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Molecular Determinants of $P2Y_2$ Nucleotide Receptor Function

Implications for Proliferative and Inflammatory Pathways in Astrocytes

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Abstract

In the mammalian nervous system, P2 nucleotide receptors mediate neurotransmission, release of proinflammatory cytokines, and reactive astrogliosis. Extracellular nucleotides activate multiple P2 receptors in neurons and glial cells, including G protein-coupled P2Y receptors and P2X receptors, which are ligand-gated ion channels. In glial cells, the $P2Y_2$ receptor subtype, distinguished by its ability to be equipotently activated by ATP and UTP, is coupled to pro-inflammatory signaling pathways. *In situ* hybridization studies with rodent brain slices indicate that $P2Y_2$ receptors are expressed primarily in the hippocampus and cerebellum. Astrocytes express several P2 receptor subtypes, including $P2Y_2$ receptors whose activation stimulates cell proliferation and migration. $P2Y_2$ receptors, via an RGD (Arg-Gly-Asp) motif in their first extracellular loop, bind to $\alpha_v\beta_3/\beta_5$ integrins, whereupon $P2Y_2$ receptor activation stimulates integrin signaling pathways that regulate cytoskeletal reorganization and cell motility. The C-terminus of the $P2Y_2$ receptor contains two Src-homology-3 (SH3)-binding domains that upon receptor activation, promote association with Src and transactivation of growth factor receptors. Together, our results indicate that $P2Y_2$ receptors complex with both integrins and growth factor receptors to activate multiple signaling pathways. Thus, $P2Y_2$ receptors present novel targets to control reactive astrogliosis in neurodegenerative diseases.

Index Entries: Astrocytes; astrogliosis; growth factor receptors; inflammation; $P2Y_2$ receptors; nucleotides; proliferation; RGD motif; SH3-binding domain; integrins.

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Introduction

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, activation of G proteins, mitogen-activated protein kinases (MAPK), integrins 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.

P2 Receptor Classification

In the early 1970s, it was reported that ATP was released into the extracellular space by stimulation of nonadrenergic, noncholinergic 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 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

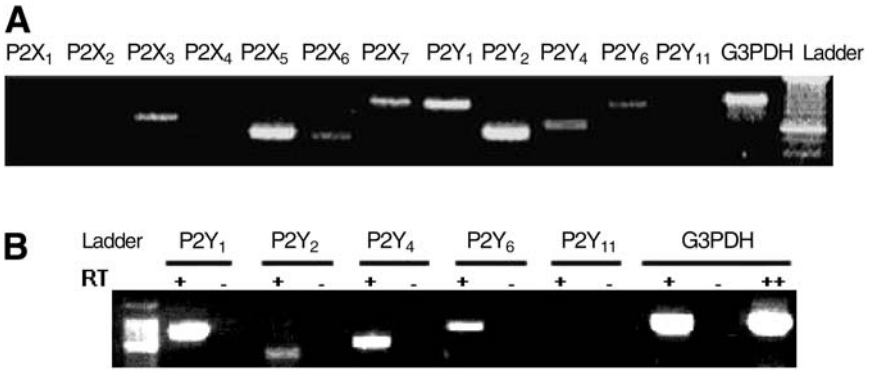

Fig. 1. Expression of P2 receptors in rat cortical astrocytes and neurons. Total RNA was isolated from primary rat cortical **(A)** astrocytes or **(B)** neurons with the RNeasy Mini Kit (Qiagen, Chatsworth, CA). DNase I (Qiagen) was added to remove genomic DNA. The first strand of cDNA was synthesized from total RNA with or without reverse transcriptase (± RT) by the First Strand cDNA Synthesis Kit for RT-PCR (polymerase chain reaction) (Roche, Indianapolis IN). Ten percent of the cDNA product (± RT) 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 *(44,109,110).* 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 taken 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. The + and – symbols indicate reaction products in the presence or absence of reverse transcriptase (only the +RT samples are shown in (A) and ++ indicates amplification of a G3PDH-positive control sample (B).

(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 cyclo-oxygenase-2, which are cellular responses associated with reactive astrogliosis *(26–32).* Astrocytes express numerous subtypes of P2 nucleotide receptor. 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 Fig. 1A, primary astrocytes isolated from rat brain 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 result 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-triphosphate (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 involved in *d*-amphetamine-induced brain sensitization and calcium signaling affect neural progenitor cell proliferation and migration during early CNS development *(4,39).* Together with $P2Y_2$ receptors, $P2Y_1$ receptor activation caused arachidonic acid (AA) release from cultured astrocytes *(40).* $P2Y_2$ and $P2X_7$ nucleotide receptors also mediate neuroinflammatory 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 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 understanding 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 nervous system and the CNS, $P2Y_2$Rs have been identified in immortalized astrocytes, NG108-15 neuroblastoma × 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 upregulated 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 correlated with the increased expression of osteopontin 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 reverse transcriptase–polymerase chain reaction (RT-PCR) to determine that $P2Y_2$R messenger RNA (mRNA) was expressed at relatively low levels in normal rodent (i.e., rat, mouse, and gerbil) brain slices, but it was most abundant in the hippocampus and cerebellum (unpublished data). In the 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. 1A), primary neurons (Fig. 1B), and microglial cells *(3).* Thus, the $P2Y_2$R is an intriguing new target for anti-inflammatory and antiproliferative therapies in neurodegenerative diseases and atherosclerosis.

$P2Y_2$Rs 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 upregulated 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, cyclo-

oxygenase, 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, P2YR-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 of the cytokine transforming growth factor-β (TGF-β) in astrocytes *(3).* In turn, TGF-β regulates cell proliferation and differentiation that is dependent on activation of p38 and c-Jun NH_2-terminal kinase (JNK) in cultured astrocytes *(3).* Our data have shown that activation of the $P2Y_2$R and the $P2X_7$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 IFN-γ increased PGE_2 production about 13-fold compared to untreated astrocytes and 2-fold over cytokines alone, indicating that cytokines and nucleotides coregulate 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 (*see* Fig. 2) *(74).* Recent studies in our laboratory have demonstrated that the wild-type $P2Y_2$R colocalizes with α_v integrins when the recombinant $P2Y_2$R is expressed in human 1321N1 astrocytoma cells, a cell line 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 colocalization with α_v integrins than the wild-type receptor. The EC_{50} for nucleotide-induced calcium mobilization was approx 1000-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 signaling paths access to the G_o- 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, lung epithelial tumor

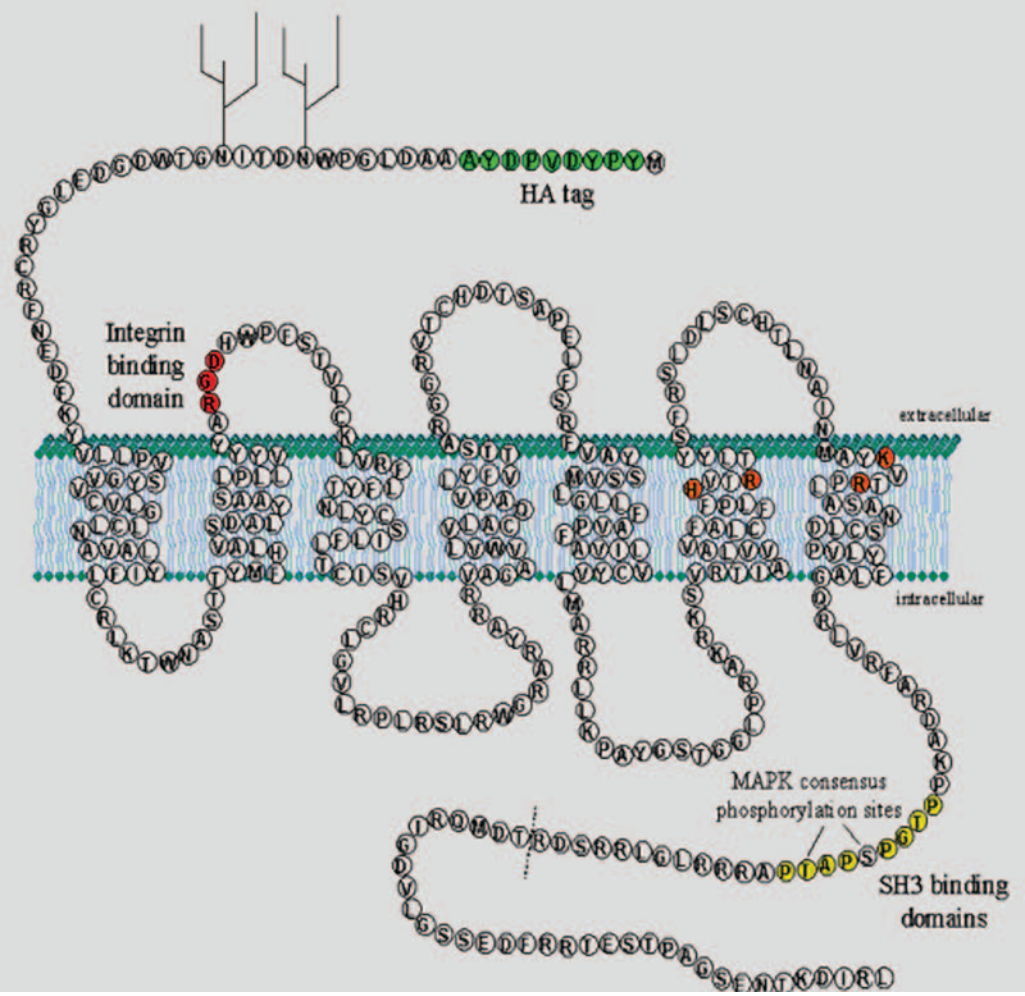

Fig. 2. Two-dimensional structure of the human $P2Y_2R$. Highlighted features include the consensus RGD integrin-binding domain, positively charged amino acid residues involved in agonist binding, two consensus PXXP SH3-binding domains, and an incorporated hemagglutinin (HA) tag used for immunofluorescence and immunoprecipitation of various $P2Y_2R$ constructs. The dotted line indicates the location of a truncation site for creation of a sequestration-resistant $P2Y_2R$.

cells, and smooth muscle cells *(79–81).* Therefore, we examined whether interactions between $P2Y_2Rs$ and $\alpha_v\beta_3/\beta_5$ integrins enabled the $P2Y_2R$ to modulate integrin-mediated cell migration by comparing responses of wild-type and RGE-mutant $P2Y_2Rs$. Results indicated that the presence of the RGD domain in the $P2Y_2R$ is necessary for the receptor to mediate nucleotide-induced chemotaxis and the formation of actin stress fibers (unpublished data). Antibodies to $\alpha_v\beta_3/\beta_5$ integrins also inhibited $P2Y_2R$-mediated stress fiber formation and cell migration (unpublished data). These data demonstrate that integrin/$P2Y_2R$ interactions are required for nucleotide modulation of integrin-associated responses such as cytoskeletal rearrangements that control cell migration. Based on findings in other laboratories on the

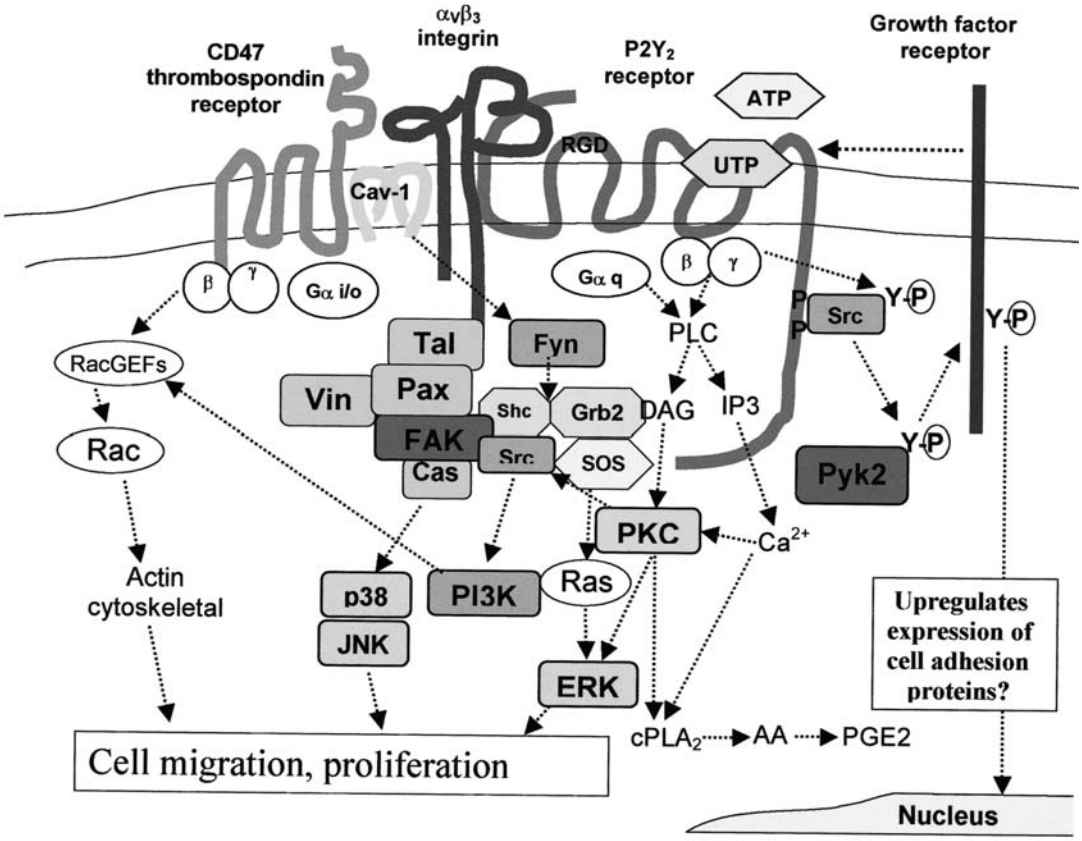

Fig. 3. A schematic outline of the signaling pathways mediated by the activated P2Y$_2$R. The activation of the P2Y$_2$R by ATP or UTP transactivates $\alpha_v\beta_3/\beta_5$ integrin and growth factor receptor signaling pathways involving ERK, JNK, p38, PI3-K, and the small GTPase Rac that regulate 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 PKC, second messengers for stimulation of intracellular calcium mobilization and PKC, respectively.

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$_2$R, the RGE-mutant P2Y$_2$R requires approx 1000-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$_2$R-mediated cell migration (unpublished data).

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).* Osteopontin (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 d of focal ischemia, astrocytes expressing $\alpha_v\beta_3$ are localized in an osteopontin-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-$\alpha_v\beta_3$ or anti-osteopontin anti-bodies (unpublished results). Other unpublished results indicate that UTP causes upregulation of $\alpha_v\beta_3/\beta_5$ and OPN in rat astrocytes in a dose- and 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 colocalization 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$_2$ Receptors Regulate 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, *(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 GFRs 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 comodulate 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$_2$R to activate calcium-dependent ERK1/2 activation. Furthermore, anti-α_v antibody and RGDs tetrapeptide partially inhibited UTP-induced ERK1/2 phosphorylation *(74)*. We also have found that cell proliferation induced by P2Y$_2$R 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$_2$R 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.

References

1. Gahtan E. and Overmier J.B. (1999) Inflammatory pathogenesis in Alzheimer's disease: biological mechanisms and cognitive sequeli. *Neurosci. Biobehav. Rev.* **23(5),** 615–633.
2. Rutka J.T., Murakami M., Dirks P.B., et al. (1997) Role of glial filaments in cells and tumors of glial origin: a review. *J. Neurosurg.* **87(3),** 420–430.
3. Gendron F.P., Newbold N.L., Vivas-Mejia P.E., et al. (2003) Signal transduction pathways for $P2Y_2$ and $P2X_7$ nucleotide receptors that mediate neuroinflammatory responses in astrocytes and microglial cells. *Biomed. Res.* **14,** 47–61.
4. Franke H., Kittner H., Grosche J., and Illes P. (2003) Enhanced $P2Y_1$ receptor expression in the brain after sensitisation with d-amphetamine. *Psychopharmacology (Berl.)* **167,** 187–194.
5. Weisman G.A., Garrad R.C., Erb L.J., Santos-Berrios C., and Gonzalez F.A. (1999) P2Y receptors in the nervous system: molecular studies of a $P2Y_2$ receptor subtype from NG108-15 neuroblastoma x glioma hybrid cells. *Prog. Brain Res.* **120,** 33–43.
6. Burnstock G. (1972) Purinergic nerves. *Pharmacol. Rev.* **24(3),** 509–581.
7. Burnstock G., Campbell G., Satchell D., and Smythe A. (1970) Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br. J. Pharmacol.* **40(4),** 668–688.
8. Burnstock G. and Wood J.N. (1996) Purinergic receptors: their role in nociception and primary afferent neurotransmission. *Curr. Opin. Neurobiol.* **6(4),** 526–532.
9. Neary J.T., Rathbone M.P., Cattabeni F., Abbracchio M.P., and Burnstock G. (1996) Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. *Trends Neurosci.* **19(1),** 13–18.
10. Vitolo O.V., Ciotti M.T., Galli C., Borsello T., and Calissano P. (1998) Adenosine and ADP prevent apoptosis in cultured rat cerebellar granule cells. *Brain Res.* **809(2),** 297–301.
11. Ostrom R.S., Gregorian C., Drenan R.M., Gabot K., Rana B.K., and Insel P.A. (2001) Key role for constitutive cyclooxygenase-2 of MDCK cells in basal signaling and response to released ATP. *Am. J. Physiol. Cell Physiol.* **281(2),** C524–C531.
12. Ahmed S.M., Rzigalinski B.A., Willoughby K.A., Sitterding H.A., and Ellis E.F. (2000) Stretch-induced injury alters mitochondrial membrane potential and cellular ATP in cultured astrocytes and neurons. *J. Neurochem.* **74(5),** 1951–1960.
13. Ciccarelli R., Di Iorio P., Giuliani P., et al. (1999) Rat cultured astrocytes release guanine-based purines in basal conditions and after hypoxia/hypoglycemia. *Glia* **25,** 93–98.
14. Bergfeld G.R. and Forrester T. (1992) Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. *Cardiovasc. Res.* **26(1),** 40–47.
15. Bodin P. and Burnstock G. (2001) Purinergic signalling: ATP release. *Neurochem. Res.* **26,** 959–969.
16. Pedersen S.F., Nilius B., Lambert I.H., and Hoffmann E.K. (1999) Mechanical stress induces release of ATP from Ehrlich ascites tumor cells. *Biochim. Biophys. Acta* **1416,** 271–284.
17. Sak K. and Webb T.E. (2002) A retrospective of recombinant P2Y receptor subtypes and their pharmacology. *Arch. Biochem. Biophys.* **397(1),** 131–136.
18. Inbe H. Watanabe S., Miyawaki M., Tanabe E., and Encinas J.A. (2004) Identification and characterization of a cell-surface receptor, P2Y15, for AMP and adenosine. *J. Biol. Chem.* **279(19),** 19,790–19,799.
19. Burnstock G. (2000) P2X receptors in sensory neurones. *Br. J. Anaesth.* **84(4),** 476–488.
20. Ciccarelli R., Ballerini P., Sabatino G., et al. (2001) Involvement of astrocytes in purine-mediated reparative processes in the brain. *Int. J. Dev. Neurosci.* **19,** 395–414.
21. O'Callaghan J.P. and Jensen K.F. (1992) Enhanced expression of glial fibrillary acidic protein and the cupric silver degeneration reaction can be used as sensitive and early indicators of neurotoxicity. *Neurotoxicology* **13(1),** 113–122.
22. Menet V., Prieto M., Privat A., and Gimenez y Ribotta M. (2003) Axonal plasticity and func-

tional recovery after spinal cord injury in mice deficient in both glial fibrillary acidic protein and vimentin genes. *Proc. Natl. Acad. Sci. USA* **100(15),** 8999–9004.
23. Eddleston M. and Mucke L. (1993) Molecular profile of reactive astrocytes—implications for their role in neurologic disease. *Neuroscience* **54(1),** 15–36.
24. Norton W.T., Aquino D.A., Hozumi I., Chiu F.C., and Brosnan C.F. (1992) Quantitative aspects of reactive gliosis: a review. *Neurochem. Res.* **17(9),** 877–885.
25. Mcgraw J., Hiebert G.W., and Steeves J.D. (2001) Modulating astrogliosis after neurotrauma. *J. Neurosci. Res.* **63(2),** 109–115.
26. Neary J.T., Baker L., Jorgensen S.L., and Norenberg M.D. (1994) Extracellular ATP induces stellation and increases glial fibrillary acidic protein content and DNA synthesis in primary astrocyte cultures. *Acta Neuropathol. (Berl.)* **87(1),** 8–13.
27. Brambilla R. and Abbracchio M.P. (2001) Modulation of cyclooxygenase-2 and brain reactive astrogliosis by purinergic P2 receptors. *Ann. NY Acad. Sci.* **939,** 54–62.
28. Brambilla R., Burnstock G., Bonazzi A., Ceruti S., Cattabeni F., and Abbracchio M.P. (1999) Cyclo-oxygenase-2 mediates P2Y receptor-induced reactive astrogliosis. *Br. J. Pharmacol.* **126(3),** 563–567.
29. Brambilla R., Neary J.T., Cattabeni F., et al. (2002) Induction of COX-2 and reactive gliosis by P2Y receptors in rat cortical astrocytes is dependent on ERK1/2 but independent of calcium signalling. *J. Neurochem.* **83(6),** 1285–1296.
30. Franke H., Krugel U., Schmidt R., Grosche J., Reichenbach A., and Illes P. (2001) P2 receptor-types involved in astrogliosis in vivo. *Br. J. Pharmacol.* **134,** 1180–1189.
31. Franke H., Krugel U., and Illes P. (1999) P2 receptor-mediated proliferative effects on astrocytes in vivo. *Glia* **28(3),** 190–200.
32. Neary J.T., Whittemore S.R., Zhu Q., and Norenberg M.D. (1994) Synergistic activation of DNA synthesis in astrocytes by fibroblast growth factors and extracellular ATP. *J. Neurochem.* **63(2),** 490–494.
33. Lenz G., Gottfried C., Luo Z., et al. (2000) P_{2Y} purinoceptor subtypes recruit different mek activators in astrocytes. *Br. J. Pharmacol.* **129(5),** 927–936.
34. Jacques-Silva M.C., Rodnight R., Lenz G., et al. (2004) P2X7 receptors stimulate AKT phosphorylation in astrocytes. *Br. J. Pharmacol.* **141(7),** 1106–1117.
35. Fumagalli M., Brambilla R., D'Ambrosi N., et al. (2003) Nucleotide-mediated calcium signaling in rat cortical astrocytes: role of P2X and P2Y receptors. *Glia* **43,** 218–230.
36. Kukley M., Barden J.A., Steinhauser C., and Jabs R. (2001) Distribution of P2X receptors on astrocytes in juvenile rat hippocampus. *Glia* **36(1),** 11–21.
37. Khakh B.S., Smith W.B., Chiu C.S., Ju D., Davidson N., and Lester H.A. (2001) Activation-dependent changes in receptor distribution and dendritic morphology in hippocampal neurons expressing $P2X_2$-green fluorescent protein receptors. *Proc. Natl. Acad. Sci. USA* **98(9),** 5288–5293.
38. King B.F., Neary J.T., Zhu Q., Wang S., Norenberg M.D., and Burnstock G. (1996) P2 purinoceptors in rat cortical astrocytes: expression, calcium-imaging and signalling studies. *Neuroscience* **74(4),** 1187–1196.
39. Scemes E., Duval N., and Meda P. (2003) Reduced expression of P2Y1 receptors in connexin43-null mice alters calcium signaling and migration of neural progenitor cells. *J. Neurosci.* **23(36),** 11,444–11,452.
40. Chen W.C. and Chen C.C. (1998) ATP-induced arachidonic acid release in cultured astrocytes is mediated by Gi protein coupled P2Y1 and P2Y2 receptors. *Glia* **22(4),** 360–370.
41. Kim S.G., Soltysiak K.A., Gao Z.G., Chang T.S., Chung E., and Jacobson K.A. (2003) Tumor necrosis factor alpha-induced apoptosis in astrocytes is prevented by the activation of P2Y6, but not P2Y4 nucleotide receptors. *Biochem. Pharmacol.* **65(6),** 923–931.
42. Moore D.J., Murdock P.R., Watson J.M., et al. (2003) GPR105, a novel Gi/o-coupled UDP-glucose receptor expressed on brain glia and peripheral immune cells, is regulated by immunologic challenge: possible role in neuroimmune function. *Brain Res. Mol. Brain Res.* **118(1–2),** 10–23.
43. Lustig K.D., Erb L., Landis D.M., et al. (1992) Mechanisms by which extracellular ATP and UTP stimulate the release of prostacyclin from bovine pulmonary artery endothelial cells. *Biochim. Biophys. Acta* **1134(1),** 61–72.
44. Seye C.I., Kong Q., Erb L., et al. (2002) Functional $P2Y_2$ nucleotide receptors mediate uridine 5′-triphosphate-induced intimal hyperplasia in collared rabbit carotid arteries. *Circulation* **106(21),** 2720–2726.
45. Pillois X., Chaulet H., Belloc I., Dupuch F., Desgranges C., and Gadeau A.P. (2002)

Nucleotide receptors involved in UTP-induced rat arterial smooth muscle cell migration. *Circ. Res.* **90(6),** 678–681.
46. Kunapuli S.P. and Daniel J.L. (1998) P2 receptor subtypes in the cardiovascular system. *Biochem. J.* **336(3),** 513–523.
47. Kim K.C., Park H.R., Shin C.Y., Akiyama T., and Ko K.H. (1996) Nucleotide-induced mucin release from primary hamster tracheal surface epithelial cells involves the P2u purinoceptor. *Eur. Respir. J.* **9(7),** 1579.
48. Berti-Mattera L.N., Wilkins P.L., Madhun Z., and Suchovsky D. (1996) P2-purigenic receptors regulate phospholipase C and adenylate cyclase activities in immortalized Schwann cells. *Biochem. J.* **314(2),** 555–561.
49. Ho C., Hicks J., and Salter M.W. (1995) A novel P2-purinoceptor expressed by a subpopulation of astrocytes from the dorsal spinal cord of the rat. *Br. J. Pharmacol.* **116(7),** 2909–2918.
50. Kirischuk S., Scherer J., Kettenmann H., and Verkhratsky A. (1995) Activation of P2-purinoreceptors triggered Ca^{2+} release from InsP3-sensitive internal stores in mammalian oligodendrocytes. *J. Physiol.* **483(1),** 41–57.
51. Miyagi Y., Kobayashi S., Ahmed A., Nishimura J., Fukui M., and Kanaide H. (1996) P2U purinergic activation leads to the cell cycle progression from the G1 to the S and M phases but not from the G0 to G1 phase in vascular smooth muscle cells in primary culture. *Biochem. Biophys. Res. Commun.* **222(2),** 652–658.
52. Muscella A., Elia M.G., Greco S., Storelli C., and Marsigliante S. (2003) Activation of P2Y2 receptor induces c-FOS protein through a pathway involving mitogen-activated protein kinases and phosphoinositide 3-kinases in HeLa cells. *J. Cell. Physiol.* **195(2),** 234–240.
53. Seye C.I., Yu N., Jain R., et al. (2003) The $P2Y_2$ nucleotide receptor mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells. *J. Biol. Chem.* **278(27),** 24,960–24,965.
54. Koshiba M., Apasov S., Sverdlov V., et al. (1997) Transient up-regulation of $P2Y_2$ nucleotide receptor mRNA expression is an immediate early gene response in activated thymocytes. *Proc. Natl. Acad. Sci. USA* **94(3),** 831–836.
55. Turner J.T., Weisman G.A., and Camden J.M. (1997) Upregulation of $P2Y_2$ nucleotide receptors in rat salivary gland cells during short-term culture. *Am. J. Physiol.* **273(3),** C1100–C1107.
56. Xu J., Chalimoniuk M., Shu Y., et al. (2003) Prostaglandin E2 production in astrocytes: regulation by cytokines, extracellular ATP, and oxidative agents. *Prostaglandins Leukotriene Essent. Fatty Acids* **69(6),** 437–448.
57. Xu J., Weng Y.I., Simonyi A., et al. (2002) Role of PKC and MAPK in cytosolic PLA2 phosphorylation and arachidonic acid release in primary murine astrocytes. *J. Neurochem.* **83(2),** 259–270.
58. Welch B.D., Carlson N.G., Shi H., Myatt L., and Kishore B.K. (2003) $P2Y_2$ receptor-stimulated release of prostaglandin E2 by rat inner medullary collecting duct preparations. *Am. J. Physiol. Renal Physiol.* **285(4),** F711–F721.
59. Mohaupt M.G., Fischer T., Schwobel J., Sterzel R.B., and Schulze-Lohoff E. (1998) Activation of purinergic P2Y2 receptors inhibits inducible NO synthase in cultured rat mesangial cells. *Am. J. Physiol.* **275(1),** F103–F110.
60. Yamakuni H., Kawaguchi N., Ohtani Y., et al. (2002) ATP induces leukemia inhibitory factor mRNA in cultured rat astrocytes. *J. Neuroimmunol.* **129(1–2),** 43–50.
61. Hou M., Moller S., Edvinsson L., and Erlinge D. (2000) Cytokines induce upregulation of vascular $P2Y_2$ receptors and increased mitogenic responses to UTP and ATP. *Arterioscler. Thromb. Vasc. Biol.* **20(9),** 2064–2069.
62. Hou M., Moller S., Edvinsson L., and Erlinge D. (1999) MAPKK-dependent growth factor-induced upregulation of $P2Y_2$ receptors in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **258(3),** 648–652.
63. Marrelli S.P., Khorovets A., Johnson T.D., Childres W.F., and Bryan R.M., Jr. (1999) P2 purinoceptor-mediated dilations in the rat middle cerebral artery after ischemia–reperfusion. *Am. J. Physiol.* **276(1),** H33–H41.
64. Kannan S. (2003) Neutrophil chemotaxis: potential role of chemokine receptors in extracellular nucleotide induced Mac-1 expression. *Med. Hypotheses* **61(5,6),** 577–579.
65. Turner J.T., Park M., Camden J.M., and Weisman G.A. (1998) Salivary gland nucleotide receptors. Changes in expression and activity related to development and tissue damage. *Ann. NY Acad. Sci.* **842,** 70–75.
66. Dennis E.A. (1994) Diversity of group types, regulation, and function of phospholipase A2. *J. Biol. Chem.* **269(18),** 13,057–13,060.
67. Balsinde J., Winstead M.V., and Dennis E.A. (2002) Phospholipase A_2 regulation of arachi-

donic acid mobilization. *FEBS Lett.* **531(1),** 2–6.
68. Martin K.A., Kertesy S.B., and Dubyak G.R. (1997) Down-regulation of P2U-purinergic nucleotide receptor messenger RNA expression during in vitro differentiation of human myeloid leukocytes by phorbol esters or inflammatory activators. *Mol. Pharmacol.* **51(1),** 97–108.
69. Gendron F.P., Chalimoniuk M., Strosznajder J., et al. (2003) P2X$_7$ nucleotide receptor activation enhances IFNγ-induced type II nitric oxide synthase activity in BV-2 microglial cells. *J. Neurochem.* **87(2),** 344–352.
70. Kopp S. (2001) Neuroendocrine, immune, and local responses related to temporomandibular disorders. *J. Orofac. Pain* **15(1),** 9–28.
71. Vancheri C., Mastruzzo C., Sortino M.A., and Crimi N. (2004) The lung as a privileged site for the beneficial actions of PGE2. *Trends Immunol.* **25(1),** 40–46.
72. Karaki S.I. and Kuwahara A. (2004) Regulation of intestinal secretion involved in the interaction between neurotransmitters and prostaglandin E2. *Neurogastroenterol. Motil.* **1,** 96–99.
73. Ferreira S.H., Moncada S., and Vane J.R. (1997) Prostaglandins and the mechanism of analgesia produced by aspirin-like drugs. 1973. *Br. J. Pharmacol.* **120(4 Suppl.),** 401–412; discussion 399–400.
74. Erb L., Liu J., Ockerhausen J., et al. (2001) An RGD sequence in the P2Y$_2$ receptor interacts with $\alpha_v\beta_3$ integrins and is required for G_o-mediated signal transduction. *J. Cell Biol.* **153(3),** 491–501.
75. Li J.M., Fan L.M., Shah A., and Brooks G. (2003) Targeting $\alpha_v\beta_3$ and $\alpha_5\beta_1$ for gene delivery to proliferating VSMCs: synergistic effect of TGF-β1. *Am. J. Physiol. Heart Circ. Physiol.* **285,** H1123–H1131.
76. Hutchings H., Ortega N., and Plouet J. (2003) Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation. *FASEB J.* **17(11),** 1520–1522.
77. Pidgeon G.P., Tang K., Cai Y.L., Piasentin E., and Honn K.V. (2003) Overexpression of platelet-type 12-lipoxygenase promotes tumor cell survival by enhancing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin expression. *Cancer Res.* **63(14),** 4258–4267.
78. Zhang H., Li Z., Viklund E.K., and Stromblad S. (2002) P21-activated kinase 4 interacts with integrin $\alpha_v\beta_5$ and regulates $\alpha_v\beta_5$-mediated cell migration. *J. Cell Biol.* **158,** 1287–1297.
79. Greig A.V., Linge C., Terenghi G., Mcgrouther D.A., and Burnstock G. (2003) Purinergic receptors are part of a functional signaling system for proliferation and differentiation of human epidermal keratinocytes. *J. Invest. Dermatol.* **120(6),** 1007–1015.
80. Schafer R., Sedehizade F., Welte T., and Reiser G. (2003) ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. *Am. J. Physiol. Lung Cell. Mol Physiol.* **285(2),** L376–L385.
81. Wilden P.A., Agazie Y.M., Kaufman R., and Halenda S.P. (1998) ATP-stimulated smooth muscle cell proliferation requires independent ERK and PI3K signaling pathways. *Am. J. Physiol.* **275(4),** H1209–H1215.
82. Gungabissoon R.A. and Bamburg J.R. (2003) Regulation of growth cone actin dynamics by ADF/cofilin. *J. Histochem. Cytochem.* **51(4),** 411–420.
83. Lin T., Zeng L., Liu Y., et al. (2003) Rho–ROCK–LIMK–cofilin pathway regulates shear stress activation of sterol regulatory element binding proteins. *Circ. Res.* **92(12),** 1296–1304.
84. Rodgers E.E. and Theibert A.B. (2002) Functions of PI 3-kinase in development of the nervous system. *Int. J. Dev. Neurosci.* **20(3–5),** 187–197.
85. Chan-Ling T. and Stone J. (1991) Factors determining the migration of astrocytes into the developing retina: migration does not depend on intact axons or patent vessels. *J. Comp. Neurol.* **303(3),** 375–386.
86. Ellison J.A., Velier J.J., Spera P., et al. (1998) Osteopontin and its integrin receptor $\alpha_v\beta_3$ are upregulated during formation of the glial scar after focal stroke. *Stroke* **29(8),** 1698–1706; discussion 1707.
87. Cao W., Luttrell L.M., Medvedev A.V., et al. (2000) Direct binding of activated c-Src to the beta 3-adrenergic receptor is required for MAP kinase activation. *J. Biol. Chem.* **275(49),** 38,131–38,134.
88. Green S.A. and Liggett S.B. (1994) A proline-rich region of the third intracellular loop imparts phenotypic beta 1-versus beta 2-adrenergic receptor coupling and sequestration. *J. Biol. Chem.* **269(42),** 26,215–26,219.
89. Tang Y., Hu L.A., Miller W.E., et al. (1999) Identification of the endophilins (SH3p4/p8/p13)

as novel binding partners for the beta1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **96(22),** 12,559–12,564.
90. Oldenhof J., Vickery R., Anafi M., et al. (1998) SH3 binding domains in the dopamine D4 receptor. *Biochemistry* **37(45),** 15,726–15,736.
91. Luttrell L.M., Della Rocca G.J., Van Biesen T., Luttrell D.K., and Lefkowitz R.J. (1997) Gβγ subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. *J. Biol. Chem.* **272(7),** 4637–4644.
92. Andreev J., Galisteo M.L., Kranenburg O., et al. (2001) Src and Pyk2 mediate G-protein-coupled receptor activation of epidermal growth factor receptor (EGFR) but are not required for coupling to the mitogen-activated protein (MAP) kinase signaling cascade. *J. Biol. Chem.* **276(23),** 20,130–20,135.
93. Soltoff S.P. (1998) Related adhesion focal tyrosine kinase and the epidermal growth factor receptor mediate the stimulation of mitogen-activated protein kinase by the G-protein-coupled $P2Y_2$ receptor. Phorbol ester or $[Ca^{2+}]_i$ elevation can substitute for receptor activation. *J. Biol. Chem.* **273,** 23,110–23,117.
94. Soltoff S.P., Avraham H., Avraham S., and Cantley L.C. (1998) Activation of $P2Y_2$ receptors by UTP and ATP stimulates mitogen-activated kinase activity through a pathway that involves related adhesion focal tyrosine kinase and protein kinase C. *J. Biol. Chem.* **273(5),** 2653–2660.
95. Liu J., Liao Z., Camden J., et al. (2004) Src homology 3 binding sites in the $P2Y_2$ nucleotide receptor interact with Src and regulate activities of Src, proline-rich tyrosine kinase 2, and growth factor receptors. *J. Biol. Chem.* **279(9),** 8212–8218.
96. Avraham H., Park S.Y., Schinkmann K., and Avraham S. (2000) RAFTK/Pyk2-mediated cellular signalling. *Cell Signal.* **12(3),** 123–133.
97. Du Q.S., Ren X.R., Xie Y., Wang Q., Mei L., and Xiong W.C. (2001) Inhibition of PYK2-induced actin cytoskeleton reorganization, PYK2 autophosphorylation and focal adhesion targeting by FAK. *J. Cell Sci.* **114(16),** 2977–2987.
98. Lim I.J., Phan T.T., Tan E.K., et al. (2003) Synchronous activation of ERK and phosphatidylinositol 3-kinase pathways is required for collagen and extracellular matrix production in keloids. *J. Biol. Chem.* **278(42),** 40,851–40,858.
99. Menu E., Kooijman R., Van Valckenborgh E., et al. (2004) Specific roles for the PI3K and the MEK–ERK pathway in IGF-1-stimulated chemotaxis, VEGF secretion and proliferation of multiple myeloma cells: study in the 5T33MM model. *Br. J. Cancer* **90(5),** 1076–1083.
100. Ding X.Z., Tong W.G., and Adrian T.E. (2003) Multiple signal pathways are involved in the mitogenic effect of 5(S)-HETE in human pancreatic cancer. *Oncology* **65(4),** 285–294.
101. Schafer M., Schafer C., Ewald N., Piper H.M., and Noll T. (2003) Role of redox signaling in the autonomous proliferative response of endothelial cells to hypoxia. *Circ. Res.* **92(9),** 1010–1015.
102. Johnson G.L. and Lapadat R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298,** 1911–1912.
103. Daub H., Wallasch C., Lankenau A., Herrlich A., and Ullrich A. (1997) Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.* **16(23),** 7032–7044.
104. Qiao M., Shapiro P., Kumar R., and Passaniti A. (2004) Insulin-like growth factor-1 regulates endogenous RUNX2 activity in endothelial cells through a PI3K/ERK-dependent and Akt-independent signaling pathway. *J. Biol. Chem.* [E-pub ahead of press].
105. Okkenhaug K. and Vanhaesebroeck B. (2003) PI3K in lymphocyte development, differentiation and activation. *Nat. Rev. Immunol.* **3(4),** 317–330.
106. Neary J.T., Kang Y., Bu Y., Yu E., Akong K., and Peters C.M. (1999) Mitogenic signaling by ATP/P2Y purinergic receptors in astrocytes: involvement of a calcium-independent protein kinase C, extracellular signal-regulated protein kinase pathway distinct from the phosphatidylinositol-specific phospholipase C/calcium pathway. *J. Neurosci.* **19(11),** 4211–4220.
107. Neary J.T., Zhu Q., Kang Y., and Dash P.K. (1996) Extracellular ATP induces formation of AP-1 complexes in astrocytes via P2 purinoceptors. *Neuroreport* **7(18),** 2893–2896.
108. Neary J.T., McCarthy M., Cornell-Bell A., and Kang Y. (1999) Trophic signaling pathways activated by purinergic receptors in rat and human astroglia. *Prog. Brain Res.* **120,** 323–332.

109. Santiago-Perez L.I., Flores R.V., Santos-Berrios C., et al. (2001) P2Y$_2$ nucleotide receptor signaling in human monocytic cells: activation, desensitization and coupling to mitogen-activated protein kinases. *J. Cell Physiol.* **187(2),** 196–208.

110. Gendron F.P., Neary J.T., Theiss P.M., Sun G.Y., Gonzalez F.A., and Weisman G.A. (2003) Mechanisms of P2X$_7$ receptor-mediated ERK1/2 phosphorylation in human astrocytoma cells. *Am. J. Physiol. Cell Physiol.* **284(2),** C571–C581.