Seye et al., 2004) and $\alpha_v\beta_3/\beta_5$ integrins (Erb et al., 2001), these results suggest that the $P2Y_2R$ participates in a multi-receptor complex to regulate the function of adherens junctions. This study elucidates a novel mechanism whereby a GPCR can modulate adherens junctions that regulate actin cytoskeletal rearrangements and leukocyte infiltration into endothelium, responses that initiate vascular inflammation in cardiovascular diseases.

Materials and Methods

Materials

Goat anti-human VE-cadherin polyclonal antibody, rabbit anti-human Flk-1(VEGFR-2) polyclonal antibody, and rabbit anti-vav2 polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-phosphotyrosine antibody was purchased from BD Bioscience (San Jose, CA). Mouse anti-HA antibody conjugated agarose beads and rabbit anti-HA antibody were purchased from Covance (Berkeley, CA). Anti-p120 catenin antibodies were purchased from Santa Cruz Biotechnology and BD Bioscience. Rabbit polyclonal anti-phospho-ERK1/2 antibody was purchased from Cell Signaling (Beverly, MA). Specific inhibitors for VEGFR-2 tyrosine phosphorylation (SU1498) and Src (PP2) were obtained from Calbiochem (Indianapolis, IN). ON-TARGETplus SMARTpool siRNA duplexes targeting the human $P2Y_2$ receptor, VE-cadherin and p120 were purchased from Dharmacon (Chicago, IL). VE-cadherin cDNA was a kind gift from Dr. Elisabetta Dejana (IFOM-IEO, Milan, Italy). All other reagents including nucleotides were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Cell Culture and Transfection

HCAEC (human coronary artery endothelial cells) were cultured in endothelial basal medium-2 (EBM-2; Clonetics, Walkerville, MD) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. HCAEC were used between the fourth and eighth passages. For transient transfections, siRNA or plasmid constructs were delivered using Targefect F-2 plus Virofect or Targefect-HUVEC from Targeting Systems (Santee, CA), respectively, according to the manufacturer's instructions. In both cases, transfection efficiency of at least 60% was achieved. Human $P2Y_2R$ ($hP2Y_2R$) cDNA encoding a hemagglutinin (HA)

tag at the N-terminus in pcDNA3.1(–) (Liao et al., 2007) or cDNA encoding the hP2Y_2R cDNA with an eGFP tag at the C-terminus in pEGFP-N1 (a kind gift from Dr. Fernando A. González, Department of Chemistry, University of Puerto Rico) were transiently expressed in HCAEC. Human 1321N1 astrocytoma cells lacking endogenous P2 receptors were also used in supplemental experiments. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, CA) containing 5% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were stably transfected with cDNA encoding either the wild-type P2Y_2Rs or a mutant P2Y_2R in which the C-terminal proline-rich SH3-binding domains where deleted (Del), as previously described (Liu et al., 2004) . These receptor constructs contained sequence encoding a HA tag at the N-terminus of the P2Y_2R, as previously described (Liu et al., 2004). In addition, the cells were transiently transfected with either pcDNA3 vector or pcDNA-VE-cadherin using the Lipofectamine 2000 reagent (Invitrogen, CA).

Confocal Laser Scanning Microscopy Visualization

HCAEC plated on glass coverslips were cultured to ~90% confluence before being transfected with hP2Y_2R-eGFP cDNA. The cell transfectants were maintained in growth medium for 72 h and transferred to serum-free medium for 12 h. Then, cells were incubated with or without 100 μM UTP for 5 min at 37 °C, washed in ice-cold PBS, fixed for 10 min in 4% (w/v) paraformaldehyde, treated with 0.1% (v/v) Triton X-100 for 5 min, and rinsed in PBS. Fixed cells were incubated with mouse anti-human VE-cadherin antibody (1:100 dilution, BD Bioscience, CA) for 1 h, washed and stained with Alexa

Fluor 594 goat anti-mouse IgG (1:200 dilution, Invitrogen) for 1 h, followed by incubation with Hoechst (Invitrogen). Coverslips were mounted on glass slides in ProLong antifade reagent (Invitrogen) and examined using a Zeiss inverted LSM 510 META confocal laser scanning microscope (CLSM) equipped with a C Apochromat 40X objective. Images were acquired, processed and analyzed with a Zeiss LSM Image Examiner.

RNA Extraction and RT-PCR

Isolation of RNA, cDNA synthesis, and RT-PCR were performed as previously described (Schrader et al., 2005). Amplification of $P2Y_2R$ cDNA was performed by RT-PCR using the following oligonucleotide primers: sense 5'-CTTCAACGAGGACTTCAAGTACGTGC-3', and antisense 5'-CATGT TGATGGCGTTGAGGGTGTGG-3'. Primers for amplification of human G3PDH cDNA were: sense 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3', and antisense 5'-CATGTGGGCCATGAGGTCC ACCAC-3'. Thirty-five amplification cycles were used, with annealing temperatures of 60 °C as previously described (Schrader et al., 2005). PCR products were resolved by 2% (w/v) agarose gel electrophoresis.

Rac activity assay

A Rac activation assay kit (Upstate Biotechnology, NY) was used to assess Rho activity according to the manufacturer's instructions. Briefly, cells were cultured in 6-well tissue culture dishes in EBM-2 with all supplements and then transferred to serum-free medium for 12 h before incubation with or without UTP for 5 min at 37 °C. Then, cells

were washed three times with ice-cold PBS, suspended in Lysis Buffer containing 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% (v/v) Igepal CA-630, 50 mM $MgCl_2$, 5 mM EDTA and 10% (v/v) glycerol, and the lysates were transferred to 1.5 ml tubes. Thirty microliters of agarose-conjugated p21 binding domain of PAK-1 that only recognizes GTP-bound Rac were added to 500 µl of lysate for 1 h at 4 °C. The beads were collected by centrifugation and washed three times with Lysis Buffer. Finally, the beads were resuspended in 40 ml of 2X Laemmli sample buffer (120 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) sucrose, 1 mM EDTA, 50 mM dithiothreitol and 0.003% (w/v) Bromophenol Blue) and Western analysis (see below) was performed with a 1:1000 dilution of mouse anti-human Rac antibody (Upstate Biotechnology, NY). In some experiments (see supplemental data), Rho activity was determined similarly except that p21-PAK-1-agarose and anti-Rac antibody were replaced with Rhotekin Rho binding domain (RBD)-agarose and mouse anti-human Rho antibody (Upstate Biotechnology, NY), respectively.

Immunoprecipitation and Immunoblotting

Immunoprecipitation (IP) and immunoblotting (IB) were performed, as previously described (Liao et al., 2007). Lysates from HCAEC were used for immunoprecipitation (IP) with the indicated antibodies, as previously described (Liao et al., 2007). The immune complexes were precipitated with protein A- or protein G-conjugated beads and analyzed by IB with antibodies against the proteins of interest. After IB, the membrane was stripped and reprobed with the same antibody used for IP. IP also was performed with lysates from cells expressing the HA-h$P2Y_2R$ using anti-HA-conjugated agarose

beads. These immunoprecipitated samples were analyzed by IB with anti-VE-cadherin or anti-HA antibody, respectively.

Internalization of HA-hP2Y$_2$R

Internalization of the HA-hP2Y$_2$R was examined indirectly by determining the uptake of HA antibodies added into 1321N1 astrocytoma cells stably expressing the wild type HA-hP2Y$_2$R, as described (Mao et al., 2007). Briefly, the cells were transfected with either pcDNA3 or pcDNA3-VE-cadherin. Then, cell transfectants were incubated at 37 °C in serum-free medium supplemented with 5 μg/ml anti-HA antibodies in the absence or presence of 1 mM UTP for 5 min to allow endocytosis of anti-HA antibody bound to the HA-hP2Y$_2$R. Cells were then placed on ice to prevent further receptor internalization, washed with ice-cold PBS, and surface-bound antibodies were removed by three washes with ice-cold acidic buffer (100 mM glycine, 20 mM magnesium acetate, 50 mM potassium chloride, pH 2.2). After an additional wash with ice-cold PBS, the cells were lysed with 2X Laemmli sample buffer, and lysates were analyzed for anti-HA antibodies by immunoblotting. Anti-HA antibodies were detected by chemiluminescence using horseradish peroxidase-conjugated antibodies (1:1000 dilution).

Figure IV-1. UTP causes clustering of the eGFP-P2Y$_2$R in intercellular junctions in HCAEC. (A) HCAEC were transiently transfected with eGFP-tagged P2Y$_2$R cDNA (shown in green) in pEGFP-N1, as described in "Materials and Methods". Then, 72 h after transfection, the cells were serum-starved for 12 h and stimulated with 100 μM UTP for 5 min at 37 °C . Cells were washed, fixed, permeabilized and stained with mouse anti-VE-cadherin antibody. Alexa Fluor 594-conjugated anti-mouse IgG and Hoechst were then used to localize VE-cadherin (shown in red) and nuclei (shown in blue), respectively. (B) A line (yellow) was drawn through the highly fluorescent cells shown in the upper panels of Fig. IV-1A (-/+ UTP). Staining densities of VE-cadherin (red curve) and eGFP-P2Y$_2$R (green curve) along the line are shown below. For each treatment, ~30 cell transfectants from 4 independent experiments were examined.

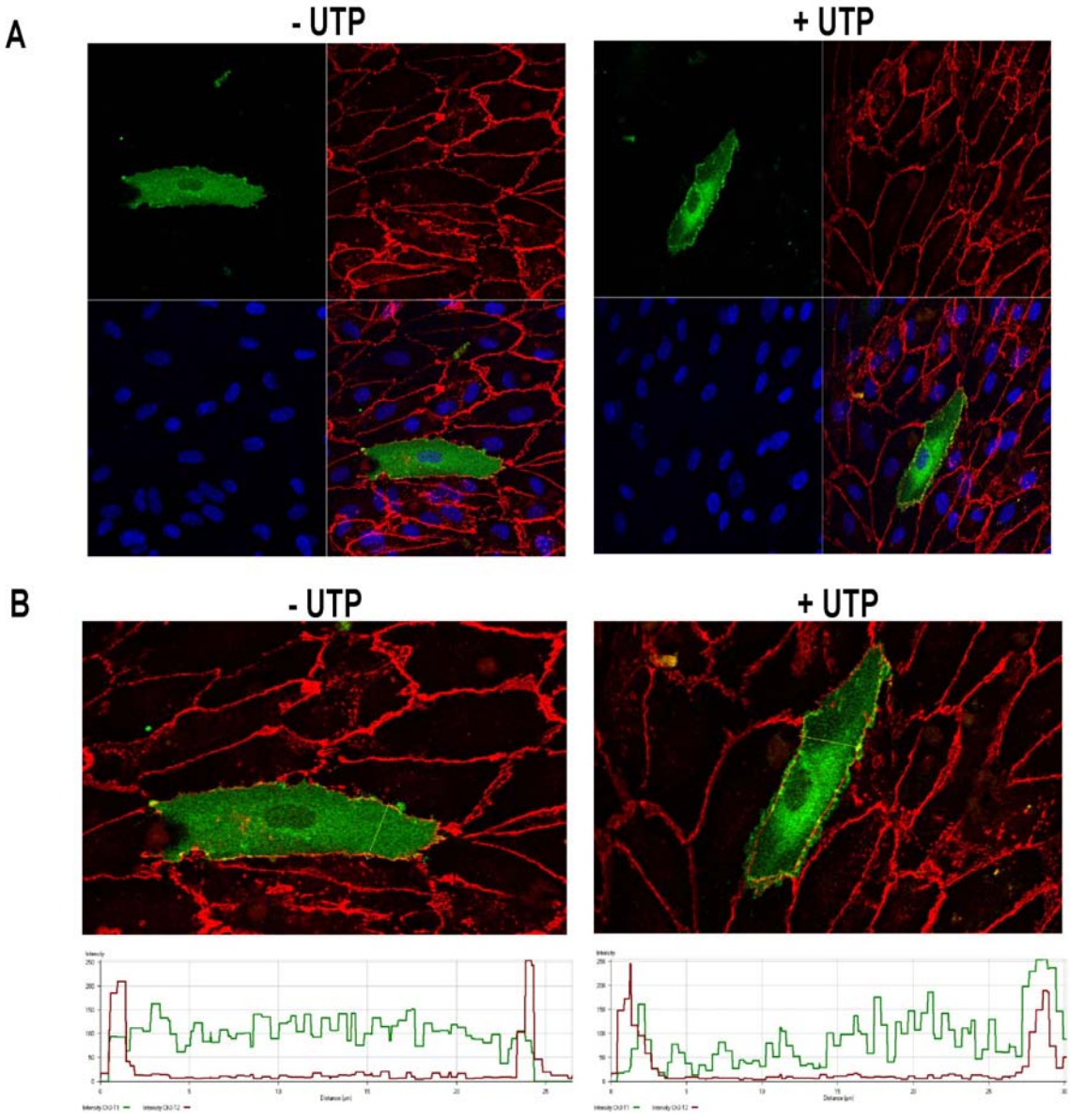

A
- UTP
+ UTP
B
- UTP
+ UTP

Figure IV-2. UTP-induced interaction between the HA-P2Y$_2$R, VE-cadherin and VEGFR-2. (A) HCAEC were transfected with HA-P2Y$_2$R cDNA in pcDNA3.1(-), as described in "Materials and Methods". Then, 36 h after transfection, the cells were serum-starved for 12 h and stimulated with 100 μM UTP at 37 °C for the indicated time. Cell lysates were prepared and subjected to IP with mouse anti-HA antibody conjugated to agarose beads and IB with anti-VE-cadherin or rabbit anti-HA antibody. (B) HCAEC were serum-starved for 12 h and stimulated with 100 μM UTP at 37 °C for the indicated time. Cell lysates were prepared and subjected to IP with anti-VEGFR-2 antibody and IB with anti-VE-cadherin antibody. The membrane was stripped and re-blotted with anti-VEGFR-2 antibody. (C) HCAEC were transfected with HA-tagged P2Y$_2$R in pcDNA3.1(-), serum-starved for 12 h and treated with PP2 (1 μM) or SU1498 (10 μM) for 1 h followed by incubation with or without 100 μM UTP for 5 min at 37 °C. Cell lysates were prepared and subjected to IP with anti-HA matrix beads and IB with anti-VE-cadherin or anti-HA antibody. Cell lysates also were subjected to IB with anti-VE-cadherin antibody to detect total VE-cadherin. Blots representative of three experiments are shown.

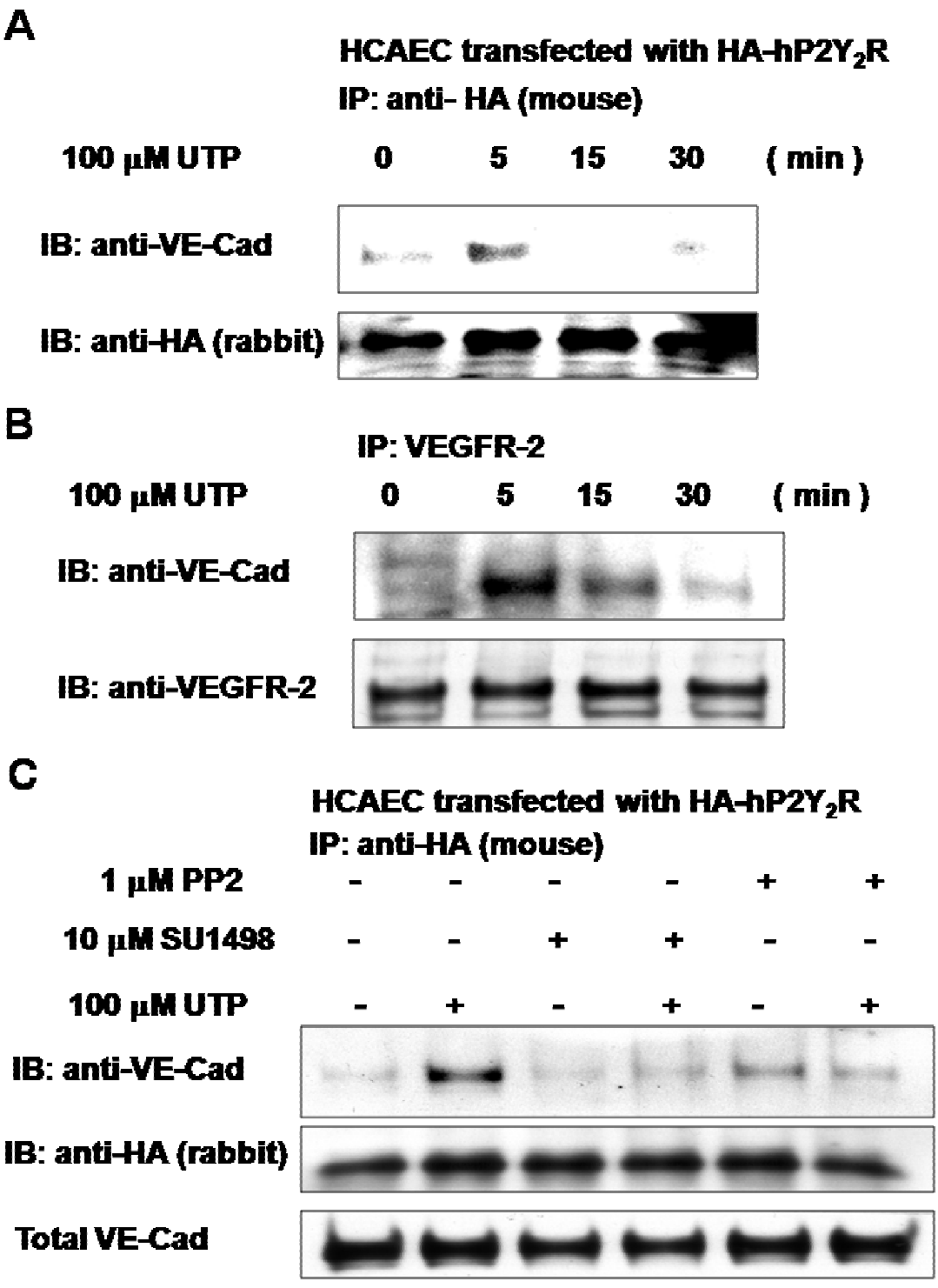

A
HCAEC transfected with HA-hP2Y2R
IP: anti- HA (mouse)
100 μM UTP
0
5
15
30
(min)
IB: anti-VE-Cad
IB: anti-HA (rabbit)
B
IP: VEGFR-2
100 μM UTP
0
5
15
30
(min)
IB: anti-VE-Cad
IB: anti-VEGFR-2
C
HCAEC transfected with HA-hP2Y2R
IP: anti-HA (mouse)
1 μM PP2
10 μM SU1498
100 μM UTP
IB: anti-VE-Cad
IB: anti-HA (rabbit)
Total VE-Cad

Figure IV-3. UTP induces tyrosine phosphorylation of VE-cadherin. (A) HCAEC were treated with UTP (100 μM) at 37 °C for the indicated time. Cell lysates were subjected to IP with anti-phosphotyrosine antibody and IB with anti-VE-cadherin antibody. Cell lysates also were subjected to IB with anti-VE-cadherin antibody to detect total VE-cadherin. (B) HCAEC were transfected with either scrambled siRNA or $P2Y_2R$-specific siRNA. Then, 36 h after transfection, total RNA was extracted from cell lysates and RT-PCR was performed with either $P2Y_2R$ primers or G3PDH primers, as described in "Materials and Methods". (C) HCAEC were transfected with either scrambled siRNA or $P2Y_2R$ siRNA. Then, 36 h after transfection, the cells were serum-starved for 12 h and incubated with or without 100 μM UTP for 5 min at 37 °C. Cell lysates were prepared and subjected to IP with anti-phosphotyrosine antibody and IB with anti-VE-cadherin antibody. The data shown are representative of results from three experiments.

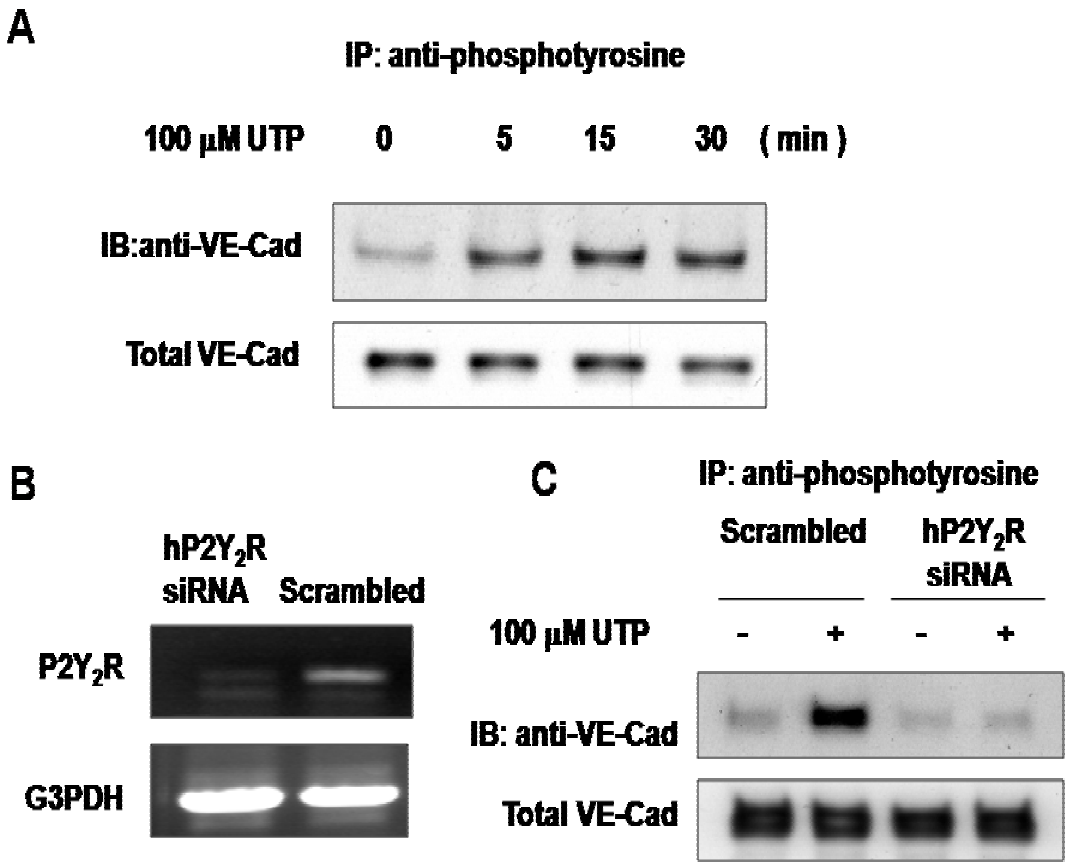

A
IP: anti-phosphotyrosine
100 μM UTP
0
5
15
30
(min)
IB:anti-VE-Cad
Total VE-Cad
B
hP2Y2R siRNA
Scrambled
P2Y2R
G3PDH
C
IP: anti-phosphotyrosine
Scrambled
hP2Y2R siRNA
100 μM UTP
-
+
-
+
IB: anti-VE-Cad
Total VE-Cad

Figure IV-4. Inhibitors of Src or VEGFR-2 prevent $P2Y_2R$-mediated tyrosine phosphorylation of VE-cadherin. HCAEC were pretreated for 1 h at 37 °C in serum-free medium with or without (A) the Src kinase inhibitor PP2 (1 μM) or (B) the VEGFR-2 inhibitor SU1498 (10 μM), and then stimulated with or without 100 μM UTP for 5 min at 37 °C. Cell lysates were prepared and subjected to IP with anti-phosphotyrosine antibody and IB with anti-VE-cadherin antibody. Cell lysates also were subjected to IB with anti-VE-cadherin antibody to detect total VE-cadherin. Blots representative of 3 experiments are shown.

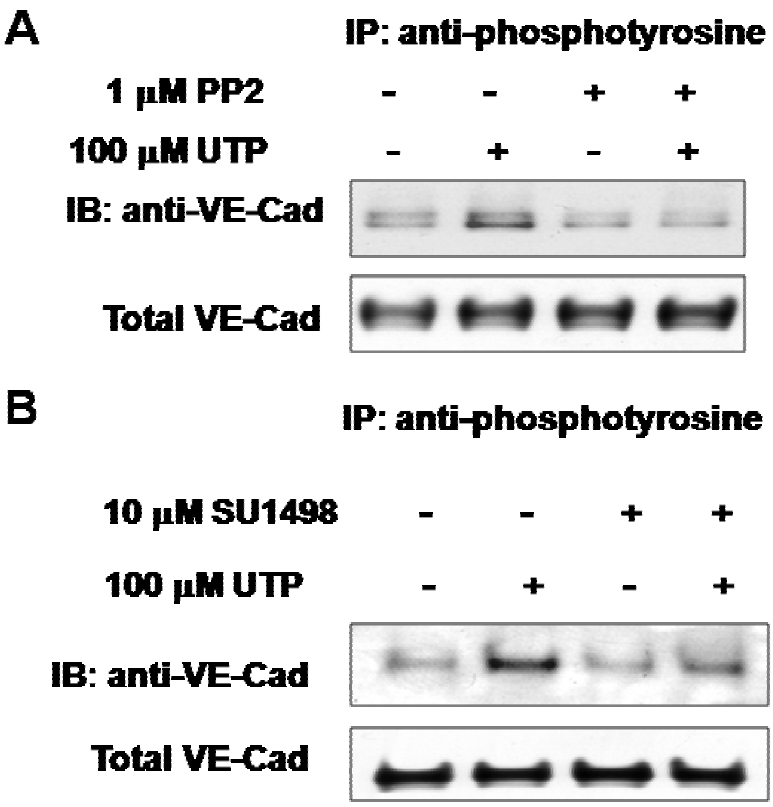

A
IP: anti-phosphotyrosine
1 μM PP2
- - + +
100 μM UTP
- + - +
IB: anti-VE-Cad
Total VE-Cad
B
IP: anti-phosphotyrosine
10 μM SU1498
- - + +
100 μM UTP
- + - +
IB: anti-VE-Cad
Total VE-Cad

Figure IV-5. VE-cadherin is necessary for Src- and VEGFR-2- dependent activation of Rac, but not MAPK, induced by UTP. (A,B) HCAEC were transfected with either scrambled siRNA or VE-cadherin-specific siRNA. Then, 36 h after transfection, (A) cells were incubated with or without 100 μM UTP at 37 °C for the indicated time. Cell lysates were prepared and analyzed by IB with either anti-VE-cadherin antibody or anti-phospho-p42/44 (ERK1/2) antibody. (B) Transfected cells were subjected to a Rac activity assay, as described in the "Materials and Methods". (C) HCAEC were serum-starved for 12 h, treated with or without PP2 (1 μM) or SU1498 (10 μM) for 1 h, and then incubated with or without 100 μM UTP for 5 min at 37 °C prior to Rac activity assay. Cell lysates also were subjected to IB with anti-Rac antibody to detect total Rac. Blots representative of 3 experiments are shown.

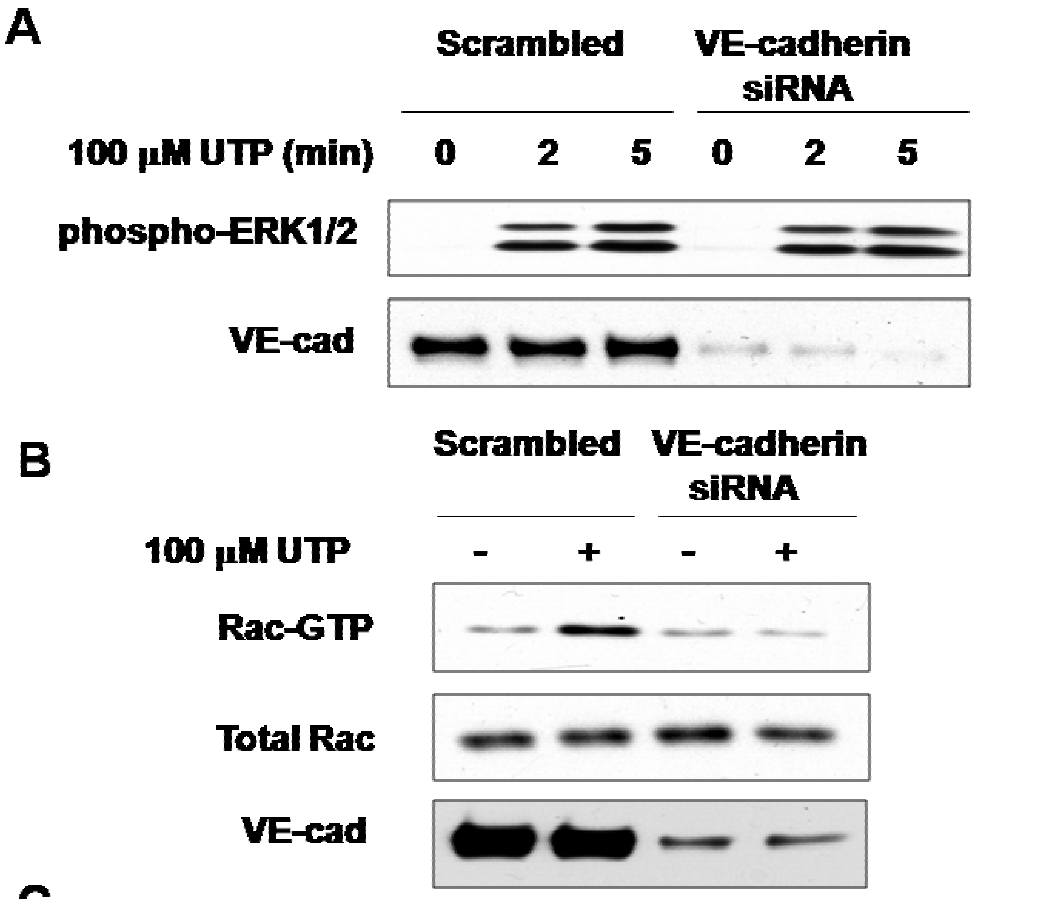

A
Scrambled
VE-cadherin siRNA
100 μM UTP (min)
0 2 5 0 2 5
phospho-ERK1/2
VE-cad
B
Scrambled
VE-cadherin siRNA
100 μM UTP
- + - +
Rac-GTP
Total Rac
VE-cad
C
1 μM PP2
10 μM SU1498
100 μM UTP
Rac-GTP
Total Rac

Figure IV-6. VE-cadherin is required for $P2Y_2R$-mediated tyrosine phosphorylation of p120 catenin that is dependent upon VEGFR-2. HCAEC were transfected with the indicated siRNA (A, D). Then, 36 h after transfection, cells were serum-starved for 12 h and incubated with or with 100 μM UTP for 5 min at 37 °C. (B,C) HCAEC were preteated with (B) PP2 (1 μM) or (C) SU1498 (10 μM) for 1 h, and then incubated with or without 100 μM UTP for 5 min at 37 °C. (A-D) Cell lysates were prepared and subjected to IP with anti-phosphotyrosine antibody and IB with anti-p120 catenin antibody. Cell lysates also were subjected to IB with anti-p120 catenin antibody to detect total p120 catenin. Blots representative of 3 experiments are shown.

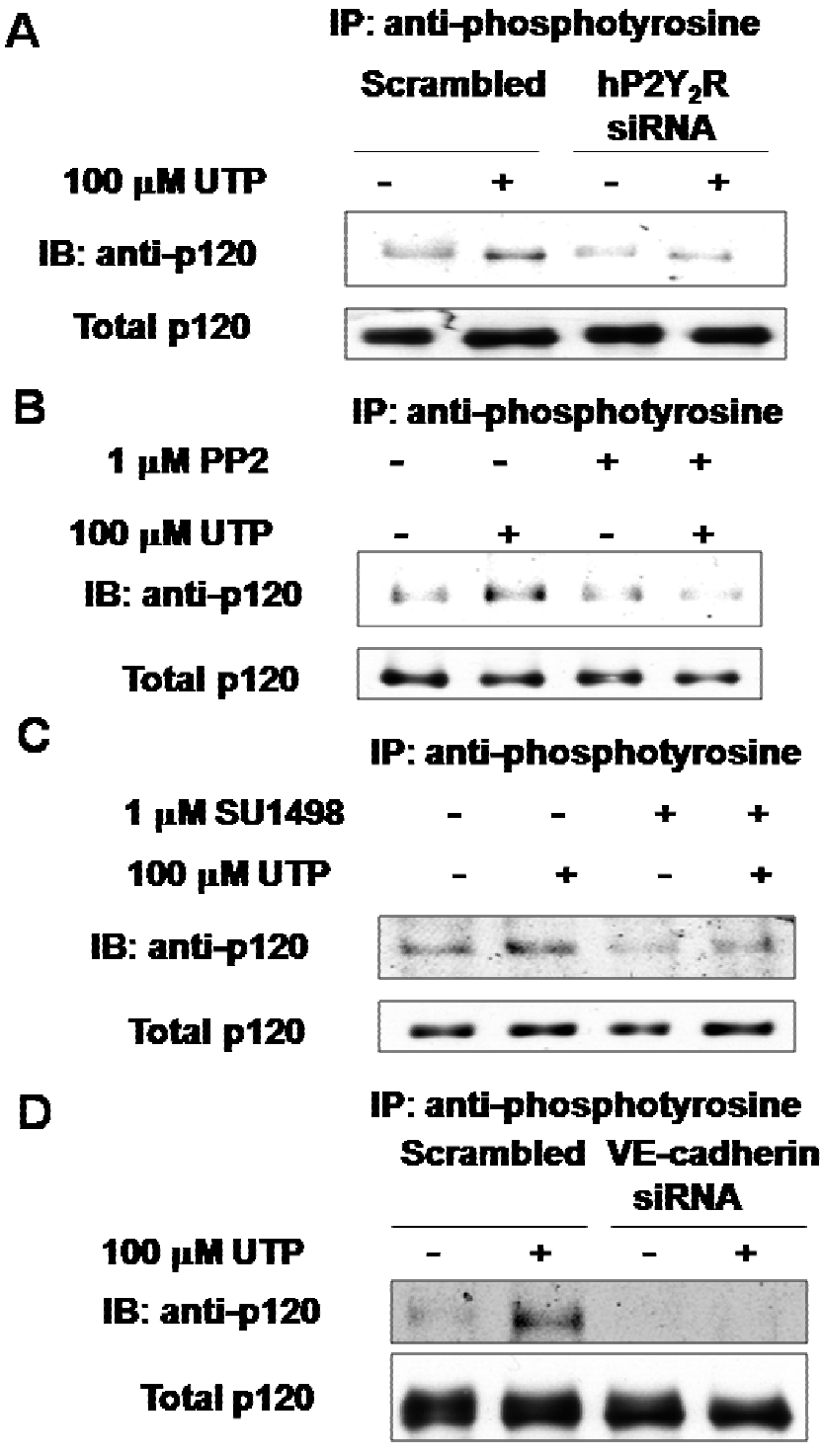

A
IP: anti-phosphotyrosine
Scrambled
hP2Y2R siRNA
100 μM UTP
- + - +
IB: anti-p120
Total p120
B
IP: anti-phosphotyrosine
1 μM PP2
- - + +
100 μM UTP
- + - +
IB: anti-p120
Total p120
C
IP: anti-phosphotyrosine
1 μM SU1498
- - + +
100 μM UTP
- + - +
IB: anti-p120
Total p120
D
IP: anti-phosphotyrosine
Scrambled
VE-cadherin siRNA
100 μM UTP
- + - +
IB: anti-p120
Total p120

Figure IV-7. p120 catenin is required for UTP-induced activation of Rac. HACEC was transfected with either scrambled siRNA or p120 catenin-specific siRNA. Then, 36 h later, cells were serum-starved for 12 h and incubated with or without 100 μM UTP for 5 min at 37 °C prior to Rac activity assay. Cell lysates were prepared and subjected to IB with anti-p120 catenin antibody. Blots representative of 3 experiments are shown.

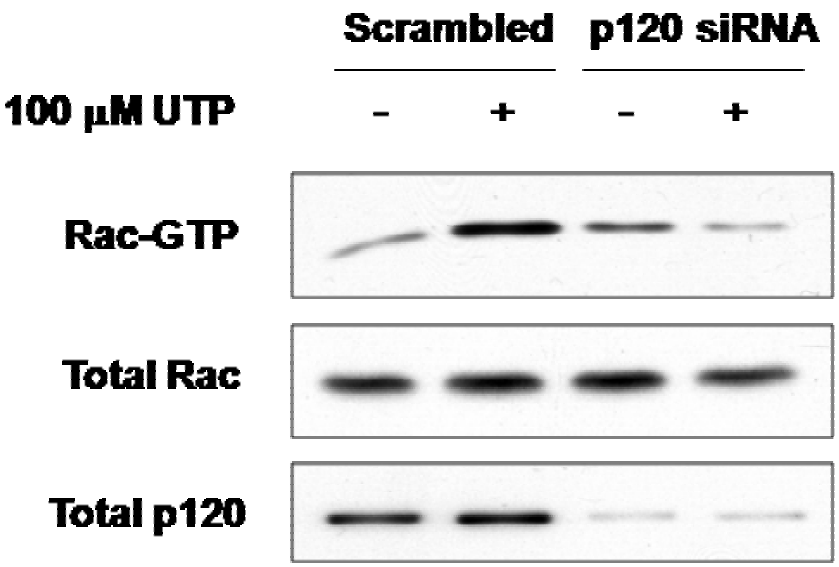
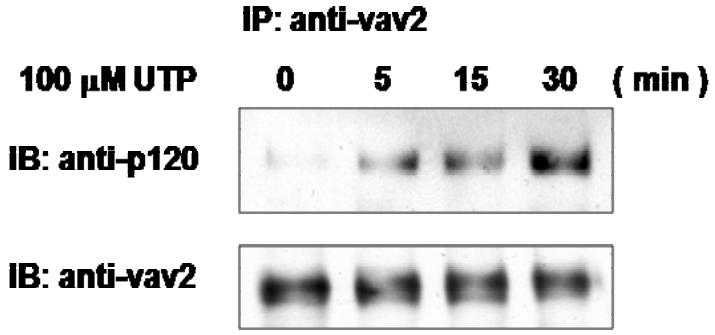

Scrambled
p120 siRNA
100 μM UTP
-
+
-
+
Rac-GTP
Total Rac
Total p120

Figure IV-8. UTP causes a VEGFR-2-dependent interaction between p120 catenin and vav2. (A) HCAEC were incubated with 100 μM UTP at 37 °C for the indicated times. (B) HCAEC were serum-starved for 12 h, treated with or without PP2 (1 μM) or SU1498 (10 μM) for 1 h, and then incubated with or without 100 μM UTP for 5 min at 37 °C. (A, B) Cell lysates were prepared and subjected to IP with anti-vav2 antibody and IB with anti-p120 catenin or anti-vav2 antibody. Blots representative of 3 experiments are shown.

A


IP: anti-vav2
100 μM UTP
0
5
15
30
(min)
IB: anti-p120
IB: anti-vav2

B

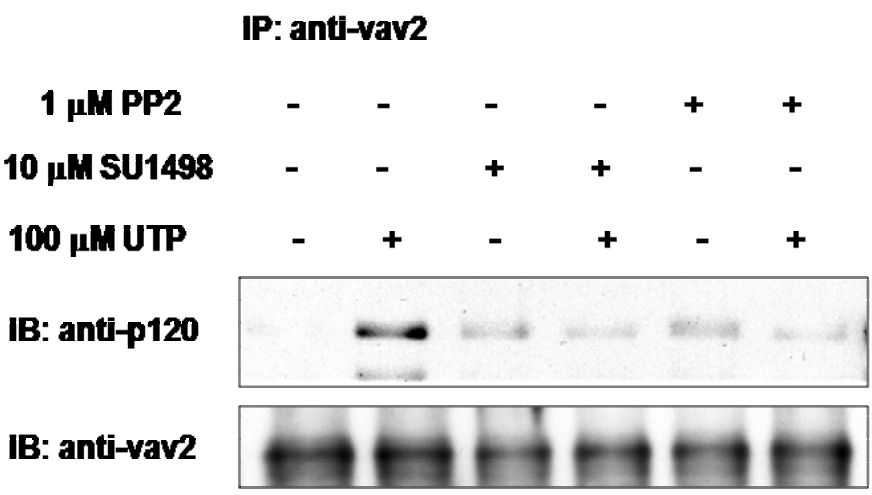
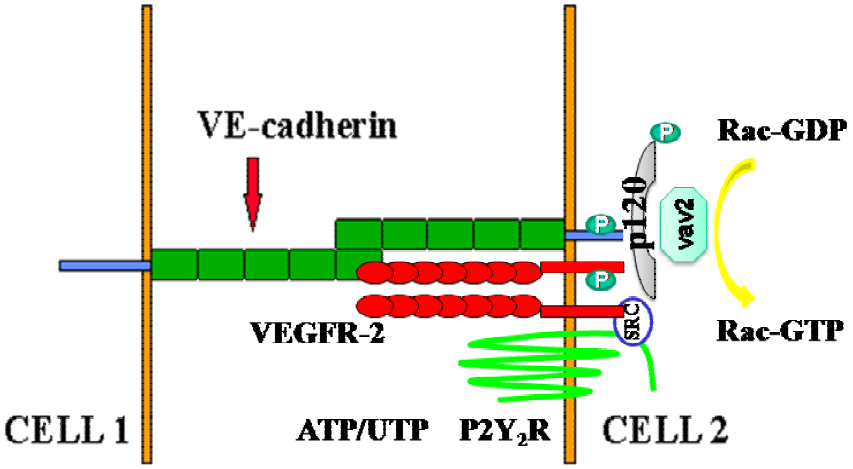
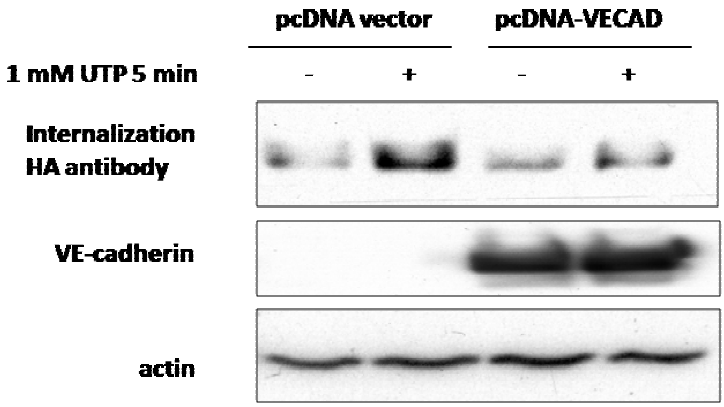

IP: anti-vav2
1 μM PP2
10 μM SU1498
100 μM UTP
IB: anti-p120
IB: anti-vav2

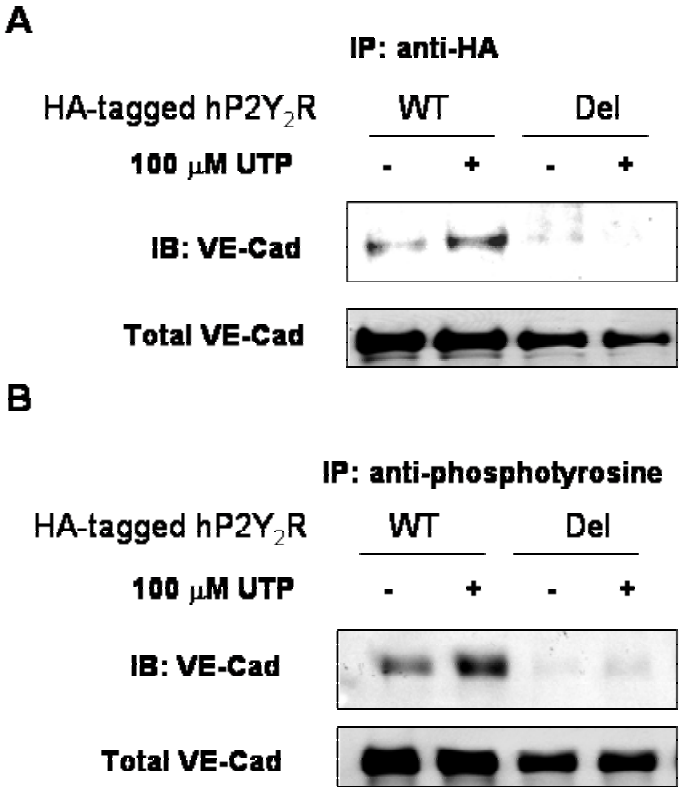
Figure IV-9. VE-cadherin is required for VEGFR-2-dependent activation of Rac mediated by $P2Y_2R$. In endothelial cells exposed to UTP/ATP, activated $P2Y_2R$ relocalizes to intercellular junctions by interacting with VE-cadherin, an endothelial specific component of intercellular adherens junctions that is necessary for UTP-indunced activation of Rac. Src-dependent VEGFR-2 activation is required for UTP/ATP-induced association of the $P2Y_2R$ with VE-cadherin as well as tyrosine phosphorylation of VE-cadherin and the VE-cadherin-associated protein p120 catenin, and the association of the Rac GTP exchange factor vav2 with p120 catenin leading to Rac activation.


VE-cadherin
Rac-GDP
P
p120
vav2
P
P
VEGFR-2
SRC
Rac-GTP
CELL 1
ATP/UTP
P2Y2R
CELL 2

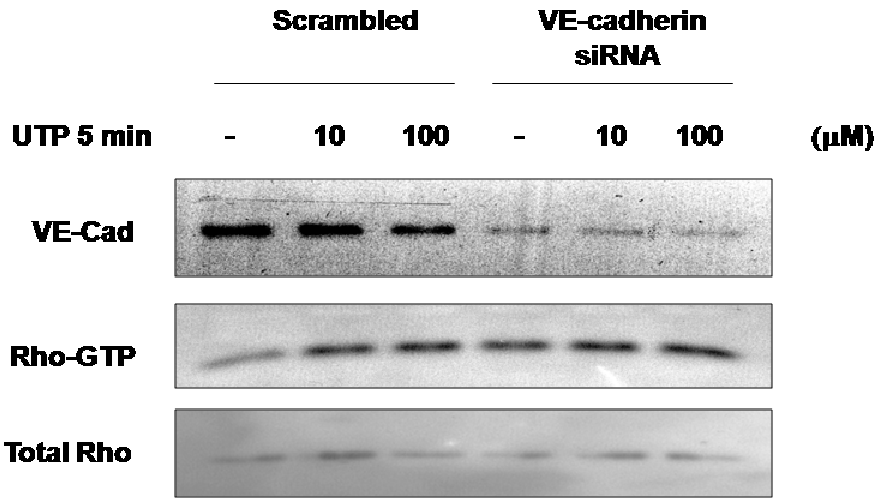
Supplemental Figure IV-S1. VE-cadherin inhibits UTP-induced internalization of the HA-hP2Y$_2$R in 1321N1 astrocytoma cells. Human 1321N1 astrocytoma cells stably expressing the wild type HA-hP2Y$_2$R were transiently transfected with pcDNA3 or pcDNA3-VE-cadherin. Anti-HA antibodies were added to the medium and the cells were incubated with or without 1 mM UTP for 5 min at 37 °C to allow endocytosis to proceed. Surface-bound antibodies were removed from cells by acidic buffer wash and internalized anti-HA antibodies were detected by immunoblotting, as described in "Materials and Methods". Total cell lysates also were subjected to immunoblotting with anti-VE-cadherin or anti-actin antibodies.


pcDNA vector
pcDNA-VECAD
1 mM UTP 5 min
-
+
-
+
Internalization
HA antibody
VE-cadherin
actin

Supplemental Figure IV-S2. The Src-binding domain of the $P2Y_2R$ is required for the $P2Y_2R$ to interact with and phosphorylate VE-cadherin. Human 1321N1 cells expressing the HA-tagged WT (wild type) or Del (deletion of prolines in the Src-binding domains) mutant $hP2Y_2R$ were transfected with VE-cadherin cDNA in pcDNA3. Cells were serum-starved for 12 h and incubated with or without 100 μM UTP for 5 min at 37 °C. Cell lysates were prepared and subjected to IP with (A) anti-HA matrix or (B) anti-phosphotyrosine antibody, and IB with anti-VE-cadherin antibody. Cell lysates also were prepared and analyzed by immunoblotting with anti-VE-cadherin antibody. Blots representative of 3 experiments are shown.


A
IP: anti-HA
HA-tagged hP2Y2R
WT
Del
100 μM UTP
- + - +
IB: VE-Cad
Total VE-Cad
B
IP: anti-phosphotyrosine
HA-tagged hP2Y2R
WT
Del
100 μM UTP
- + - +
IB: VE-Cad
Total VE-Cad

Supplemental Figure IV-S3. Effect of down-regulation of VE-cadherin on UTP-induced activation of Rho in HCAEC. HCAEC were transfected with either scrambled or VE-cadherin-specific siRNA. Then, 36 h after transfection, cells were serum-starved for 12 h and incubated with or without the indicated concentration of UTP for 5 min at 37 °C prior to performing a Rho activity assay, as described in "Materials and Methods". Cell lysates also were prepared and analyzed by immunoblotting with anti-Rho antibody. Blots representative of 3 experiments are shown.


Scrambled
VE-cadherin siRNA
UTP 5 min
-
10
100
-
10
100
(μM)
VE-Cad
Rho-GTP
Total Rho

CHAPTER V

Summary and future directions

1. Summary

Extracellular nucleotides have been recognized for their roles in the development of various chronic inflammatory diseases including vascular inflammation. Leukocyte emigration from the blood stream to the site of infection or injury is a major step in the early stages of many inflammatory diseases, a step that involves the processes of endothelium-dependent leukocyte adhesion, transendothelial migration, and chemotaxis. The fact that mechanical and pathological insults cause release of extracellular nucleotides from vascular endothelial cells and leukocytes triggers interest in research to define the role of extracellular nucleotides in mediating leukocyte recruitment into vascular tissue.

Effects of extracellular nucleotides are mediated by cell surface receptors belonging to two families – the ligand-gated ion channels, P2XRs, and the G protein-coupled receptors, P2YRs. In a study of stress-induced vascular injury, a silicone collar was placed around a rabbit carotid artery causing a dramatic increase in $P2Y_2R$ mRNA expression in endothelial and smooth muscle cells (Seye et al., 2002). Introduction of the $P2Y_2R$ agonist UTP into the collar, caused intimal thickening, indicative of atherogenesis, as well as a 4-fold increase in leukocyte migration into the intima, suggesting a potential role for the endothelial cell $P2Y_2R$ in monocytes recruitment in the inflammatory process (Seye et al., 2002).

As reviewed in Chapter I, the $P2Y_2R$ contains a consensus integrin-binding domain

(RGD sequence) in the first extracellular loop, enabling it to interact with $\alpha_v\beta_{3/5}$ integrins (Erb et al., 2001). It also has consensus Src-homology-3 (SH3) binding sites in the intracellular C-terminal tail that bind directly to Src and allow the $P2Y_2R$ to interact with and transactivate several growth factor receptors including the vascular endothelial growth factor receptor-2 (VEGFR-2/Flk-1) (Liu et al., 2004; Seye et al., 2004).

The overall aim of the research described in this dissertation is to understand the mechanisms involved in extracellular nucleotide-induced leukocyte emigration during vascular inflammation. It is important to learn how the $P2Y_2R$ mediates cell cytoskeletal rearrangements and subsequent chemotaxis, and how the $P2Y_2R$ coordinates with adherens junction proteins to regulate endothelial cell signaling and leukocyte transendothelial migration in response to extracellular nucleotides released from stressed or damaged tissue. Towards this aim, the results presented in this dissertation have led to the following novel conclusions:

1) Interaction with α_v integrin via the RGD motif in the $P2Y_2R$ is required for the $P2Y_2R$ to mediate cell chemotaxis and accompanying cytoskeletal rearrangements.

2) Interaction with α_v integrin is required for the $P2Y_2R$ to access and activate G_o and G_{12}, but not G_q proteins.

3) Coupling to G_o and G_{12} proteins enables the $P2Y_2R$ to mediate activation of Rac and Rho small GTPases, respectively, thereby regulating the activities of different downstream effectors that coordinately contribute to cell chemotaxis.

4) Activation of the $P2Y_2R$ in endothelial cells leads to tyrosine phosphorylation of adherens junction proteins, including VE-cadherin and p120 catenin, via the Src-dependent transactivation of VEGFR-2.

5) Upon stimulation, the $P2Y_2R$ transiently translocates to endothelial intercellular junctions and interacts with VE-cadherin, suggesting a potential role for the $P2Y_2R$ in regulating leukocyte transendothelial migration.

6) VE-cadherin is required for $P2Y_2R$-mediated activation of Rac in endothelial cells, possibly by regulating p120 interaction with the RhoGEF, vav2.

Although the cell chemotaxis study was performed in an astrocytoma cell line where no endogenous P2 receptors are present, the ability to express wild type or mutant $P2Y_2Rs$ in a null background has shed light on the general mechanism involved in $P2Y_2R$-mediated cell chemotaxis. Since neutrophils express both $P2Y_2Rs$ and $\alpha_v\beta_3$ integrins (Rainger et al., 1999; Chen et al., 2006), it is likely that extracellular nucleotide-induced neutrophil chemotaxis during inflammation is also regulated by interactions between these two receptors. Immune cells have to transmigrate quickly through the endothelium via paracellular junctions before the migration to extravascular tissues occurs. Interestingly, in the presence of extracellular nucleotides, the endothelial $P2Y_2R$ transiently interacts with VE-cadherin and, instead of being internalized, is recruited to endothelial adherens junctions. The interaction between the $P2Y_2R$ and VE-cadherin in endothelial cells is also required for activation of Rac, a Rho GTPase that modulates cellular structures regulating the permeability of endothelium to macro-molecules and leukocytes (van Wetering et al., 2003), suggesting a direct role for the

$P2Y_2R$ in vascular inflammation. Taken together, the results presented in this dissertation demonstrate that the $P2Y_2R$ associates within a multi-protein complex that includes α_v integrin, growth factor receptors, and VE-cadherin to regulate cell chemotaxis and cytoskeletal reorganizations by controlling Rho GTPase activities.

2. Future directions

A. Further delineate the mechanism of $P2Y_2R$-mediated chemotaxis

In chapters II and III, we have demonstrated that the $P2Y_2R$ requires α_v integrins to activate certain G proteins required for cell migration (*i.e.*, G_o, which activates Rac, and G_{12}, which activates Rho). However, it is unclear how these signals are temporally and spatially regulated. For example, it is known that the $P2Y_2R$ remains evenly distributed along the peripheral membrane in a migrating cell (Chen et al., 2006), whereas Rac redistributes to the front and Rho redistributes to the rear of a migrating cell (Sander et al., 1999). Therefore, it is very likely that the $P2Y_2R$ regulates these downstream signals via temporal and spatial controls. Our preliminary results indicate that G_o and G_{12} do not distribute differentially along cells exposed to a UTP gradient, but we have technical concerns that our method did not detect the endogenous active form of these G proteins. However, other activated signaling molecules (*e.g.,* PI3K, vav2, p115 RhoGEF), including those associated with the $P2Y_2R$ (α_v integrin) could be detected using specific antibodies raised against their activated forms and this approach should be pursued. Follow-up studies also should be conducted with isolated neutrophils, or a differentiated neutrophil cell line, since astrocytoma cells do not polarize during migration to the same extent as neutrophils.

To explore spatial signaling during chemotaxis, various molecules could be labeled with antibodies against their activated forms in a neutrophil model (*e.g.*, DMSO-differentiated HL-60 cells). Those antibodies could include anti-phospho-PI3K, anti-phospho-vav2, anti-phospho-FAK, anti-phospho-PAK, anti-phospho-β_3 integrin, and WOW-1 (anti-activated $\alpha_v\beta_3$ integrin antibody). The cells would be examined at uniform concentrations of UTP/ATP or in a UTP/ATP gradient achieved by micropipette delivery. Meanwhile, PHAKT-GFP (the GFP-tagged pleckstrin homology domain of the Akt protein kinase) or Gβ-GFP could be transfected into the cells to indicate the direction of polarization and migration.

Furthermore, neutrophils isolated from wild type and $P2Y_2R$ knockout ($P2Y_2R^{-/-}$) mice could be used to unambiguously determine the role of the $P2Y_2R$ in regulating the activation and distribution of these signaling molecules.

B. Identification and characterization of novel $P2Y_2R$-associated proteins in endothelial cells and leukocytes.

Different cells have different profiles of gene expression and therefore may use different signaling pathways to the same downstream protein target. For example, the $P2Y_2R$ inhibits cell spreading and migration in human keratinocytes via $G_{q/11}$ (Taboubi et al., 2007), rather than $G_{i/o}$ or $G_{12/13}$. Since the $P2Y_2R$ interacts with growth factor receptors and α_v integrins, we speculate that the $P2Y_2R$ may be a component of a large signaling complex containing integrins and integrin-associated proteins such as pyk2, Src, FAK, growth factor receptors and actin cytoskeletal proteins. A better understanding of the nature of these multi-protein interactions and signaling events involving $P2Y_2Rs$ will

likely identify intervention points for selectively controlling $P2Y_2R$ activities that mediate inflammatory responses, leading to new treatments for inflammatory disorders, such as atherosclerosis, diabetes, neurodegenerative disorders, cancer, and ulcers.

To identify novel $P2Y_2R$-associated proteins, plasmids contain HA-tagged or GFP-tagged $P2Y_2R$ cDNA would be transfected into neutrophils or endothelial cells. After immunoprecipitation with specific antibodies (*e.g.*, anti-HA or anti-GFP antibody), the immunoprecipitated products can be analyzed by SDS-PAGE or 2D electrophoresis. By use of mass spectrometry, we may be able to identify proteins having transient or sustained interactions with the activated $P2Y_2R$.

To characterize the function of these interacting proteins in $P2Y_2R$-mediated signaling and cell migration, specific inhibitors or RNA interference could be used to functionally down-regulate these proteins. In addition, these $P2Y_2R$-interacting proteins can be examined for their post-translational modifications in response to activation of the $P2Y_2R$. Immunostaining of these proteins in migrating cells could give insight into how the $P2Y_2R$ regulates chemotactic signals spatially.

C. Determine whether the $P2Y_2R$ mediates transendothelial migration of immune cells in stressed, injured, or infected arteries.

The $P2Y_2R$ is up-regulated in vascular endothelium in response to arterial injury or stress (Seye et al., 2002). Furthermore, *in vivo* application of the $P2Y_2R$ ligand UTP to rabbit carotid arteries undergoing perivascular collar-induced stress has been found to enhance intimal hyperplasia and monocyte infiltation into the neointima (Seye et al., 2002) . These responses suggest that the $P2Y_2R$ plays a critical role in initiating an

inflammatory response, and studies should be performed to conclusively determine whether this subtype of nucleotide receptors contributes significantly to vascular inflammation in humans.

To also address the importance of the $P2Y_2R$ in vascular inflammation *in vivo*, $P2Y_2R$ knockout ($P2Y_2R^{-/-}$) mice could be used to determine whether nucleotides still promote immune cell migration into stressed or injured arteries. Arterial injury in normal versus $P2Y_2R^{-/-}$ mice can be induced by placement of a perivascular silicone collar around the carotid artery and immunohistochemical analysis would be used to assess accumulation of immune cells in the injured artery, including monocytes/macrophages, granulocytes, T cells, and dendrocytes. The effect of the $P2Y_2R$ agonists (ATP and UTP) on transendothelial migration of immune cells could be evaluated by administering these ligands extravascularly through a pump attached to the collar. In addition, vascular inflammatory phenotypes thought to be associated with $P2Y_2R$ activation (*i.e.,* monocyte chemotaxis, adhesion, transendothelial migration, and expression of chemotactic/adhesive proteins including E-selectin, MCP-1, VCAM-1, VE-cadherin, osteopontin, and vitronectin) could be analyzed in primary monocytes and endothelial cells isolated from normal versus $P2Y_2R^{-/-}$ mice or in cultured cells in which endogenous $P2Y_2R$ expression has been down-regulated with RNAi. In addition, monocytes and vascular endothelial cells isolated from normal and $P2Y_2R^{-/-}$ mice could be used to assess the role of the $P2Y_2R$ in ATP/UTP-stimulated chemotaxis, monocyte adhesion to endothelial cells, and transendothelial migration of monocytes.

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