ROLE OF P2Y$_2$ NUCLEOTIDE RECEPTORS

IN REACTIVE ASTROGLIOSIS

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Of the Requirements for the Degree

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by

MIN WANG
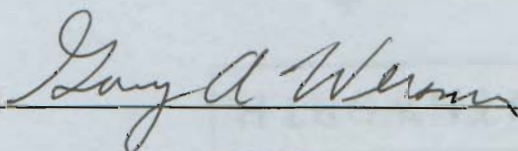
Dr. Gary A. Weisman, Thesis Supervisor

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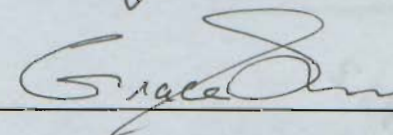The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled.

ROLE OF P2Y$_2$ NUCLEOTIDE RECEPTORS

IN REACTIVE ASTROGLIOSIS

Presented by Min Wang

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance.

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ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS:

AA, Arachidonic acid;

ATP, Adenosine 5'-triphosphate;

$[Ca^{2+}]_I$, Concentration of cytoplasmic free calcium;

$cPLA_2$, Ca^{2+}-dependent cytosolic PLA_2;

Cas, Crk-associated substrate, $p130^{Cas}$;

Cav-1, Caveolin-1;

COX1/2, Cyclooxygenase-1/2;

DAG, Diacylglycerol;

EC_{50}, Half maximal effective concentration;

EGFR, Epidermal growth factor receptor;

ERK, Extracellular-signal regulated kinase;

FAK, Focal adhesion kinase;

Fyn, Feline yes-related protein;

GFAP, Glial fibrillary acidic protein;

GFR, Growth factor receptor;

GPCR, G protein-coupled receptor;

Grb2, Growth factor receptor bound protein 2;

HA, Hemagglutinin;

IB, Immunoblot;

$IFN\gamma$, Interferon-γ;

IL-1β, Interleukin-1 beta;

iNOS, Inducible nitric oxide synthase;

IP, Immunoprecipitate;

IP_3, Inositol-1,4,5-trisphosphate;

JNK, c-Jun NH_2-terminal kinase;

MAPK, Mitogen-activated protein kinase;

MCP-1, Monocyte chemoattractant protein-1;

NTPDase, Nucleoside triphosphate diphosphohydrolase;

OPN, Osteopontin;

P, Proline;

$P2Y_2R$, $P2Y_2$ nucleotide receptor;

Pax, Paxillin;

PBS, Phosphate buffered saline;

PDGFR, Platelet derived growth factor receptor;

PGI_2, Prostacyclin;

PGE_2, Prostaglandin E_2;

PI, Propidium iodide;

PI3-K, Phosphatidylinositol-3 kinase;

PIP2, Phosphatidylinositol 4,5-bisphosphate;

PKC, Protein kinase C;

PLC, Phospholipases C;

PLA_2, Phospholipases A2;

PMN cells, Polymorphonuclear leukocytes;

Pyk2, Proline-rich tyrosine kinase-2;

RacGEF, Rac guanine exchange factor;

RGD, Arginine-glycine-aspartic acid;

RGE, Arginine-glycine-glutamic acid;

Rho A, Member of the Rho GTPase family;

ROCK, Rho associated kinase;

RT-PCR, Reverse transcriptase-polymerase chain reaction;

SAPK, Stress-activated protein kinase;

SH3, Src-homology-3;

Shc, *shc* gene products;

siRNA, Small inhibitory ribonucleic acid;

Sos, Guanine nucleotide exchange factor; Son of sevenless;

Src, Non-receptor tyrosine kinase;

Tal, Talin;

TNFα, Tumor necrosis factor-α;

UDP, Uridine diphosphate;

UTP, Uridine 5'-triphosphate;

UV, Ultraviolet;

VCAM-1, Vascular cell adhesion molecule-1;

VEGF, Vascular endothelial growth factor;

VEGFR-2, Vascular endothelial growth factor receptor-2;

Vin, Vinculin;

X, Any amino acid.

ROLE OF P2Y$_2$ NUCLEOTIDE RECEPTORS IN REACTIVE ASTROGLIOSIS

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ABSTRACT

Astrocytes become activated in response to brain injury characterized by increased expression of GFAP and increased rates of cell migration and proliferation. Damage to brain cells causes the release of cytoplasmic nucleotides, such as ATP and UTP, ligands for P2 nucleotide receptors. Results in this study with primary rat astrocytes indicate that activation of a G protein-coupled P2Y$_2$ receptor for ATP and UTP increases GFAP expression and both chemotactic and chemokinetic cell migration. UTP-induced astrocyte migration was inhibited by silencing of P2Y$_2$R expression with P2Y$_2$R siRNA. UTP also increased the expression in astrocytes of $\alpha_V\beta_{3/5}$ integrins that are known to interact directly with the P2Y$_2$R to modulate its function. Anti-α_V integrin antibodies prevented UTP-stimulated astrocyte migration, suggesting that P2Y$_2$R/α_V interaction mediates the activation of astrocytes by UTP. P2Y$_2$R-mediated astrocyte migration required the activation of the PI3-K/Akt and MEK/ERK signaling pathways, responses that also were inhibited by anti-α_V integrin antibody. These results suggest that P2Y$_2$Rs and their associated

signaling pathways may be important factors regulating astrogliosis in brain disorders.

CHAPTER 1: LITERATURE REVIEW

<u>Introduction</u>

Astrocytes and microglia, two types of glial cells in the brain, are activated under a variety of pathological conditions and can contribute to neurodegeneration {1}. Activated astrocytes undergo reactive astrogliosis characterized by cell hypertrophy and hyperplasia and increased motility {2}. Extracellular nucleotides activate P2 nucleotide receptors in glial cells and neurons that regulate inflammatory responses and neurotransmission, respectively {3, 4}. We have investigated the function of a $P2Y_2$ nucleotide receptor for ATP and UTP expressed in glial cells that can regulate cell proliferation and motility through stimulation of signal transduction pathways including intracellular calcium mobilization, and activation of G proteins, mitogen-activated protein kinases (MAPK), and integrin and growth factor receptors {5}. This review will focus on the structure and function of $P2Y_2$ receptors and their role in inflammatory and proliferative responses in astrocytes.

<u>P2 receptor classification</u>

In the early 1970s, it was reported that ATP was released into the extracellular space by stimulation of non-adrenergic, non-cholinergic nerves to activate responses postulated to be mediated by P2 purinergic receptors for nucleotides {6, 7}. Over the next few decades, it has been recognized that activation of P2 nucleotide receptors can modulate a variety of responses in cells of the mammalian central nervous system (CNS) including neurotransmission,

cell growth and apoptosis {8~10}. It is known that nucleotides can be released from excitatory neurons, injured cells, cells undergoing mechanical or oxidative stress, aggregating platelets, degranulating macrophages, and astrocytes {11~14} by mechanisms including exocytosis from ATP/UTP-containing vesicles, facilitated diffusion via putative ABC transporters, cytoplasmic leakage, or by poorly understood electrodiffusional movements through ATP/nucleotide channels {15, 16}. Extracellular nucleotides act as signaling molecules by activating cell surface P2 receptors belonging to two structurally distinct families: the G protein-coupled receptors (P2YR) and the ligand-gated ion channels (P2XR). Nine P2Y receptors have been cloned and identified as G protein-coupled receptors (GPCRs), including $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$, and $P2Y_{15}$ {17, 18}, although the $P2Y_{15}$ receptor has not been conclusively established to be a P2 nucleotide receptor. Seven P2X receptors have been cloned and identified as ligand-gated ion channels, including $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ {19}. Functional responses to activation of these P2 receptor subtypes in neurons and glial cells under normal and pathological conditions include cell apoptosis, proliferation, migration, inflammation, ion transport, and neurotransmission {19, 20}. Therefore, P2 receptors in the CNS might be potential targets for pharmaceutical therapies in neurological disorders. Among these P2 receptors, our research has focused on the $P2Y_2R$ and its signaling pathways in the regulation of responses in astrocytes associated with reactive astrogliosis, a process that contributes to neurodegeneration.

P2 receptors in astrocytes

Previous studies revealed that ATP acts as a neurotransmitter when it is released from presynaptic neuronal vesicles and stimulates electrical responses in postsynaptic neurons, although the physiological role of P2 receptors in astrocytes is less well studied. Under pathological conditions such as neurodegenerative diseases or brain trauma, nucleotides released from damaged neurons can stimulate glial cells to become reactive and undergo a series of changes that are collectively termed reactive astrogliosis {2}. Depending on the conditions, reactive astrogliosis can have either neurotoxic or neuroprotective effects {21-23}. Reactive astrogliosis is characterized by increased astrocyte proliferation and morphological changes, including extensive cellular hypertrophy, fiber extension and increased expression of GFAP (glial fibrillary acidic protein) {2, 24}. Moreover, activated astrocytes migrate to the edge of an injury similar to macrophages in the cardiovascular system and they form a ''reactive astrocyte wall'' to separate healthy from injured brain cells and to secrete cytokines, chemokines, growth factors, antigens, and cell adhesion molecules {25}. Recent studies have demonstrated that extracellular nucleotides can stimulate GFAP expression, astrocyte proliferation, and activation of cyclooxygenase-2, which are cellular responses associated with reactive astrogliosis {26~32}. Astrocytes express numerous subtypes of P2 nucleotide receptors. For example, studies with primary cultures of cortical astrocytes obtained from 1-d-old rat pups revealed the presence of $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors {33} as well as $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_6$, and $P2X_7$ receptors {34}.

Studies with cortical astrocyte cultures from 7-d-old rats indicated the presence of all P2 nucleotide receptors except $P2X_6$ {35}. As shown in Figure 1, we found that primary astrocytes isolated from the brain of 2~3 days old rat express several P2 nucleotide receptor subtypes, including $P2X_3$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$, but not $P2Y_{11}$, $P2X_1$ or $P2X_2$. Differences in subtype expression might be the results of differences in developmental patterns of receptor expression or variations in culture conditions. *In vivo* studies also have demonstrated the expression of P2 nucleotide receptors in astrocytes from different brain regions {30, 36}.

As modulators of cell-to-cell communication in brain astrocytes, ATP and other nucleotides can increase the intracellular calcium concentration ($[Ca^{2+}]_i$) by stimulating calcium influx from the extracellular milieu through activation of P2X receptors {37} or by stimulation of inositol-1,4,5-trisphosphate (IP_3)-mediated calcium mobilization from intracellular stores through G protein-coupling to phospholipase C (PLC) via activation of P2Y receptors {38}. $P2Y_1$ receptors involve in *d*-amphetamine-induced brain sensitization {4} and calcium signaling which affects neural progenitor cell proliferation and migration during early central nervous system development {39}. Together with $P2Y_2$ receptors, $P2Y_1$ receptor activation causes arachidonic acid (AA) release from cultured astrocytes {40}. $P2Y_2$ and $P2X_7$ nucleotide receptors also mediate neuro-inflammatory responses in astrocytes and microglial cells {3}. Other studies suggest that $P2Y_6$ receptor activation prevents astrocyte apoptosis {41}. The recently identified $P2Y_{14}$ receptor for UDP-glucose in astrocytes was found to be upregulated by

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lipopolysaccharide, suggesting that these receptors might regulate immune responses in the brain {42}. Elucidation of the signaling pathways regulated by P2 receptors in astrocytes will help in understand the inflammatory/proliferative responses that occur in reactive astrogliosis, leading to neuronal loss in neurodegenerative diseases.

P2Y$_2$ nucleotide receptor

Activation of G protein-coupled P2Y$_2$Rs stimulates PLC and leads to the production of IP$_3$ and diacylglycerol (DAG) {5, 43}, second messengers for calcium release from intracellular storage sites and protein kinase C (PKC) activation, respectively. The P2Y$_2$R is expressed in epithelial cells, smooth muscle cells, endothelial cells, leukocytes and cardiomyocytes {43~47}. In cells derived from the peripheral and the CNS, P2Y$_2$Rs have been identified in immortalized astrocytes, NG108-15 neuroblastoma $\times$ glioma hybrid cells, Schwann cells, dorsal horn and cortical astrocytes, astrocytoma cells, rat cortical neurons, and oligodendrocytes {3, 5, 48~50}. P2Y$_2$Rs mediate pro-inflammatory responses, including increased cell proliferation, cell motility, and expression of cell adhesion molecules and cytokines {44, 51~53}. The P2Y$_2$R subtype is distinguished in its ability to be up-regulated under conditions of stress or injury in activated thymocytes, salivary gland epithelial cells, and models of vascular tissue injury {44, 54, 55}. For example, a vascular collar placed around a rabbit carotid artery was found to cause a dramatic increase in P2Y$_2$R expression in smooth muscle cells and endothelium leading to intimal thickening that is significantly enhanced by local application of the P2Y$_2$R agonist UTP and

correlates with the increased expression of osteopontin (OPN) and the proliferation of smooth muscle cells {44}. In addition, binding of monocytes to endothelium because of P2Y$_2$R-mediated upregulation of vascular cell adhesion molecule-1 (VCAM-1) promotes monocyte infiltration into neointima, suggesting that P2Y$_2$R activation can mediate an inflammatory response {53}. Very few studies have investigated the role of P2Y$_2$Rs in the brain *in vivo*. We utilized *in situ* hybridization and RT-PCR to determine that P2Y$_2$R mRNA was expressed at relatively low levels in normal rodent (i.e., rat, mouse and gerbil) brain slices, but was most abundant in hippocampus and cerebellum (unpublished data of Kong, Q.). In hippocampus, P2Y$_2$R mRNA was highly expressed in the dentate gyrus, and we also detected P2Y$_2$R mRNA in rat primary astrocytes (Fig. 1), primary neuron (unpublished data of Kong, Q.) and microglial cells {3}. Thus, the P2Y$_2$R is an intriguing new target for anti-inflammatory and anti-proliferative therapies in neurodegenerative diseases and atherosclerosis.

P2Y$_2$R and inflammatory responses

P2Y$_2$Rs mediate the synthesis of pro-inflammatory mediators and cell adhesion molecules, and the release of AA {53, 56, 57}. P2Y$_2$Rs also regulate the synthesis of prostaglandins, nitric oxide, and cytokines {56, 58~60}. P2Y$_2$R expression is up-regulated by agents that mediate inflammation, including cytokines, interleukin (IL)-1β, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α {61, 62}. In several cell types, upregulation of P2Y$_2$Rs is associated with nucleotide-induced stimulation of PKC, cyclooxygenase, and MAPKs {55, 63~65}. In primary murine astrocytes, P2Y$_2$Rs mediate the activation of calcium-

dependent and calcium-independent PKCs and extracellular-signal regulated protein kinases 1 and 2 (ERK1/2) to regulate the activity of cytosolic phospholipase A_2 (cPLA$_2$) {3, 57}, which hydrolyzes cell membrane phospholipids to produce AA {66}, a precursor of pro-inflammatory chemokines such as eicosanoids, prostaglandins, and leukotrienes {67}. Furthermore, P2Y$_2$R-mediated release of AA and activation of type 2 cyclooxygenase (COX-2) play a role in inflammation and reactive astrogliosis in neurodegenerative diseases {27, 28}. Consistent with a role for P2Y$_2$Rs in the proliferative phenotype, P2Y$_2$R mRNA expression has been found to be down-regulated during cell differentiation {68}. The P2Y$_2$R agonist UTP stimulates expression of mRNA to the cytokine transforming growth factor-β (TGF-β) in astrocytes {3}. In turn, TGF-β regulates cell proliferation and differentiation that is dependent upon activation of p38 and JNK in cultured astrocytes {3}. Our lab has shown that activation of P2Y$_2$R and P2X$_7$R induces phosphorylation of ERK, JNK and p38 in astrocytes and microglial cells, suggesting that P2Y$_2$Rs regulate pro-inflammatory responses in these cells {3, 69}.

Prostaglandin E_2 (PGE$_2$) is a mediator of inflammation in the neuroendocrine and immune systems {70, 71} and a potent vasodilator that acts with other chemokines to increase microvascular permeability in the peripheral nervous system {72, 73}. AA is the rate-limiting substrate for PGE$_2$ synthesis by COX-1 and/or COX-2 {67}. Phospholipase A_2 (PLA$_2$), the enzyme primarily responsible for hydrolyzing membrane phospholipids to yield free AA, is activated by ATP/UTP via P2Y$_2$Rs in primary murine astrocytes in a PKC- and MAPK-

dependent manner {57}. ATP in combination with TNF-α, IL-1β and INF-γ increased PGE$_2$ production about 13-fold compared to untreated astrocytes and 2-fold over cytokines alone, indicating that cytokines and nucleotides co-regulate PGE$_2$ production in astrocytes {56}. PGs, including PGE$_2$, have been shown to promote inflammatory responses associated with a number of diseases {70, 71}, and the ability of ATP and UTP to enhance cytokine-induced inflammation strongly suggests that P2Y$_2$ receptors modulate the actions of cytokines in neurodegenerative diseases.

P2Y$_2$R and integrin interactions: functional consequences

The P2Y$_2$R contains the consensus integrin-binding motif, Arg-Gly-Asp (RGD) in its first extracellular loop that binds selectively to $\alpha_V\beta_3/\beta_5$ integrins (see Fig. 2) {74}. Recent studies in our lab have demonstrated that the wild type P2Y$_2$R co-localizes with α_V integrins when the recombinant P2Y$_2$R is expressed in human 1321N1 astrocytoma cells, a cell was devoid of endogenous G protein-coupled P2Y receptors. In contrast, a mutant P2Y$_2$R in which the RGD motif was replaced with Arg-Gly-Glu (RGE) that does not have high affinity for integrins, exhibited 10-fold less co-localization with α_V integrins than the wild type receptor. The EC$_{50}$ for nucleotide-induced calcium mobilization was approximately 1,000-fold greater for the RGE mutant than the wild-type P2Y$_2$R, suggesting that the RGD-dependent association between the P2Y$_2$R and $\alpha_V\beta_3/\beta_5$ integrins is necessary to maintain the P2Y$_2$R in a high-affinity ligand-binding state. It also was noted that the activated RGE-mutant P2Y$_2$R lost the pertussis toxin sensitivity of the wild type P2Y$_2$R, suggesting that association with $\alpha_V\beta_3/\beta_5$

integrins enables access to the G_o signaling pathway, possibly through interaction with the integrin-associated thrombospondin receptor (CD47) that is known to interact with both $\alpha_V\beta_3$ integrins and G proteins in the $G_{i/o}$ family {74}.

The $\alpha_V\beta_3/\beta_5$ integrins are widely expressed in cells of the cardiovascular system and play critical roles in angiogenesis and inflammatory responses, including cell proliferation, migration, adhesion and infiltration {75~78}. Activation of P2Y$_2$Rs by UTP or ATP induces proliferation and/or migration of human epidermal keratinocytes, epithelial cells, lung epithelial tumor cells and smooth muscle cells {79~81}. Therefore, we examined whether interactions between P2Y$_2$Rs and $\alpha_V\beta_3/\beta_5$ integrins enabled the P2Y$_2$R to modulate integrin-mediated cell migration by comparing responses of wild type and RGE-mutant P2Y$_2$Rs. Results indicated that the presence of the RGD domain in the P2Y$_2$R is necessary for the receptor to mediate nucleotide-induced chemotaxis and the formation of actin stress fibers (unpublished data of Liao, Z and Bagchi, S.). Antibodies to $\alpha_V\beta_3/\beta_5$ integrins also inhibited P2Y$_2$R-mediated stress fiber formation and cell migration (unpublished data of Liao, Z and Bagchi, S.). These data demonstrate that integrin/P2Y$_2$R interactions are required for nucleotide modulation of integrin-associated responses such as cytoskeletal rearrangements that control cell migration. Based on findings in other labs on the regulation of cytoskeletal rearrangements {82, 83}, it can be predicted that small GTPases such as Rho, Cdc42, and Rac play a role in nucleotide-induced cell migration (see Fig. 3). Other studies indicate that phosphatidylinositol 3-kinase (PI3-K) and other intracellular kinases including mitogen-activated protein

(MAP) and stress-activated protein (SAP) kinases are key regulators of integrin-mediated actin stress fiber formation and cell migration {84}. Our studies have found that compared with the wild-type $P2Y_2R$, the RGE-mutant $P2Y_2R$ requires approximately 1,000-fold higher UTP concentration to activate ERK, downstream kinase of PI3-K (Akt), and isoforms of p54 Jun kinase (JNK), a SAP kinase {74}. Inhibitors of ERK, JNK and PI3-K decreased UTP-induced chemotaxis, supporting a role for these kinases in $P2Y_2R$-mediated cell migration (unpublished data of Liao, Z and Bagchi, S.).

Glial cell migration in the CNS under physiological conditions facilitates axonal growth during development {85}. In the adult brain, astrocyte migration is critical for the structural plasticity and repair of damaged brain cells {2}. Under pathological conditions involving neuronal cell loss, reactive astrocytes migrate to the site of damage in a wound healing response {25}. It has been reported that in response to focal ischemia, expression of the integrin $\alpha_V\beta_3$ is increased in astrocytes in the peri-infarct region {86}. OPN, a ligand of $\alpha_V\beta_3$, also is elevated after ischemia in both the peri-infarct region and the infarct area, and within 15 days of focal ischemia, astrocytes expressing $\alpha_V\beta_3$ are localized in an OPN-rich region within the core infarct, which is concomitant with the astrocyte barrier. These studies suggest that OPN and $\alpha_V\beta_3$ play roles in reactive astrocyte migration and wound healing in neurodegenerative diseases. We have found that the $P2Y_2R$ agonist UTP is chemotactic for primary rat astrocytes, a response that is inhibited by anti-α_V antibodies (see Fig. 7). Other results indicate that UTP causes upregulation of $\alpha_V\beta_3/\beta_5$ in rat astrocytes in a time-dependent manner.

These data suggest that interactions between $P2Y_2Rs$ and $\alpha_V\beta_3/\beta_5$ integrins are essential for nucleotide-induced astrocyte migration during wound healing and tissue remodeling in the CNS.

SH3 binding domains in the $P2Y_2R$ regulate transactivation of growth factor receptors

Proline-rich peptide sequences have been shown to play important roles in protein-protein interactions that occur in signal transduction pathways. For example, the proline-rich consensus Src-homology-3 (SH3)-binding sequences, PXXP (P is proline and X is any amino acid), in the β3 adrenergic receptor interact directly with Src to activate ERK1/2 {87}. PXXP motifs in the β1 adrenergic and dopamine D4 receptor interact with endophilins, SH3 domain-containing proteins, to mediate receptor internalization and receptor coupling to G proteins {88, 89}, adenylyl cyclase and MAP kinase {90}. Activation of GPCRs often causes the concomitant activation of growth factor receptors, although the mechanism controlling this transactivation is unclear {91, 92}. Previous studies have indicated that Src and Pyk2 are involved in the signaling pathway for growth factor receptor (GFR) transactivation by some GPCRs, including $P2Y_2Rs$ {91~93}, although researchers are divided on the role of this pathway in the activation of downstream mitogenic signaling by GPCRs. For example, studies with EGFR/Src/Pyk2 dominant negative mutants or an EGFR kinase inhibitor demonstrated the importance of EGFR/Src/Pyk2 in $P2Y_2R$-mediated MAPK activation in rat-1 fibroblasts and PC12 cells {94}. In contrast, studies with embryonic fibroblasts derived from Src^{-/-}, Pyk2^{-/-}, or Src^{-/-}Pyk2^{-/-} mice suggested

that Src/Pyk2 are essential for GPCR-mediated transactivation of the EGFR but are dispensable for GPCR-mediated MAPK activation {92}.

We have identified two PXXP motifs in the intracellular carboxy-terminal tail of the human $P2Y_2R$ that mediate GFR transactivation induced by ATP or UTP {95}. Immunofluorescence experiments showed that the SH3 binding domains in the $P2Y_2R$ facilitates its colocalization with the EGFR in response to $P2Y_2R$ activation by nucleotides. Although activation of the $P2Y_2R$ mediates ERK1/2 phosphorylation, our studies indicate that deletion of the SH3 binding motifs of the $P2Y_2R$ does not suppress ERK1/2 activation when the mutant receptors are expressed in 1321N1 astrocytoma cells {95}, most likely because of the ability of the $P2Y_2R$ to also activate Src and ERK1/2 via alternative routes (see Fig. 3). Consistent with this conclusion, the Src inhibitor PP2 prevented nucleotide-induced $P2Y_2R$/EGFR co-localization and ERK1/2 activation in 1321N1 cells expressing the wild-type $P2Y_2R$. Furthermore, immunoprecipitation experiments with UTP-treated cells showed that the wild-type $P2Y_2R$, but not the SH3 binding domain deletion mutant $P2Y_2R$, coprecipitates with Src. These results strongly suggest that activation of the $P2Y_2R$ promotes Src binding to the PXXP motifs in the $P2Y_2R$ to transactivate the EGFR or other GFRs and enables extracellular nucleotides to stimulate GFR-mediated signaling pathways that upregulate expression of cell adhesion proteins.

Our results also suggest that the $P2Y_2R$ mediates Src-dependent Pyk2 phosphorylation at Tyr881. A SH2 binding site is formed in Pyk2 after its

activation at Tyr881, which enhances association of Pyk2 with several SH2 domain-containing proteins, including Src {92} and the adaptor protein Grb2 {96}. Because activation of Pyk2 has been associated with cytoskeletal reorganization and cell proliferation {96, 97}, P2Y$_2$R-mediated Pyk2 activation is postulated to have similar physiological consequences.

P2Y$_2$R regulates cell proliferation

Both ATP and UTP induce cell cycle transition from the G1 to S and M phases in primary vascular smooth muscle cell cultures, indicating that P2Y$_2$R activation can regulate cell proliferation {51}. The P2Y$_2$R has been shown to mediate smooth muscle cell proliferation that leads to the development of neointimal hyperplasia, the precursor of atherosclerotic plaques {44}. Proliferation of smooth muscle cells by ATP was reported to require independent activation of PI3-K and ERK1/2 {81}. In HeLa cells, activation of the P2Y$_2$R causes PI3-K-dependent activation of ERK1/2, which induces cell proliferation through activation and expression of the early response protein c-fos {52}. These cell-dependent differences might reflect the ability of ATP to activate multiple P2 receptors in some cell types. In primary astrocytes, extracellular ATP increases GFAP protein expression and DNA synthesis {26, 32}, whereas injection of ATP or its analogs in the rat nucleus accumbens causes upregulation of GFAP-positive and GFAP/BrdU-positive cells, suggesting that nucleotides regulate astrocyte proliferation *in vivo* {31}.

ERK1/2 regulates cell proliferation in HeLa cells, human dermal microvascular endothelial cells, keloids, pancreatic tumor cells and myeloma

cells {52, 98~101}. Various extracellular agents, including cytokines, growth factors, and agonists of GPCRs, activate ERK1/2 signaling pathways {102, 103}. PI3-K transduces the signal between growth factor receptors (GFR) and ERK1/2 to regulate cell proliferation in endothelial cells and tumor cells {99, 104}. PI3-K also regulates diverse cellular processes including cell apoptosis, survival and migration {105}.

The signaling cascade for ATP-induced astrocyte proliferation has been investigated {32, 106}, and it has been shown that ERK1/2 induces gene expression via activation of AP-1 transcriptional complexes {107}. ATP also causes sequential activation of phospholipase D and calcium-independent PKCδ in astrocytes, and the activation of the SAP kinase p38 has been shown to regulate AP-1-mediated astrocyte proliferation {108}. Recent studies in our laboratory have investigated the pathways whereby P2Y$_2$Rs mediate nucleotide-stimulated activation of ERK1/2 and PI3-K. Inhibitors of c-Src and calcium-independent isoforms of PKC, but not calcium-dependent PKC, were found to prevent UTP–induced ERK1/2 phosphorylation {95, 109}, suggesting that calcium-independent PKC and c-Src lie upstream of ERK1/2 activation in P2Y$_2$R-mediated signaling pathways. The inhibitors of PI3-K, LY294002 and wortmannin, and the G$_{i/o}$ inhibitor pertussis toxin also decreased P2Y$_2$R-mediated ERK1/2 activation, but not G$_q$- and PLC-dependent calcium mobilization, suggesting that both the integrin-binding and SH3-binding domains of the P2Y$_2$R co-modulate ERK1/2 activation. We have found that ERK1/2 activation by the wild type but not the RGE mutant P2Y$_2$R, is partially inhibited by

the calcium chelator BAPTA, suggesting that interactions with integrins are required for the $P2Y_2R$ to activate calcium-dependent ERK1/2 activation. Furthermore, anti-αv antibody and RGDS tetrapeptide partially inhibited UTP-induced ERK1/2 phosphorylation {74}. We also have found that cell proliferation induced by $P2Y_2R$ activation in primary astrocytes is dependent on PI3-K and ERK1/2 and that transactivation of the EGFR could play a role in this process (unpublished data). Taken together, our studies indicate that activation of the $P2Y_2R$ promotes cell proliferation through transactivation of integrin and GFR signaling pathways that stimulate MAP and SAP kinases, although G protein-mediated activation of PLC might contribute to signal amplification (Fig. 3). Further studies on protein-protein interactions within the multiple signaling pathways of the $P2Y_2R$ complex should lead to a better understanding of nucleotide-induced astrocyte proliferation and the inflammatory response, information that should yield new insight into treatments for Alzheimer's and other neurological diseases.

CHAPTER 2: ROLE OF P2Y$_2$ NUCLEOTIDE RECEPTORS

IN REACTIVE ASTROGLIOSIS

Introduction

Astrocytes, a type of glial cell in the central nervous system, regulate water and electrolyte transport, local pH and ionic equilibrium, and neurotransmitter uptake {109}. Astrocytes can become reactive under a variety of pathological conditions, a process termed astrogliosis characterized by increased expression of GFAP and enhanced cell migration and proliferation {24}. In cerebral ischemia, reactive astrocytes migrate to the edge of an injured area and form a barrier between damaged and healthy tissue {86}. Although there are indications that reactive astrocytes can protect undamaged tissue and limit secondary injury, excessive or chronic accumulation of astrocytes can produce deleterious effects and prevent neuronal regeneration within the damaged area {2, 110}. Reactive astrogliosis is associated with increased production of cytokines and other pro-inflammatory agents that can damage neurons {25}. Release from astrocytes of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) has been shown to precede neuronal degeneration {111, 112}. Therefore, a greater understanding of the mechanisms involved in reactive astrogliosis should provide new insights into ways to prevent irreversible brain damage in neurological disorders.

Astrocyte migration during reactive astrogliosis requires cell cytoskeletal rearrangements involving the extracellular matrix {25}. It has been shown that

osteopontin (OPN), an extracellular matrix protein, is upregulated during the formation of glial scars after focal ischemia {113}. A receptor for OPN, the integrin $\alpha_V\beta_3$ is also upregulated in reactive astrocytes that localize to the peri-infarct area 5 days after ischemia and to an OPN-rich, glial barrier 15 days post-ischemia, suggesting that $\alpha_V\beta_3$ and its extracellular ligands are involved in reactive astrogliosis {86}. The $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins play essential roles in cell migration by interacting with extracellular ligands containing an arginine-glycine-aspartic acid (RGD) motif including OPN, vitronectin, fibronectin, and thrombospondin {114~117}.

When tissues are damaged, cytoplasmic nucleotides such as ATP and UTP are released from injured cells. These nucleotides can activate cell surface P2 nucleotide receptors and trigger cell proliferation, migration or apoptosis {79, 118, 119}. Previous studies indicate that stretch- or stab-induced injury causes the activation of a G protein-coupled P2Y nucleotide receptor in astrocytes and in turn, increases extracellular signal-regulated kinase (ERK) activation, GFAP expression, and astrocyte proliferation {26, 31, 32, 108, 120}. The present study investigated the role of the $P2Y_2$ nucleotide receptor ($P2Y_2R$) subtype in the activation of primary rat astrocytes. Among the human nucleotide receptor subtypes, G protein-coupled $P2Y_2Rs$ are unique in that they are activated by either ATP or UTP, and contain a RGD motif in the first extracellular loop that enables $P2Y_2Rs$ to interact directly with $\alpha_V\beta_3/\beta_5$ integrins {74}. In this study, we tested the hypothesis that activation of $P2Y_2Rs$ is associated with astrogliosis. The results indicate that activation of the $P2Y_2R$ in primary astrocytes increases

the expression of GFAP and $\alpha_V\beta_3/\beta_5$, and induces astrocyte migration. Furthermore, activation of intracellular signaling pathways involving phosphatidylinositol-3-kinase (PI3-K) and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) were required for P2Y$_2$R-mediated astrocyte migration, and these responses were inhibited by anti-α_V antibodies. These results demonstrate a pathway whereby activation of P2Y$_2$Rs by nucleotides released from damaged or stressed cells can trigger astrogliosis associated with brain injuries, suggesting potential targets for the prevention of neurodegeneration.

Materials and Methods

Antibodies and Reagents

Goat anti-rat α_V, anti-rat α_4, anti-rat β_3, anti-rat β_5 and rabbit anti-rat ERK, anti-rat GFAP (H50), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated monkey anti-goat IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LY294002, U0126, rabbit anti-rat phospho-p44/42 ERK1/2 and rabbit anti-rat Akt (Ser473) antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Rabbit anti-rat α_V antibody was obtained from Chemicon (Temecula, CA). Rabbit anti-rat actin antibody was obtained from Cytoskeleton (Denver, CO). Nucleotides and other chemicals were from Sigma (St. Louis, MO).

Primary Rat Astrocyte Cell Culture

Astrocytes were isolated from the cerebral cortices of postnatal 2- to 3-day old Sprague Dawley rat pups and cultured as previously described (McCarthy and de Vellis, 1980) {121} with minor modifications. Briefly, dissected cerebral cortices were placed in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsdad, CA). The meninges were removed, and the cortices were cut into small pieces and incubated with 0.25% (w/v) trypsin-EDTA at 37°C for 7 min. The suspension was then filtered through 85 μm nylon mesh and centrifuged at 1000 rpm (~250 g) for 5 min. The cell pellet was re-suspended in culture medium, comprised of DMEM plus 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 7.5 μg/ml fungizone, and transferred to T75 culture flasks (Techno Plastic Products, Trasadingen,

Switzerland). Cells were maintained in an atmosphere of 5% CO_2 and 95% air at

37°C and the medium was changed twice a week. When cells reached ~80-90%

confluence, flasks were shaken at 225 rpm for 6 h at room temperature to

remove microglial cells. Then, cells were washed with phosphate buffered saline

(PBS), removed from flasks by treatment with 0.05% (w/v) trypsin-EDTA at 37°C

for 4 min, and seeded at 1×10^6 cells in 60 mm culture dishes for RT-PCR

experiments. Approximately 1×10^5 cells/well were cultured in 12-well plates for

immunoblot analysis. Astrocytes were identified by GFAP staining (more than

95% of the total cells were GFAP positive).

Astrocyte Treatments

Primary rat astrocytes (1×10^5 cells/well) were maintained in 12-well plates

with culture medium for 2 days followed by serum-free medium for 1 day.

Astrocytes were subsequently incubated with 10 μg/ml anti-α_V or anti-α_4 antibody

for 2 h, and stimulated with UTP at the concentrations and times indicated in the

figure legends. For immunoblot analysis, cells were washed with ice-cold PBS

and lysed with 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). The cell lysate was used for immunoblot analysis.

P2Y$_2$ Receptor siRNA Transfection

Vector-based, P2Y$_2$R-specific siRNAs were designed using Genscript's

siRNA design target and construct builder (http//www.genscript.com).   The

consecutive sequences for *P2Y$_2$R antisense*, loop and <u>P2Y$_2$R sense cDNA</u>, 5'-

*AGGCCGCATACAGTGCATCAG*TTGATATCCG<u>CTGATGCACTGTATGCGGCC</u>

T-3', were cloned into the pRNA-U6.1/Neo plasmid vector. Empty vector was used as a negative control. Transfection of primary rat astrocytes was performed using Lipofectamine 2000 (Invitrogen, Carlsdad, CA) according to the manufacturer's instructions. Briefly, astrocytes at ~70-80% confluence were cultured for 1 day in culture medium without antibiotics. Lipofectamine 2000 was mixed in DMEM for 5 min at room temperature, incubated for 20 min with plasmid DNA diluted in DMEM, and incubated with astrocytes for 24 h at 37°C in a cell culture incubator. The efficiency of siRNA transfection of astrocytes was determined by the reduction in P2Y$_2$R mRNA levels detected by RT-PCR.

RNA Extraction and RT-PCR

Total RNA was isolated from primary astrocytes using the Qiagen Rneasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized from total RNA using the First Strand cDNA Synthesis Kit and an oligo-dT primer (Roche Diagnostics Corporation, Indianapolis, IN). Specific primers were used to amplify P2Y$_2$R, P2Y$_4$R and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA by RT-PCR, as previously described {122}. PCR was performed as follows: 94°C for 2 min, 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min with a final elongation step at 72°C for 7 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels with 10 μg/ml ethidium bromide and bands were visualized under ultraviolet (UV) light. The ratio of P2Y$_2$R/G3PDH product was determined using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Chemokinesis Assay

This assay was adapted from a protocol described by Albrecht-Buehler (1977) {123}. Cover slips (22×22 mm) and gold particles were prepared as described. Primary rat astrocytes (1×10^5 cells/ml) in serum-free medium were seeded onto cover slips. Then, cells were incubated with or without UTP or ATP at the concentration indicated in the figure legends for 18 h at 37°C. When indicated, inhibitors or other agents were added to the astrocytes 2 h before addition of UTP or ATP. Then, cells were washed with PBS and fixed with 4% (v/v) formaldehyde for 15 min. Fixed cells were observed under an inverted microscope and images were captured by a digital camera (Qimaging, Burnaby, BC, Canada) and analyzed with Northern Eclipse 6.0 software (Empix Imaging Inc. Mississauga, ON, Canada). As cells migrated on the cover slips, they displaced and endocytosed gold particles leaving white phagokinetic tracks that were recorded.

Chemotaxis Assay

Primary rat astrocytes (transfected or untransfected) were suspended with trypsin, washed and re-suspended in serum-free medium at 5×10^5 cells/ml. Transwell chambers (Becton Dickinson Labware, Franklin Lakes, NJ) with 8 μm pore size polycarbonate membrane inserts were placed into wells of 24-well culture plates. The lower Transwell chamber was filled with 600 μl of serum-free medium containing UTP or ATP, as indicated, and 100 μl of cell suspension was added to the upper chamber. In some cases, antibodies were added to the lower chamber and inhibitors to both chambers, 2 h prior to nucleotide addition. After 18 h at 37°C in a 5% CO_2 incubator, the cells remaining in the upper chamber

were removed by scraping the membrane with a cotton swab. Cells attached to the bottom of the membrane were fixed with ice-cold methanol for 15 min, washed with PBS and stained with hematoxylin for 15 min. Stained cells observed under a microscope were counted in 10 fields and represented chemotactic cells that had migrated from the upper to the lower chamber in response to nucleotides in the lower chamber.

Immunoblot Analysis

The detection of integrin subunits, GFAP, phospho-ERK1/2, ERK1/2, phospho-Akt, and actin was achieved by immunoblot analysis. For these assays, primary rat astrocytes were grown to ~70-80% confluence in 6-well plates, cultured in serum-free medium for two days and stimulated with UTP in the absence or presence of inhibitors (added 2 h before the UTP), as indicated in the figure legends. Then, cells were washed with ice-cold PBS and lysed in 100 µl of 2X Laemmli sample buffer. Cell lysates were sonicated with 2 sec blasts of 4 pulses using a Sonic Dismembrator (Fisher, PA), heated for 5 min at 96°C, subjected to 10% SDS-PAGE, and transferred to nitrocellulose membrane. Western blot analysis was performed using 1:1000 dilution of the indicated primary antibody at 4°C overnight followed by 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG antibody for 1 h at room temperature. Chemiluminescence of antibody-labeled proteins was detected on x-ray films and quantitated with Quantity One software (Bio-Rad Laboratories, Hercules, CA). Then, the antibodies were stripped from the membranes by a 30 min incubation at 60°C in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM 2-

mercaptoethanol, and 2% (w/v) SDS), and the membranes were re-probed with anti-actin antibody to determine the relative amounts of protein loading in each lane.

Immunoprecipitation

Primary rat astrocytes grown to ~80% confluence in 100 mm tissue culture dishes were cultured in serum-free medium for 48 h and stimulated with 100 μM UTP for 0, 8, 18 or 24 h. Then, cells were lysed for 15 min at 4°C in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% (v/v) NP-40, 0.25% (v/v) Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with proteinase inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN). Insoluble membrane and unlysed cells were removed from the lysate by centrifugation at 13,000 g for 20 min at 4°C. Then, 5 μg/ml anti-α_V antibody was added to 800 μl of lysate and incubated at 4°C with gentle rocking overnight, followed by incubation with 100 μl of Protein A agarose beads (Upstate, MA) at 4°C with gentle rocking overnight. The mixture was washed 3 times with 800 μl of PBS and immunoblot analysis was performed with anti-α_V, anti-β_3 or anti-β_5 antibodies, as described above.

Statistical Analysis

Data indicate the means $\pm$ S.E.M. of results from at least three experiments. Results were analyzed by one-way ANOVA with a Bonferroni post-test for comparing more than two groups and unpaired Student's *t*-test for comparing two groups, where $p < 0.05$ was considered to be significant.

Results

P2Y$_2$Rs mediate migration of primary rat cerebral cortical astrocytes

GFAP, an intermediate filament protein expressed in astrocytes of the central nervous system, is a specific marker of astrocytes {124}. Increased expression of GFAP in astrocytes has been used as an indicator of reactive astrogliosis {2}. Stimulation of primary rat astrocytes with UTP (Fig. 4a) or ATP {26} caused a time-dependent increase in GFAP expression (Fig. 4a), suggesting that extracellular nucleotides induce reactive astrogliosis. Incubation of primary astrocytes with ATP or UTP also caused an increase in the area of chemokinetic tracks (Fig. 4b), an indication of migration of reactive astrocytes. In addition, ATP or UTP added to the lower chamber of cell culture dishes containing Transwells with semi-permeable polycarbonate membrane inserts caused a dose-dependent increase in the transmembrane chemotactic migration of astrocytes that were plated in the upper chamber (Fig. 4c). UDP, which is not a P2Y$_2$R agonist {125}, had no significant effect on astrocyte migration in either assay (data not shown). Together, these data demonstrate that ATP and UTP, equipotent agonists of the P2Y$_2$R subtype {125}, activate the migration of primary astrocytes.

To examine the role of the P2Y$_2$R in extracellular nucleotide-induced astrocyte migration, we used P2Y$_2$R siRNA to inhibit the expression of the receptor. RT-PCR showed that P2Y$_2$R siRNA significantly reduced P2Y$_2$R but not P2Y$_4$R mRNA expression in astrocytes (Fig. 5a). Pre-treatment of astrocytes with P2Y$_2$R siRNA also resulted in the inhibition of UTP-induced astrocyte

migration (Fig. 5b). This result demonstrates that P2Y$_2$Rs mediate UTP-induced migration of primary rat astrocytes.

UTP-induced astrocyte migration is dependent on α_V integrins

The $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins play essential roles in mediating cell migration in various cell types {114, 119}. Increased expression of $\alpha_V\beta_3$ integrin was detected in reactive astrocytes in the peri-infarct region after focal cerebral ischemia {86, 113}. Incubation of primary rat astrocytes with 100 μM UTP induced a time-dependent increase in the expression of α_V, β_3 and β_5 proteins (Fig. 6a). To confirm that the expressed α_V, β_3 and β_5 integrin subunits were capable of forming $\alpha_V\beta_3$ and $\alpha_V\beta_5$ complexes, we determined that an 8 to 24 h treatment of primary rat astrocytes with 100 μM UTP significantly increased α_V subunit association with β_3 and β_5 subunits, since incubation of cell lysates with anti-α_V antibody could be used to immunoprecipitate β_3 and β_5 (Fig. 6b). These results indicate that UTP induces upregulation of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin complexes associated with reactive astrogliosis. To demonstrate the involvement of α_V integrins in UTP-induced astrocyte migration, we showed that addition of anti-α_V antibodies to the lower chamber of Transwells nearly abolished the UTP-induced transmembrane migration of astrocytes from the upper chamber (Fig. 7).

MEK/ERK and PI3-K/Akt activation are required for UTP-induced astrocyte migration

It has been reported that MEK/ERK and PI3-K/Akt are two important signaling pathways that regulate cell migration {119, 126, 127} and P2Y$_2$Rs are

known to mediate the activation of ERK and PI3-K in a variety of cell types {52, 74, 95, 128, 129}. Results in Fig. 8 show that U0126, an inhibitor of MEK, and LY294002, an inhibitor of PI3-K, prevented UTP-induced chemokinesis (Fig. 8a) and chemotaxis (Fig. 8b) of primary rat astrocytes, whereas SB203580, an inhibitor of p38, did not (data not shown). In primary astrocytes, U0126 and LY294002 also inhibited UTP-induced phosphorylation of ERK1/2 (substrates of MEK) and Akt (a substrate of PI3-K), but not p38 (data not shown). These results suggest that $P2Y_2R$-mediated MEK/ERK and PI3-K/Akt activation regulate UTP-induced astrocyte migration through overlapping pathways.

Results shown in Fig. 9 indicate that anti-α_V, but not anti-α_4, antibody inhibited ERK and Akt activation induced by UTP in primary rat astrocytes. Although anti-α_V antibody inhibited UTP-induced ERK phosphorylation by only 30% (Fig. 9a), UTP-induced Akt phosphorylation was almost completely inhibited (Fig. 9b). The partial inhibition of ERK phosphorylation by anti-α_V antibody is consistent with previous findings showing that $P2Y_2R$-mediated ERK activation can occur through several distinct pathways involving $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins {74}, phospholipase C {130}, and the Src-dependent transactivation of growth factor receptors {93, 95, 131}. Taken together, these results strongly suggest that α_V integrin and $P2Y_2R$ interactions mediate UTP-induced PI3-K/Akt activation, and partially mediate ERK activation, responses required for astrocyte migration.

Discussion

Reactive astrogliosis is critical for tissue remodeling and repair in the central nervous system {25} and occurs in various brain pathologies such as trauma, stroke and Alzheimer's disease {2}. Chronic astrogliosis is also thought to have deleterious effects such as the inhibition of neuronal regeneration in brain injury {2}. Previous studies have indicated that nucleotides can increase GFAP expression in astrocytes, indicative of reactive astrogliosis {26, 31}. This study was undertaken to determine the P2 receptor subtype and signaling pathways involved in nucleotide-induced reactive astrogliosis.

Extracellular nucleotides are released from aggregating platelets, degranulating macrophages, excitatory neurons, and injured or stressed cells in response to ischemia, hypoxia or mechanical stretch {11~13, 16, 132, 133}. Release of nucleotides has been proposed to occur by exocytosis of ATP/UTP-containing vesicles, facilitated diffusion by putative ABC transporters, cytoplasmic leakage, and by electrodiffusional movements through ATP/nucleotide channels {134}. The released nucleotides can activate cell surface P2 nucleotide receptors that mediate physiological functions such as neurotransmission and cell proliferation, migration and apoptosis {20, 133}. It has been found that extracellular ATP and UTP can induce smooth muscle and endothelial cell migration {44, 45, 119, 135} and ATP and ADP have been shown to induce chemotaxis in a microglial cell line {136}. However, it has not been established whether extracellular nucleotides can stimulate the migration of primary astrocytes. Furthermore, the subtype of P2 receptor that promotes

reactive astrogliosis is not known and the mechanisms involved have not been fully elucidated. The results of the present study indicate that extracellular UTP activates a G protein-coupled $P2Y_2R$ subtype in primary rat astrocytes and induces the phenotype of reactive astrogliosis that is characterized by increased expression of GFAP and an enhanced rate of cell migration. In addition, we have identified novel and complex signaling pathways that regulate nucleotide-induced astrocyte migration through the interaction of the $P2Y_2R$ with $\alpha_v\beta_3/\beta_5$ integrins and the stimulation of the downstream signaling molecules PI3-K and MEK.

The $P2Y_2R$ subtype of G protein-coupled P2Y nucleotide receptor has been proposed to play an essential role in immune responses and injury {44, 55, 65, 137}. $P2Y_2R$ upregulation occurs in response to vascular injury and leads to neointimal hyperplasia and inflammation in arteries through processes involving smooth muscle cell migration/proliferation and the endothelial-dependent adherence of monocytes, respectively, responses associated with atherogenesis and atherosclerosis {44, 53, 131}. In the present study, we investigated the ability of $P2Y_2Rs$ to mediate reactive astrogliosis. Results indicate that the equipotent and equi-efficacious $P2Y_2R$ agonists ATP and UTP {5, 138} stimulated astrocyte chemokinesis and chemotaxis (Fig. 4). Among the P2 nucleotide receptors, only the $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor subtypes can be activated by uridine nucleotides {139, 140} and primary astrocytes express mRNA for these three P2YR subtypes {35, data not shown}. Among these receptors, only the $P2Y_2R$ can be fully activated by ATP as well as UTP, whereas $P2Y_6$ receptors are more sensitive to UDP than UTP {140}. We have determined

that UDP does not induce astrocyte migration (data not shown), eliminating a role for the $P2Y_6R$ in responses to UTP that could have occurred upon UTP degradation to UDP by ecto-NTPDases {141}. $P2Y_4Rs$ are preferentially activated by UTP, and are relatively insensitive to ATP {142, 143}. Therefore, the ability of similar concentrations of UTP and ATP to induce astrocyte migration is most characteristic of the pharmacological profile of $P2Y_2Rs$. Nonetheless, the finding that $P2Y_2R$ siRNA, which suppresses $P2Y_2R$ but not $P2Y_4R$ mRNA expression, can prevent UTP-induced astrocyte migration (Fig. 5) unambiguously demonstrates the role of $P2Y_2Rs$ in this process. Unfortunately, the unavailability of specific anti-$P2Y_2R$ antibodies precludes an attempt to evaluate whether $P2Y_2R$ siRNA inhibits $P2Y_2R$ protein expression in primary astrocytes.

The $\alpha_V\beta_{3/}\beta_5$ integrins are receptors for RGD-containing extracellular matrix proteins {144} that have important roles in angiogenesis and inflammation. The $\alpha_V\beta_3$ integrin is upregulated in reactive astrocytes, and plays a key role in tissue remodeling and limits the extent of brain injury {86, 113}. The $\alpha_V\beta_5$ integrin and its extracellular ligands also have been linked to cell migration in astrocytes, breast carcinoma cells, endocrine progenitor cells, and smooth muscle cells {114, 116, 119, 145}. Inhibition of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin activities by peptidic or nonpeptidic antagonists was found to decrease UTP-induced smooth muscle cell migration associated with OPN expression {119}, which is mediated by $P2Y_2Rs$ {45}. Recently, the $P2Y_2R$ was shown to interact directly with $\alpha_V\beta_{3/}\beta_5$ integrins via an RGD motif in its first extracellular loop {74}. Furthermore, our studies indicate that interactions between $P2Y_2R$ and α_V integrins are critical for the

P2Y$_2$R to activate G$_{12}$ and G$_o$ and stimulate G$_{12}$- and G$_o$-mediated signaling events that lead to cell migration in human astrocytoma 1321N1 cells expressing a recombinant P2Y$_2$R {74; unpublished data}. Results in the current study indicate that UTP induced expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin complexes (Fig. 6) and anti-α_V antibody significantly inhibited P2Y$_2$R-mediated migration of primary astrocytes (Fig. 7). Thus, these results indicate for the first time that UTP induces the upregulation of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ in astrocytes and demonstrate a role for these integrins in P2Y$_2$R-mediated astrocyte migration.

PI3-K/Akt and ERK are critical signaling molecules that regulate cell migration {126, 127}, and integrin-mediated cell migration {146}. It has been reported that PI3-K is involved in the migration of reactive astrocytes {147}, and a PI3-K inhibitor significantly prevented P2Y$_2$R-mediated astrocyte migration (Fig. 8). We also found that a MEK inhibitor prevented UTP-stimulated astrocyte chemokinesis and chemotaxis (Fig. 8). UTP-induced phosphorylation of Akt was completely inhibited by anti-α_V antibody (Fig. 9), whereas the antibody partially inhibited UTP-induced ERK phosphorylation. These results are consistent with a role for P2Y$_2$Rs and $\alpha_V\beta_{3/}\beta_5$ integrin interactions in both Akt and ERK activation, although P2Y$_2$R activation of ERK can also occur through G protein-dependent activation of phospholipase C and Src-dependent transactivation of growth factor receptors {74, 93, 95}. Nonetheless, it appears that astrocyte migration in response to UTP-induced activation of PI3-K/Akt and MEK/ERK is dependent upon P2Y$_2$R-mediated interactions with $\alpha_V\beta_{3/}\beta_5$ integrins.

Previous studies have shown that extracellular nucleotides cause rapid release from astrocytes of the wound-related factor TGF-β {3}. Our recent studies also indicate that P2Y$_2$R activation by UTP rapidly induces the transactivation of vascular endothelial growth factor receptors that mediate sustained increases in the expression of pro-inflammatory vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells {53, 131}. Similarly, P2Y$_2$Rs and extracellular nucleotides cause increases in GFAP and $\alpha_V\beta_{3/}\beta_5$ integrin expression in primary astrocytes. These results suggest that P2Y$_2$Rs may represent an early mediator of reactive astrogliosis, and may prove to be novel targets for therapies that minimize the deleterious effects of chronic astrogliosis associated with brain injury or disease.

The effects of reactive astrogliosis on neurological functions have been described in both positive and negative terms. In the initial stages, reactive astrogliosis can be beneficial in limiting brain damage in Alzheimer's disease by promoting the clearance of β-amyloid {148}. In the chronic stages, reactive astrogliosis leads to the formation of glial scars that prevent neuronal cell regeneration or have neurotoxic effects that promote the formation of astrocyte-derived amyloid plaques {149}. Although the long-term effects of reactive astrogliosis require further elucidation, our data provide insights into the mechanisms underlying the initiation of reactive astrogliosis due to P2Y$_2$R activation. We also have found that activation of P2Y$_2$Rs in astrocytic cells promotes cell survival mechanisms and conditioned medium from these UTP-treated cells stimulates outgrowth of neurites in PC-12 cells {150}. Thus, the

dual role of $P2Y_2Rs$ in promoting neuroprotection and neurodegeneration warrants further investigation.

Figure 1. Expression of P2 receptors in rat cortical astrocytes. Total RNA was isolated from primary rat cortical astrocytes with the RNeasy Mini Kit (Qiagen, Chatsworth, CA). DNase I (Qiagen, Chatsworth, CA) was added to remove genomic DNA. The first strand of cDNA was synthesized from total RNA with by the First Strand cDNA Synthesis Kit for RT-PCR (Roche, Indianapolis IN). Ten percent of the cDNA product was used as the template for PCR amplification with the Expand High Fidelity PCR System (Roche, Indianapolis IN). Oligonucleotide primers were designed to selectively amplify cDNA for specific subtypes of P2Y and P2X receptors and G3PDH (Glyceraldehyde 3-phosphate dehydrogenase), as previously described {44, 122, 128}. Thirty-five amplification cycles were used with annealing temperatures of 60°C. PCR products were resolved by 1.5% agarose gel electrophoresis with 10 μg/ml ethidium bromide in the gel solution. Pictures of the gels were photographed using an electrophoresis system (Fisher Scientific, Pittsburgh, PA) under ultraviolet illumination. A 1-Kb DNA extension ladder (Invitrogen, CA) was used to indicate the size of an amplified PCR product.
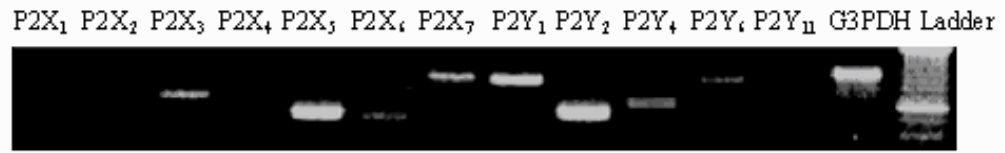
Figure 1

P2X₁ P2X₂ P2X₃ P2X₄ P2X₅ P2X₆ P2X₇ P2Y₁ P2Y₂ P2Y₄ P2Y₆ P2Y₁₁ G3PDH Ladder

Figure 2. Two-dimensional structure of the human P2Y$_2$R. Highlighted features include the consensus RGD integrin-binding domain (in red), positively-charged amino acid residues involved in agonist binding (in orange), two consensus PXXP SH3-binding domains (in yellow), and an incorporated hemagglutinin (HA) tag (in green) used for immunofluorescence and immunoprecipitation of various P2Y$_2$R constructs. The dotted line indicates the location of a truncation site for creation of a sequestration-resistant P2Y$_2$R.
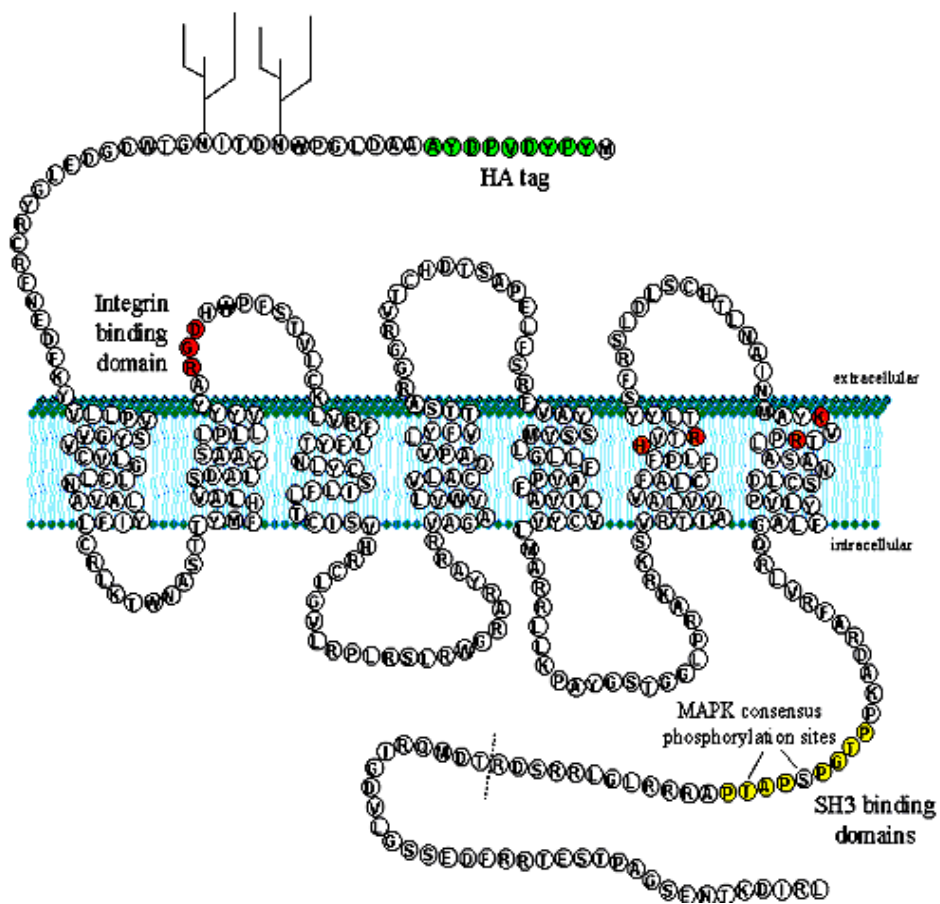
Figure 2

Figure 3. A schematic outline of the signaling pathways mediated by the activated P2Y$_2$R. The activation of the P2Y$_2$R by ATP or UTP transactivates $\alpha_v\beta_3/\beta_5$ integrin and growth factor receptor signaling pathways involving ERK, JNK, p38, PI3-K, and the small GTPase Rac that regulate cell proliferation and/or migration. Also shown is the classical P2Y$_2$R coupling to the G$_q$-protein that mediates activation of PLC and the formation of IP$_3$ and DAG, second messengers for stimulation of intracellular calcium mobilization and PKC, respectively.
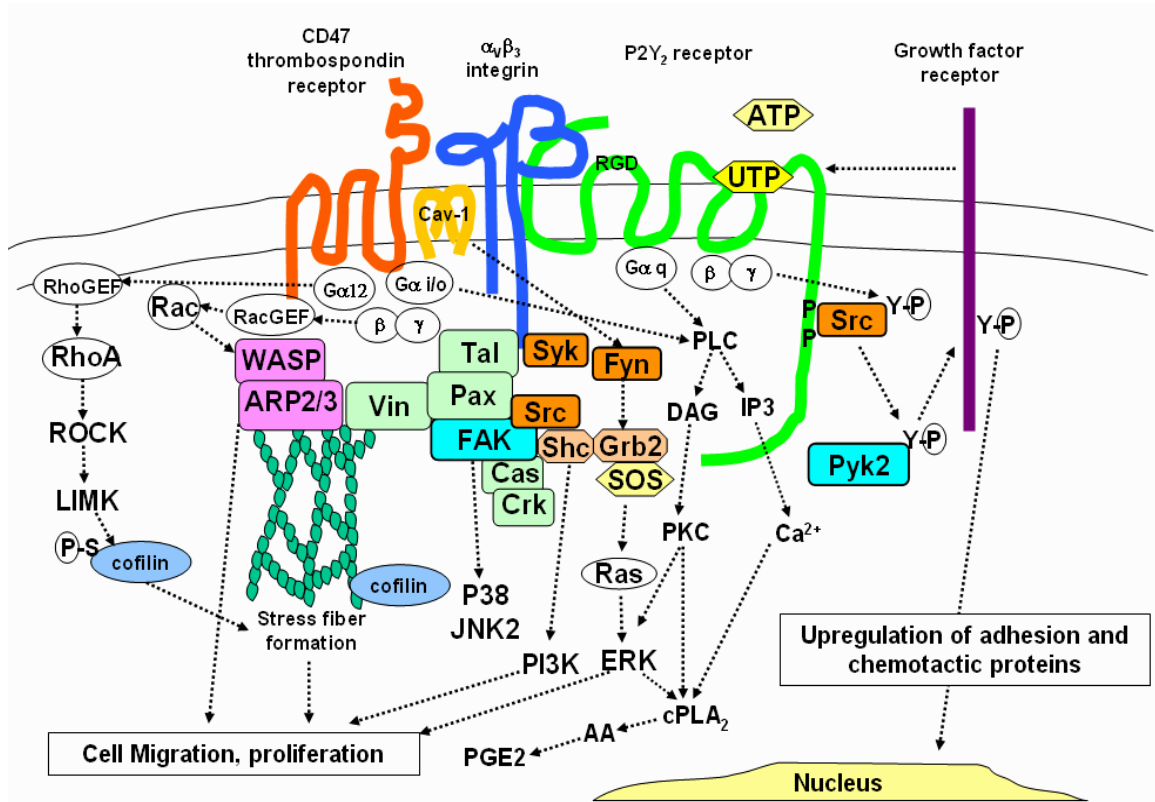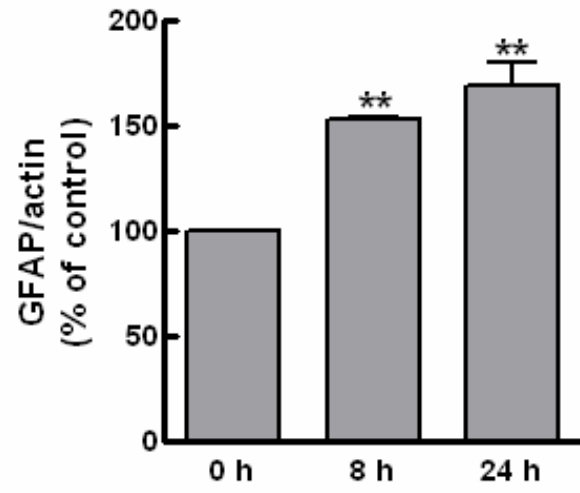
Figure 3

Figure 4: Extracellular UTP or ATP induces astrocyte migration. (a). UTP

stimulates GFAP expression. Quiescent astrocytes (maintained in serum-free

medium for 48 h at 37°C) were stimulated with 50 µM UTP for the indicated times

at 37°C. Then, cell lysates were prepared and subjected to 10% SDS-PAGE, and

immunoblot analysis was performed as described in "Materials and Methods".

GFAP was detected with anti-GFAP antibody and membranes were stripped and

re-probed with anti-actin antibody to verify the equivalence of protein loading

between lanes. Bands were quantitated and expressed as the relative intensities

of GFAP/actin in UTP-treated cells, as compared to 0 h controls, where **$p <$

0.01 indicates a significant difference. **(b).** UTP or ATP stimulates astrocyte

chemokinesis. Primary rat astrocytes (black dots) were seeded on cover slips

coated with gold particles and incubated in serum-free medium with or without

100 µM ATP or UTP for 18 h at 37°C. Then, cells were washed with PBS, fixed,

observed under a microscope and the images were captured with a digital

camera and analyzed by Northern Eclipse software. The area of the white

phagokinetic tracks indicates the extent of cell migration. Bar = 100 µm. **(c).**

UTP or ATP stimulates astrocyte chemotaxis. A modified, open-well Transwell

chamber (Falcon) with a polycarbonate membrane insert was used to separate

two chambers in 24-well plates. Primary rat astrocytes (5×10^4 cells per chamber)

in serum-free medium were seeded onto the membrane in the upper chamber

whereas the lower chamber contained 0, 1, 10 or 100 µM UTP or ATP. Cells

were incubated in a cell culture incubator for 18 h at 37°C. Then, membranes

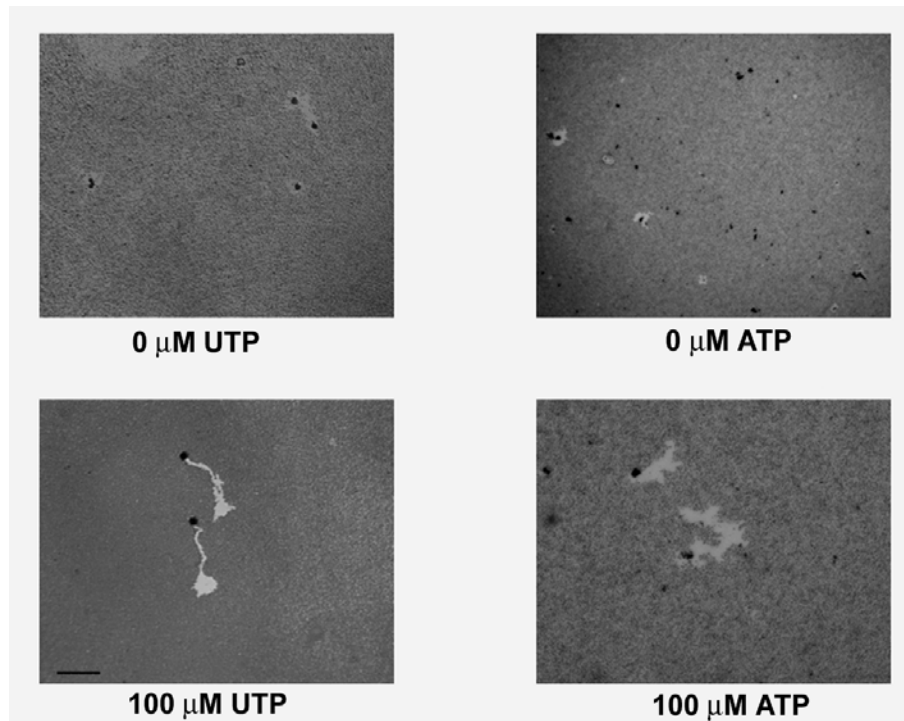were washed with PBS and cells that had migrated from the upper to the lower

chamber were quantitated as described in "Materials and Methods". Data

represent the means ± S.E.M. of results from at least three experiments where *p

< 0.05 and **p < 0.01 indicate significant differences from untreated controls.
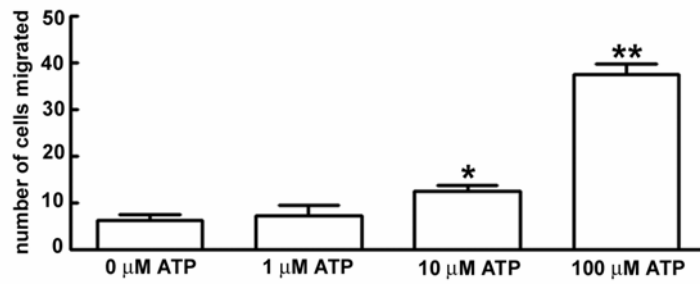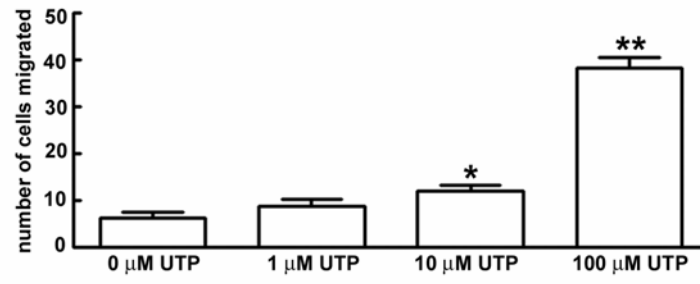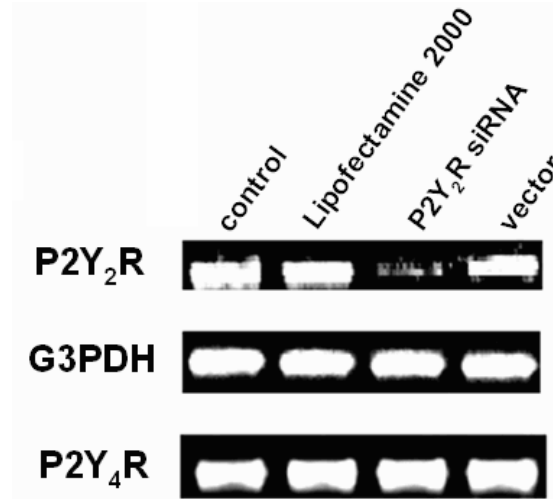
Figure 4

(a)

(b)

0 μM UTP

0 μM ATP

100 μM UTP

100 μM ATP

(c)

Figure 5: P2Y$_2$R siRNA inhibits UTP-induced astrocyte migration. (a). P2Y$_2$R siRNA decreases P2Y$_2$R mRNA expression. Primary rat astrocytes incubated for 1 day in DMEM without antibiotics were treated with Lipofectamine 2000 with or without 3 μg of P2Y$_2$R siRNA or empty vector, as described in "Materials and Methods". After 24 h, total RNA was isolated and expression of P2Y$_2$R, P2Y$_4$R (negative control) or G3PDH mRNA was determined by RT-PCR, as described in "Materials and Methods". Lane 1: untreated control; Lane 2: Lipofectamine 2000 control; Lane 3: P2Y$_2$R siRNA; Lane 4: empty vector control. **(b)**. P2Y$_2$R siRNA inhibits UTP-induced astrocyte migration. Cells treated with P2Y$_2$R siRNA or empty vector in Lipofectamine 2000 for 24 h as in (a) were removed with trypsin, washed 3 times with serum-free medium, and used for chemotactic assays with or without 100 μM UTP, as described for Figure 1c. After 18 h, cell migration was determined, as described in "Materials and Methods". Data represent the means $\pm$ S.E.M. of results from three experiments, where **$p < 0.01$ indicates a significant difference from UTP-treated controls.
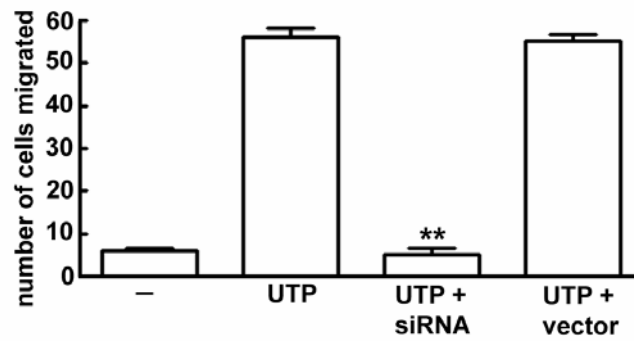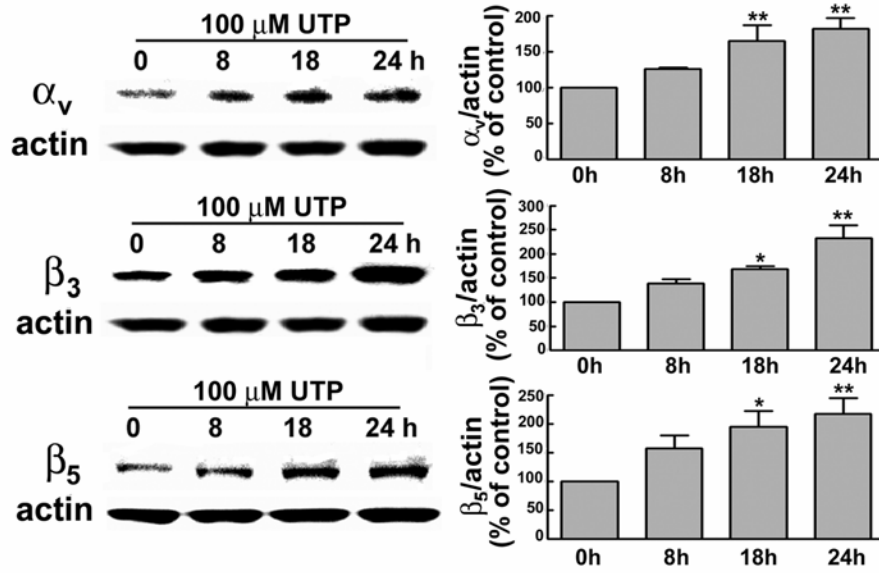
Figure 5

(a)

(b)

Figure 6: UTP increases the expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin complexes. **(a)**. UTP increases α_V, β_3 and β_5 expression in time-dependent manner. Primary rat astrocytes in serum-free medium were stimulated with 100 µM UTP for the indicated times at 37°C. Cell lysates were prepared and subjected to SDS-PAGE and immunoblot analysis, as described in "Materials and Methods". Detection of α_V, β_3 or β_5 in immunoblots was performed with anti-α_V, anti-β_3 or anti-β_5 antibodies, respectively. Membranes were stripped and re-probed with anti-actin antibody to verify the equivalence of protein loading between lanes. Bands were quantitated and expressed as the relative intensities of integrin/actin in UTP-treated cells, as compared to 0 h controls, where $*p < 0.05$ and $**p < 0.01$ indicate significant differences. **(b)**. UTP stimulates the expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin complexes in primary astrocytes. Cell lysates were prepared from astrocytes incubated in serum-free medium with 100 µM UTP for the indicated times at 37°C. Lysates were immunoprecipitated (IP) with anti-α_V antibody and subjected to immunoblot (IB) analysis for α_V, β_3 or β_5, as described in "Materials and Methods". Anti-actin antibody was used to detect actin expression to verify the equivalence of protein loading between lanes.
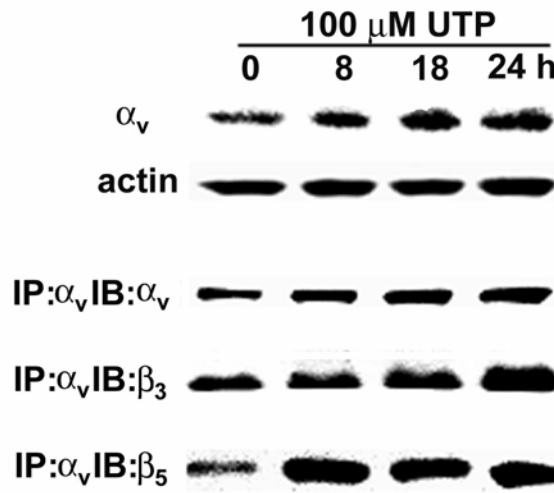
Figure 6

(a)

(b)

Figure 7: Anti-α_V antibody inhibits UTP-stimulated astrocyte chemotaxis.
Anti-α_V antibody or an irrelevant IgG was added to the lower chamber of Transwells with membrane inserts and primary rat astrocytes were added to the upper chamber and incubated for 2 h at 37°C. When indicated, 100 μM UTP was added to the lower chamber, cells were incubated for 18 h at 37°C and the number of astrocytes that migrated into the lower chamber was determined, as described in "Materials and Methods". Data represent means $\pm$ S.E.M. of results from at least three experiments where **p < 0.01 indicates significant differences from UTP-treated controls.
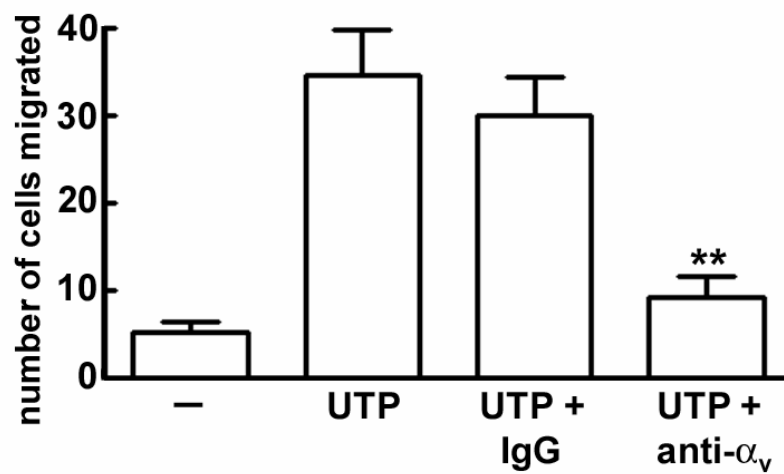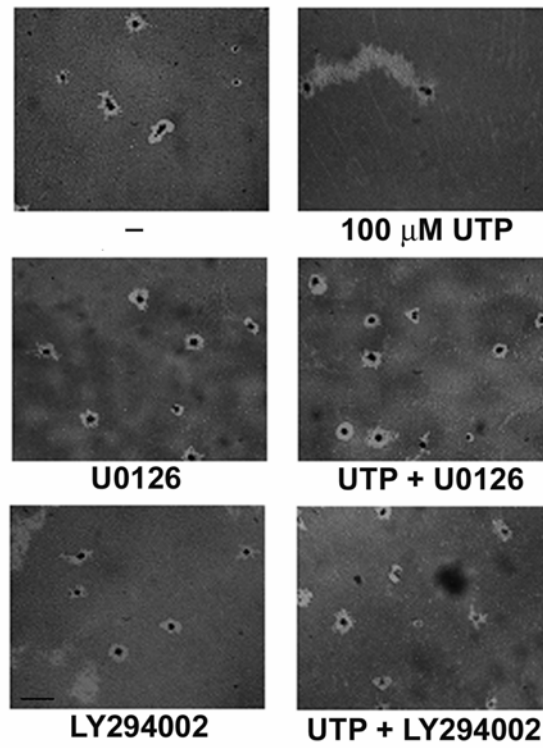
Figure 7

Figure 8: MEK and PI3-K activities are required for UTP-stimulated astrocyte migration. **(a)**. Inhibitors of MEK and PI3-K prevent UTP-stimulated astrocyte chemokinesis. Primary rat astrocytes were incubated with 10 μM U0126 or LY294002 in serum-free medium for 2 h at 37°C and 100 μM UTP was added, when indicated. After 18 h, cells were washed, fixed, and astrocyte chemokinesis was determined, as described in "Materials and Methods". Bar in lower left panel = 50 μm. **(b)**. Inhibitors of MEK and PI3-K prevent UTP-stimulated astrocyte chemotaxis. Primary rat astrocytes were placed in the upper chamber of Transwells and 10 μM U0126 or LY294002 was added to both chambers for 2 h at 37°C. When indicated, 100 μM UTP was added to the lower chamber, cells were incubated for 18 h at 37°C and the number of astrocytes that migrated to the lower chamber was determined, as described in "Materials and Methods". Data represent the means $\pm$ S.E.M. of results from at least three experiments where **$p < 0.01$ indicates significant differences from UTP-treated controls.

Figure 8

(a)

(b)

Figure 9: Anti-α_V antibody inhibits UTP-induced ERK1/2 and Akt phosphorylation. Primary rat astrocytes were incubated with or without 10 μg/ml anti-α_V or anti-α_4 (negative control) antibody in serum-free medium for 2 h at 37°C. When indicated, 100 μM UTP was added for 5 min and the phosphorylation of **(a)** ERK1/2 or **(b)** Akt was determined by Western blot analysis using anti-phospho-ERK1/2 or anti-phospho-Akt (Ser473) antibody, as described in "Materials and Methods". Membranes were stripped and re-probed with anti-actin antibody to verify the equivalence of protein loading between lanes. Bands were quantitated and expressed as the relative intensities of pERK/actin or pAkt/actin in UTP- or UTP plus antibody-treated cells, as compared to untreated controls. Data represent the means $\pm$ S.E.M. of results from four experiments where *$p < 0.05$ and **$p < 0.01$ indicate significant differences from UTP-treated controls.
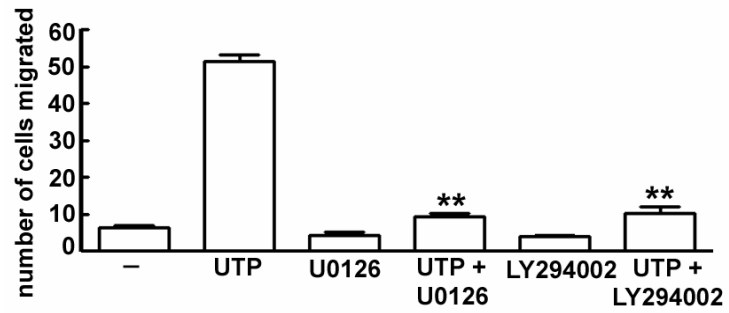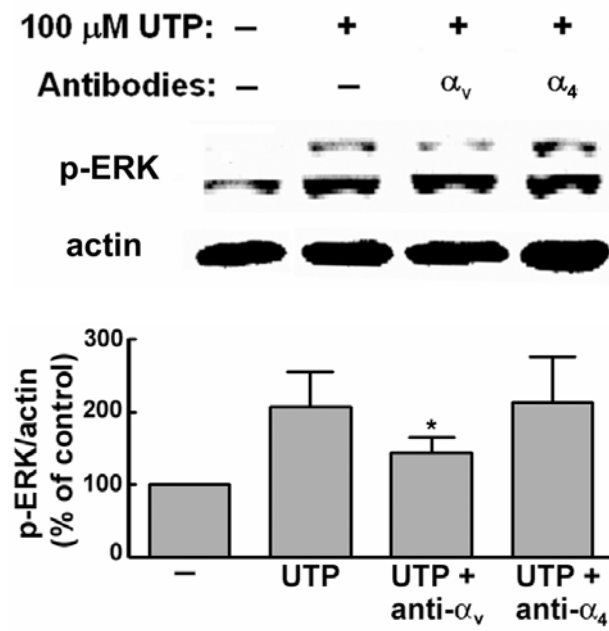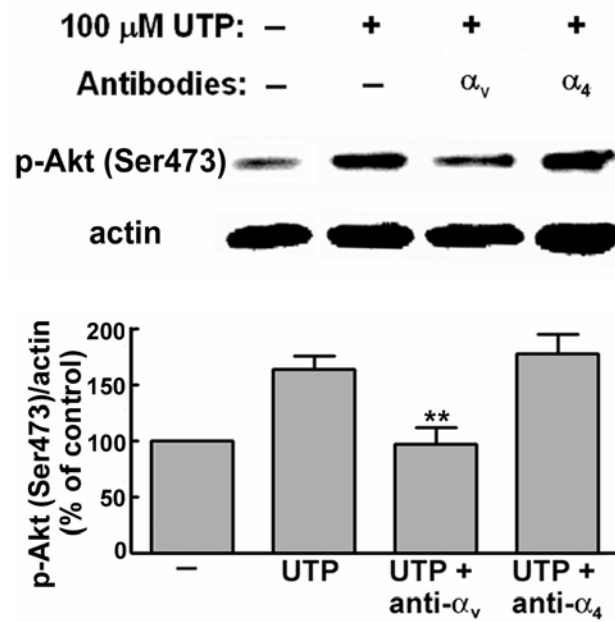
Figure 9

(a)

(b)

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