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Mechanisms for Inhibition of P2 Receptors Signaling in Neural Cells

Fernando A. González,*[,1,2] Gary A. Weisman,[3] Laurie Erb,[3] Cheikh I. Seye,[3] Grace Y. Sun,[3] Betty Velázquez,[1] Melvin Hernández-Pérez,[1] and Nataliya E. Chorna[1]

[1]Department of Chemistry, University of Puerto Rico, Río Piedras Campus, Puerto Rico; [2]Department of Biochemistry, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico; and [3]Department of Biochemistry, University of Missouri–Columbia, Columbia, MO

Abstract

Trophic factors are required to ensure neuronal viability and regeneration after neural injury. Although abundant information is available on the factors that cause the activation of astrocytes, little is known about the molecular mechanisms underlying the regulation of this process. Nucleotides released into the extracellular space from injured or dying neural cells can activate astrocytes via P2 nucleotide receptors. After a brief historical review and update of novel P2 receptor antagonists, this article focuses on recent advancements toward understanding molecular mechanisms that regulate G protein-coupled P2Y receptor signaling. Among P2Y receptor subtypes, the heptahelical $P2Y_2$ nucleotide receptor interacts with vitronectin receptors via an RGD sequence in the first extracellular loop, and this interaction is required for effective signal transduction to activate mitogen-activated protein kinases ERK1/2, to mobilize intracellular calcium stores via activation of phospholipase C, protein kinase C isoforms, and to activate focal adhesion kinase and other signaling events. Ligation of vitronectin receptors with specific antibodies caused an inhibition of $P2Y_2$ receptor-induced ERK1/2 and p38 phosphorylation and $P2Y_2$ receptor-induced cytoskeleton rearrangement and DNA synthesis. Structure–function studies have identified agonist-induced phosphorylation of the C-terminus of the $P2Y_2$ receptor, an important mechanism for receptor desensitization. Understanding selective mechanisms for regulating $P2Y_2$ receptor signaling could provide novel targets for therapeutic strategies in the management of brain injury, synaptogenesis, and neurological disorders.

Index Entries: Purinergic signaling; antagonists; nucleotide receptors; astrocytes; astrogliosis; extracellular nucleotides.

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* Author to whom all correspondence and reprint requests should be addressed. E-mail: fgonzal@u.pracd.upr.clu.edu

Introduction

Trophic factors are required to ensure neuronal viability and regeneration after neural injury. Neural injury leads to increased release of growth factors, especially those for polypeptides that act via receptor tyrosine kinases (e.g., fibroblast growth factor, epidermal growth factor, and platelet-derived growth factor) *(1,2).* After mechanical or ischemic trauma to the central nervous system (CNS), the release of nucleotides together with neurotransmitters (also released during normal electrical nerve stimulation) into the extracellular space generates micromolar concentrations of ATP that activate P2Y nucleotide receptors and can, alone or in combination with growth factors, stimulate astrocyte proliferation. Therefore, extracellular nucleotides have been implicated in astrogliosis, an injury-induced hypertrophic response of astrocytes that results in "glial scarring" *(3,4).* Although much is known about the factors that cause the activation of astrocytes, very little is known about the molecular mechanisms underlying the activation process. It is our conviction that a better understanding of the molecular mechanisms that induce astrocyte activation will lead to novel therapeutic strategies for neurological disorders related to astrogliosis, including trauma, stroke, seizure, aging, and degenerative and demyelinative diseases.

P2 nucleotide receptors are abundantly distributed in mammals and elicit a multitude of responses in diverse tissues and cell types, including neurons, glia, epithelia, endothelia, bone, muscle, and hematopoietic tissues. Several excellent recent reviews that explore the physiological functions of P2 nucleotide receptors have been recently published *(5–8).* P2 nucleotide receptors comprise two families of membrane receptors: ionotropic P2X receptors that are ATP-gated ion channels, and metabotropic G-protein-coupled P2Y receptors activated by a variety of nucleotides and their analogs *(5).*

To date, seven P2X receptor subtypes ($P2X_1$–$P2X_7$) that are 40 to 50% identical in amino acid sequence have been cloned and characterized in vertebrate species *(9,10).* Each P2X receptor has two transmembrane domains, separated by an extracellular domain (approx 280 amino acids) *(11).* P2X receptors are mostly involved in synaptic transmission in the peripheral and CNS *(11–13),* but they have also been reported to play a role in initiating certain primary afferent signals *(14,15).* Homomeric $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, and $P2X_7$ channels and heteromeric $P2X_{2/3}$ and $P2X_{1/5}$ channels have been most fully characterized *(11).* P2X receptors can form multimers, as is the case of $P2X_{2/3}$ receptors that have been shown to maintain a functional heterotrimer $P2X_2$-$P2X_3$-$P2X_2$ with "head-to-tail" structure *(10).* P2X receptors form channels permeable to small monovalent cations and some have significant calcium or anion permeability *(11).* An exceptional nonselective ion channel is formed by homomeric $P2X_7$ receptors that mediate an increase in the membrane permeability of large, normally impermeant compounds, including some calcium chelators that promote actin disaggregation *(16),* leading to rapid cytoskeletal rearrangements such as membrane blebbing *(17).*

Presently, nine P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$, and $P2Y_{15}$) have been cloned and functionally defined *(9,18–21).* P2Y receptors have seven transmembrane domain structures and act via G protein coupling to phospholipases C, (PLC) leading to the formation of inositol-1,4,5-triphosphate (IP_3) and mobilization of intracellular Ca^{2+}, or adenylyl cyclase, leading to changes in the intracellular concentration of cAMP. The response time for activation of P2Y receptors is typically slower than for activation of P2X receptors because opening of ligand gated ion channels typically occurs faster than the generation of second messengers by G protein-coupled receptors (GPCRs) *(5).* P2Y receptors have diverse functions, including the regulation of platelet aggregation, muscle contraction, inflammation, neurotransmission, insulin secretion, and epithelial ion transport. It has been suggested that nucleotides acting through P2Y receptors can play trophic roles in the development and regeneration of tissue in the nervous system *(22).*

This review focuses on signal transduction pathways coupled to $P2Y_2$ receptors *(23)*, including (1) G_q-mediated activation of PLC that generates second messengers for intracellular calcium mobilization and protein kinase C (PKC) activation *(4,5,24–26)*, (2) activation of $\alpha_v\beta_3/\beta_5$ integrin signaling cascades as a result of the presence of an arg-gly-asp (RGD) domain in the $P2Y_2$ receptor that couples the receptor to focal adhesion kinases (FAK) and G_i/G_o proteins *(24)*, and (3) the src-dependent transactivation of growth factor receptors that is mediated by SH3-binding sites in the intracellular C-terminus of the $P2Y_2$ receptor *(27)*. The tripartite signaling pathways of the $P2Y_2$ receptor enables the complex integration of extracellular nucleotide signals that regulate the activities of mitogen- and stress-activated protein kinases, including p38, JNK, and extracellular signal-regulated kinase: 1 and 2 (ERK1/2) *(4,24,27–30)* and phosphoinositol 3-kinase (PI 3-kinase).

Although extensive evidence documents the potential importance of P2X and P2Y receptor subtypes in the regulation of neural responses, there are surprisingly few examples of selective antagonists for these receptors. One reason for this might be the complex and sometimes overlapping pharmacologies of these receptors for agonists and antagonists. A recent review describes in detail the subtype selectivity profile of P2 receptor antagonists such as suramin, NF023 [symmetrical 3′-urea of 8-(benzamido)naphthalene-1,3,5-trisulfonic acid], NF279 [8, 8′-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino))*bis*(1,3,5-naphthalenetrisulfonica cid)], pyridoxal-5-phosphate (P5P), pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), iso-PPADS, reactive blue 2, reactive red, trypan blue, Evans blue, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), arylazidoaminopropionyl ATP, 2-alkylthio derivatives of ATP and 5′-p-fluorosulfonyl benzoyladenosine *(5)*. In this review, we describe the development of new P2 receptor antagonists and discuss recent discoveries on the mechanisms of P2 receptor signaling that provide the framework for novel strategies for the modulation of P2 receptor functions.

New Antagonists for P2X Receptors

TNP-ATP

2′(3′)-*O*-(2,4,6-Trinitrophenyl)-adenosine-5′-triphosphate) (TNP-ATP) has been known to be an effective antagonist of P2X receptors for more than three decades *(31–33)*. More recently, this compound was reported to display selective antagonism against $P2X_1$, $P2X_3$, and $P2X_{2/3}$ receptors with effective concentrations in the low nanomolar range *(34)*. Furthermore, TNP-ATP appears to be a noncompetitive antagonist at rat $P2X_3$ receptors *(35)*. Unfortunately, there are limitations in the use of TNP-ATP in whole-tissue preparations or under in vivo conditions because of its susceptibility to degradation by ectonucleotidases *(36)*.

Basilen Blue

Basilen blue, an isomer of reactive blue 2 and an antagonist of P2Y and P2X nucleotide receptors, was found to prevent cell death evoked by hypoglycemia, chemical hypoxia, mitochondrial dysfunction, glutamate-dependent excitotoxicity, and low potassium-induced apoptosis in rat cerebellar neurons *(37–39)*. Recently, it was found that hypoglycemia induces overexpression of $P2X_7$ and $P2Y_4$ receptors and that basilen blue suppressed hypoglycemia-induced expression of $P2X_7$ and $P2Y_4$, but not $P2X_4$ or $P2Y_1$ receptors *(40)*. Furthermore, basilen blue acting through $P2X_2$ and $P2X_4$ receptors prevented cell death evoked by glucose/oxygen deprivation *(41)*. It was shown that ischemic conditions induced specific neuronal loss in hippocampal, cortical, and striatal organotypic cultures, which was prevented by basilen blue and suramin. Interestingly, glucose/oxygen deprivation upregulates $P2X_2$ receptors in neuronal cell bodies and in fibers of the CA1 pyramidal cell layer, the strata oriens and radiatum, and $P2X_4$ proteins in microglial cells *(41)*. Basilen blue

(5–10 μ*M*) reversibly inhibited neural growth factor (NGF)-dependent neurite outgrowth in PC-12 cells, and suramin, oxidized-ATP and DIDS, other P2 receptor antagonists were also effective in this regard *(42).* In addition, basilen blue caused a reduction in the amplitude of currents induced by kainate *(43).* The maximal response to kainate, but not its EC_{50}, decreased in the presence of basilen blue, indicating a noncompetitive mechanism of inhibition *(43).* These results demonstrate that P2 receptor antagonists can modulate kainate-induced currents in central neurons, suggesting a potential use of these compounds as neuroprotective agents in oxidative stress.

A-317491

It was recently shown that A-317491 is a potent and selective antagonist (K_i = 22-92 n*M*) of recombinant human and rat $P2X_3$ and $P2X_{2/3}$ receptor-mediated calcium flux *(44,45).* A-317491 was highly selective for $P2X_3$ receptors (IC_{50} > 10 μ*M*) over other P2 and neurotransmitter receptors, ion channels, and enzymes *(44).* Furthermore, [^{3}H]A-317491 has been used to specifically label human $P2X_{2/3}$ and $P2X_3$ receptors *(46).* Activation of $P2X_3$ receptors are likely to be involved in chronic pain conditions, particularly chronic inflammatory and neuropathic pain, and therefore $P2X_3$ receptors selective antagonists such as A-317491 might be a useful as novel analgesics *(47).*

Periodate-Oxidized ATP

Periodate-oxidized ATP (oATP) is a Schiff-base-forming reagent that has been used as an irreversible antagonist of the $P2X_7$ receptor *(48).* However, oATP has recently been shown to suppress pro-inflammatory signaling via P2 receptor-independent mechanisms *(49,50).* In addition, oATP can inhibit nuclear factor (NF)-κB activation and interleukin (IL)-8 release caused by tumor necrosis factor (TNF)-α in wild-type HEK293 cells lacking $P2X_7$ receptors, suggesting that some anti-inflammatory effects of oATP might not be the result of blockade of the $P2X_7$ receptors *(49,50).*

NF449

The novel suramin analog 4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid (NF449) was reported to be highly specific as an antagonist of $P2X_1$ nucleotide receptors. At ATP concentrations that activate human $P2X_1$ (1 μ*M*) and human $P2X_7$ (100 μ*M*) receptors, NF449 exhibited IC_{50} values of 0.05 n*M* and 40 μ*M*, respectively *(51).* NF449 has the following rank order of antagonist potencies for P2 receptors: $P2X_1$ >> $P2X_3$ > $P2Y_1$ > $P2Y_2$ > ecto-nucleotidases; this is unique among the P2 receptor antagonists reported to date *(52).*

KN-62

The tyrosine derivative KN-62 (1-[*N*,*O*-*bis*(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine) is an inhibitor of calcium/calmodulin-dependent proteins and has been described as a potent antagonist of a $P2X_7$-like receptor *(53).* Evaluation of the functional antagonistic properties of a novel series of KN-62-related compounds characterized by the presence of different phenyl-substituted piperazine moieties identified new potent inhibitors of ATP-stimulated secretion of IL-1β in monocyte-derived human macrophages, whereas KN-62 (the parent compound) could not completely inhibit ATP-induced cytokine secretion even at concentrations exceeding 100 n*M (54).*

L-Tyrosine Derivatives

Chemical analogs that act as antagonists of the $P2X_7$ receptor have been synthesized as tools for biophysical studies, including the L-tyrosine derivative [*N*-benzyloxycarbonyl-*O*-(4-arylsulfonyl)-L-tyrosyl]benzoylpiperazine (MRS2409). The general structure of MRS2409 and related compounds is *R*(1)-Tyr(*O*-*R*(2))-piperazinyl-*R*(3) in which the three R positions can be systematically changed by introducing

different reactive chemical groups. Interestingly, dimeric compounds linked at the R(2) position were potent antagonists displaying IC_{50} values of approx 100 n*M* for inhibition of $P2X_7$ receptor-mediated K^+ flux *(55).*

Trichloroethanol

Trichloroethanol (TCE) acts as a potent noncompetitive antagonist of $P2X_3$ receptor-dependent membrane currents and changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) *(56).* In addition, it was also shown that TCE moderately antagonizes G protein-coupled $P2Y_1$ and $P2Y_4$ receptors. Such effects of TCE may be relevant to the reduction of pain transmission in dorsal root ganglion neurons following ingestion of chloral hydrate or trichloroethylene.

New Antagonists for P2Y Receptors

Studies on P2Y receptor pharmacology are hampered by a lack of subtypeselective antagonists. However, recent studies have evaluated several compounds that might prove useful for selective inhibition of specific P2Y receptor subtypes.

Clopidogrel (Plavix)

Clopidogrel is a $P2Y_{12}$ receptor antagonist that inhibits platelet aggregation when administered together with aspirin and has been shown to have promise in the prevention of recurrent strokes and heart attacks in recent clinical trials *(57).*

Coenzyme A and Synthetic Derivatives

Endogenous coenzyme A (CoA-SH) and synthetic CoA-derivatives reversibly antagonize ATP-gated currents evoked by the human $P2Y_1$, but not the $P2Y_2$ receptors expressed in *Xenopus laevis* oocytes *(58).* This work proposed the use of two potent antagonists of the $P2Y_1$ receptor, nafenopin-CoA and ciprofibroyl-CoA, as hypolipidemic drugs.

Reactive Blue 2-Related Compounds

Acid blue 129, acid blue 80, acid blue 25, and acid violet 34 were found to be potent antagonists of $P2Y_1$ receptor-mediated inositol phosphategeneration in bovine endothelial cells, but only weak or ineffective $P2Y_2$ receptor antagonists *(59).* At 10 μ*M,* acid violet 34 enhanced the $P2Y_2$ receptor-mediated responses to UTP. These compounds are relatively selective for P2Y over P2X receptor, except for acid blue 25 *(60).*

N⁶-Methyl-2′-Deoxyadenosine-3′,5′-Bisphosphate and Its Derivatives

*N*⁶-methyl-2′-Deoxyadenosine-3′,5′-bisphosphate (MRS2179) and 2-chloro *N*⁶-methyl-*(N)*-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2279) are promising antithrombotic agents that inhibit ADP-induced platelet aggregation and increases $[Ca^{2+}]_i$, suggesting that these compounds might be selective antagonists of $P2Y_1$ receptors *(61).* MRS2279 has been shown to be a high-affinity, competitive antagonist of the endogenous $P2Y_1$ receptors in human platelets ($pK_B = 8.05$) or of human $P2Y_1$ receptors stably expressed in 1321N1 human astrocytoma cells ($pK_B = 8.10$), but had no effect on activation of the human $P2Y_2$, $P2Y_4$, $P2Y_6$, or $P2Y_{11}$ receptors by their cognate agonists *(62).* MRS2179 and MRS2279 did not antagonize the G_i-coupled ADP receptor whose activation leads to inhibition of adenylyl cyclase and decreases in cAMP levels in platelets. Recently, a 2,*N*⁶-dimethyl-2′-deoxyadenosine-3′,5′-bisphosphate derivative of MRS2179 has been reported to be fourfold more potent than MS2179 as an antagonist of ADP-induced platelet aggregation mediated by the $P2Y_1$ receptor, demonstrating the affinity-enhancing effects of the 2-methyl group *(63).*

AR-C69931MX

AR-C69931MX has been reported to be a selective $P2Y_{12}$ receptor antagonist whose intravenous administration in a canine coronary electrolytic injury model blocked ADP-induced

Table 1
Specificity of New P2 Receptor Antagonists

Antagonist	P2X receptors subtypes	P2Y receptors subtypes
TNP-ATP	$P2X_{1,3,2/3}$	—
Basilen blue	$P2X_{2,4,7}$	$P2Y_{1,4}$
A-317491	$P2X_{3,2/3}$	—
NF449	$P2X_{1,3}$	$P2Y_{1,2}$
KN-62	$P2X_7$	—
L-Tyrosine derivatives	$P2X_7$	—
TCE	$P2X_3$	$P2Y_{1,4}$
Plavix	—	$P2Y_{12}$
CoA-SH and synthetic CoA derivatives	—	$P2Y_1$
Reactive blue 2-related compounds	—	$P2Y_1$
MRS2179	—	$P2Y_1$
AR-C69931MX	—	$P2Y_{12}$

platelet aggregation and recruitment and prevented platelet-mediated thrombosis *(64).* The use of this $P2Y_{12}$ antagonist during myocardial tissue perfusion resulted in a decrease in reperfusion and cyclic flow variations. Table 1 summarizes the most recently developed P2 receptor antagonists and their specificity.

Novel Approaches in the Development of P2 Receptor Inhibitors

The mitogenic and neurotrophic effects of P2 receptors have been postulated to be mediated by intracellular calcium mobilization, activation of PLC, production of prostaglandins, and activation of mitogen-activated protein kinase (MAPK) *(4,65–72).* Elucidation of the molecular determinants and the mechanisms by which P2 nucleotide receptors trigger signal transduction pathways should be beneficial for the development of new classes of inhibitors/activators of P2 receptors, as these provide novel targets for the development of therapeutic strategies for the management of pathological conditions and human diseases. In this section, we will focus on molecular and pharmacological studies on $P2Y_2$ receptor signaling because these have allowed the development of novel modes of signaling modulation based on receptor structure–function relationships.

Inhibition of P2Y Receptor Signaling by Desensitization

Desensitization is a process by which activated receptors become resistant or insensitive to subsequent agonist exposure *(73).* Two major types of desensitization have been identified: homologous and heterologous. Homologous desensitization is an agonist-specific response in which the targeted receptor only becomes desensitized to its own agonists, whereas heterologous desensitization results in decreased responsiveness of a receptor because of activation of other receptors by their agonists or a variety of nonreceptor ligands. It has been shown that receptor phosphorylation and receptor degradation are involved in the desensitization of GPCRs *(74).* An early event in the homologous desensitization of a GPCR is its phosphorylation by GPCR kinases *(73),* which promotes interaction of the receptor with arrestins to attenuate

receptor-mediated activation of an effector system *(73);* that is, the receptor becomes "uncoupled" from its signaling pathway. Receptor phosphorylation by other protein kinases has been implicated in heterologous desensitization *(75,76).* Internalization of receptors also plays a role in receptor desensitization and can lead to receptor dephosphorylation and subsequent resensitization upon recycling to the plasma membrane *(77).*

There has been some progress made toward understanding the regulation of P2 receptor signaling and the molecular basis for its desensitization. The $P2Y_2$ receptor can be rapidly and transiently desensitized by a short exposure to nucleotides *(78,79).* Resensitization from this short-term effect does not appear to require protein synthesis. A chronic desensitization state is obtained when $P2Y_2$ receptors are treated with an agonist for prolonged times (>4 h). Resensitization from chronic desensitization requires several hours in the absence of an agonist *(80).* The chronic desensitization of $P2Y_2$ receptors in human U937 monocytic cells is associated with a decrease in cellular levels of $P2Y_2$ receptor messenger RNA (mRNA) *(81).* Agonist-induced desensitization of P2 receptors also has been reported in other cell systems *(82,83)* and does not appear to involve receptor phosphorylation by a phorbol-sensitive PKC isoform *(76,82,83).*

Recently, the importance of the C-terminus of the $P2Y_2$ receptor in its agonist-induced desensitization and internalization has been established *(84).* A series of $P2Y_2$-receptor cDNA were synthesized by polymerase chain reaction (PCR) complementary DNA, (cDNAs) to express epitope-tagged $P2Y_2$ receptors with truncations at their C-terminus of up to 40 amino acid residues. Sequence analysis of the wild-type $P2Y_2$ receptor cDNA indicated that the C-terminus contained several potential phosphorylation sites for protein kinases. The $P2Y_2$-receptor mutants were expressed in human 1321N1 astrocytoma cells that lack endogenous G protein-coupled P2 receptors to investigate the receptor domains that regulate receptor desensitization and internalization. The elimination of the last 14 C-terminal residues had no effect on receptor signaling or desensitization, but elimination of more than 21 residues decreased homologous desensitization without affecting receptor signaling. Agonist-independent PKC activation heterologously desensitized the receptor but had no effect on receptor internalization, consistent with the ideas that receptor desensitization and internalization are distinct events and that agonist occupancy is required for receptor internalization *(84).*

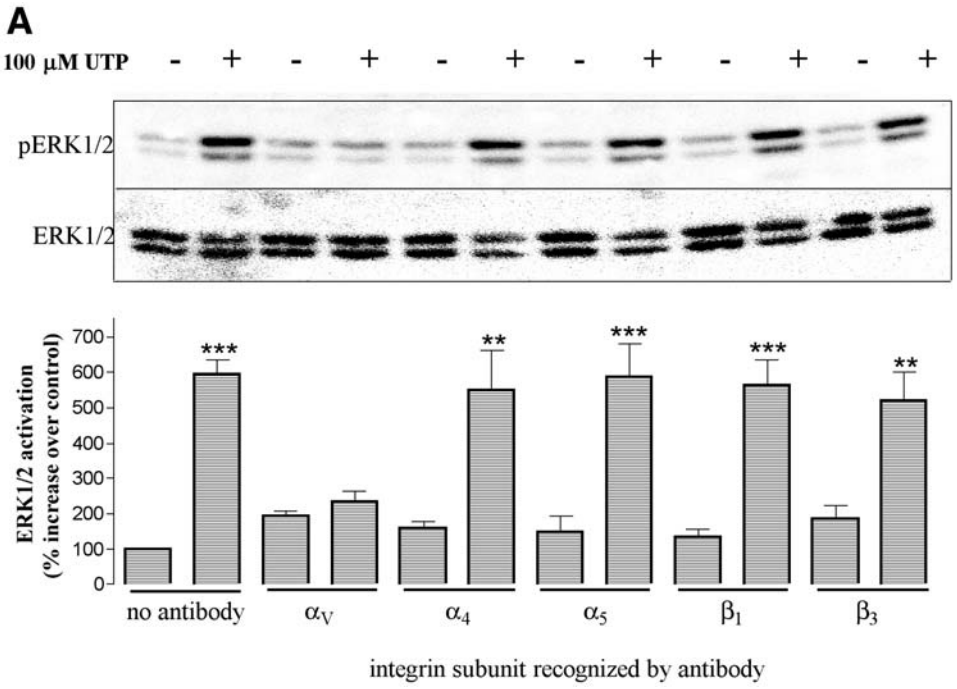
Our current hypothesis is that agonist-induced desensitization of the $P2Y_2$ nucleotide receptor involves covalent modification (i.e., phosphorylation) of the receptor that cause "uncoupling" of the receptor from PLC and the IP3-induced calcium mobilization pathway. The realization of a role for protein phosphorylation in the regulation of P2Y receptor signaling prompted the use of protein phosphatase inhibitors to modulate nucleotide receptor signaling. Thus, inhibition of protein phosphatase activity with okadaic acid has been shown to prevent resensitization of agonist-desensitized $P2Y_2$ receptors *(76,81).* Because $P2Y_2$ receptor internalization is distinct from receptor desensitization *(84),* it seems evident that distinct and independently regulated signaling pathways underlie these two processes, which would provide multiple intervention points to modulate $P2Y_2$ receptor signaling.

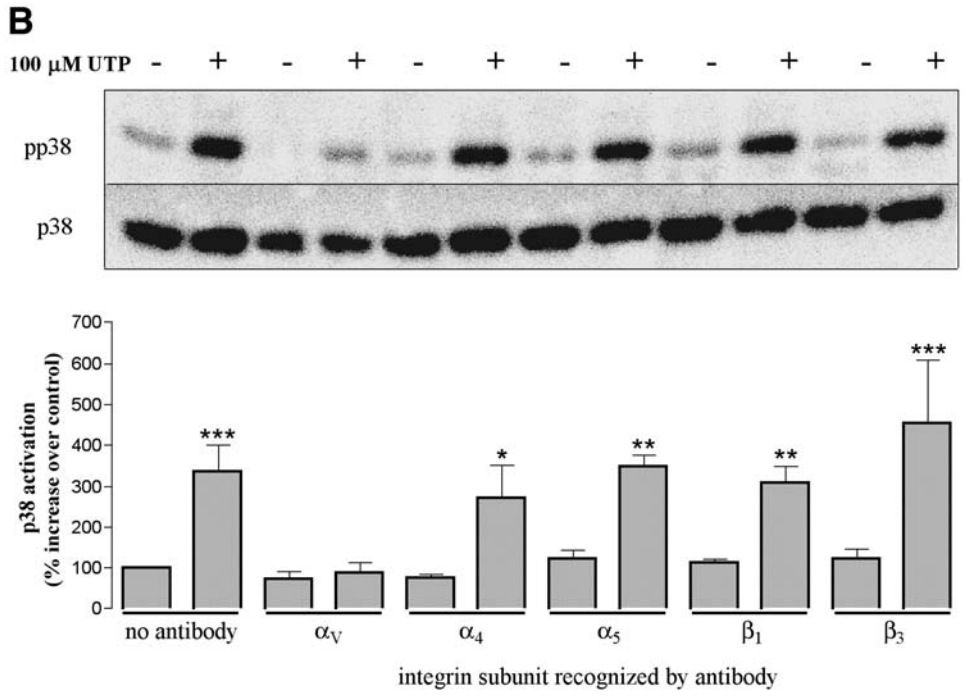
$P_{2Z}/P2X_7$ nucleotide receptors are also regulated by desensitization. A $P_{2Z}/P2X_7$ receptor in mouse fibroblasts that induces plasma membrane pore formation is desensitized by prolonged exposure to ATP *(85).* $P2X_7$ receptor desensitization is a reversible process (unpublished results), but no detailed analysis of the receptor resensitization process has been made. Different types of P2 receptors (e.g., $P2Y_2$ and $P_{2Z}/P2X_7$) coexpressed in the same cell line can be distinguished on the basis of the nucleotide concentration required to induce their desensitization *(86).* The desensitization of P2X receptors has been thoroughly described elsewhere *(87).*

Inhibition of $P2Y_2$ Receptor/Protein Interactions

$P2Y_2$ receptors in astrocytes can couple to the mobilization of intracellular calcium stores and the activation of the MAPK, ERK1/2, PLC, PKC isoforms, FAK, c-Src kinase, and other signaling molecules *(88).* Recently, it has been demonstrated that the heptahelical G protein-coupled $P2Y_2$ receptor contains an integrin-binding arg-gly-asp (RGD) sequence in its first extracellular loop that interacts with $\alpha_v\beta_3$ integrins *(24).* Immunofluorescence of 1321N1 astrocytoma cells transfected with epitope-tagged $P2Y_2$ receptors indicated that α_v integrins colocalized with the wild-type $P2Y_2$ receptor, but not with a $P2Y_2$ receptor mutant in which the RGD sequence was replaced with a nonintegrin binding motif, arg-gly-glu, (RGE). Compared with the wild-type $P2Y_2$ receptor, the RGE mutant receptor required 1000-fold higher agonist concentration to induce the PLC-dependent mobilization of intracellular free calcium and the phosphorylations of FAK and ERK1/2 *(24).* Furthermore, interaction between $\alpha_v\beta_3$ integrins and $P2Y_2$ receptors could be disrupted by anti-$\alpha_v\beta_3$-integrin monoclonal antibodies (i.e., clone 23C6 from Santa Cruz Biotechnology, Santa Cruz, CA) *(89).* The 23C6 antibodies are widely used for their capacity to block RGD-containing ligands from binding to the RGD-binding site of $\alpha_v\beta_3$ integrins *(89,90).* Recently, we demonstrated that ligation of $\alpha_v\beta_3$ integrins with 23C6 antibodies for 18 h inhibited $P2Y_2$ receptor-mediated phosphorylation of both p38 and ERK1/2 kinases in monocytic U937 cells *(91).* Similar effects were observed for 1321N1-$P2Y_2$ cells (data not shown). Furthermore, we used antibodies against integrin subunits in order to determine which subunit was primarily involved in mediating the $P2Y_2$ receptor–$\alpha_v\beta_3$ integrin interaction. We found that ligation of the α_v integrin was sufficient to inhibit $P2Y_2$ receptor signaling to MAPK. Antibodies against other integrin subunits (Fig. 1A,B) were not effective. These data confirm our previous results *(24)* that $P2Y_2$ receptor-mediated signaling, especially coupling to ERK1/2 and p38, was dependent on the interaction with α_v subunit. We also found that engagement $\alpha_v\beta_3$ integrins with 23C6 antibodies in astrocytic 1321N1-$P2Y_2$ cells affected cytoskeletal reorganization (Fig. 2). Pretreatment of 1321N1-$P2Y_2$ cells with MEK1/2 inhibitor PD98059 caused a similar inhibition suggesting a role for

Fig. 1. Inhibition of $P2Y_2$ receptor signaling by anti-$\alpha_v\beta_3$-integrin antibodies. Human 1321N1 astrocytoma cells expressing the $P2Y_2$ receptor were plated at a density 0.5×10^6 cells/well in six-well plates. Cells were pretreated overnight with medium containing antibodies (10 μg/mL) against integrin subunits (Chemicon, Temecula, CA) in serum-free Dulbecco's modified Eagle's media (DMEM) supplemented with 0.5% bovine serum albumin (BSA) with or without 100 μ*M* UTP for 5 min. Then, ERK1/2 **(A)** and p38 **(B)** phosphorylation were determined, as described *(81).* Phosphorylation of ERK1/2 (pERK1/2) and p38 (pp38) were normalized to total ERK1/2 and p38. The means ± SEM ($n = 3$) were expressed as a percentage of the response in the absence of UTP. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (one-way ANOVA). **(C)** Human 1321N1-$P2Y_2$ cells were seeded on a 96-well plate at a density 0.1×10^4 cells/well in DMEM supplemented with 5% Fetal Clone III serum (Invitrogen). After attachment of the cells, serum in the medium was substituted with 0.5% BSA and the cells were further incubated with or without 10 μg/mL anti-$\alpha_v\beta_3$ antibodies. After an overnight incubation at 37°C, 100 μ*M* UTP was added to some cultures and the cells were further incubated for an additional 24 h. Cell proliferation was measured using a 5-bromo-2'-deoxyuridine incorporation immunoassay kit following the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN). Culture media was not changed during the entire experiment. Activation of $P2Y_2$ receptors by UTP resulted in a significant ($p = 0.0167$) increase in DNA synthesis (0.14 ± 0.01, $n = 14$) compared to untreated cells (0.090 ± 0.015, $n = 14$), and treatment with anti-$\alpha_v\beta_3$ antibodies decreased UTP-induced DNA synthesis (0.089 ± 0.014, $n = 14$; $p = 0.0177$). Data are the absorbencies at 450 nm ± SEM of samples from three independent experiments.





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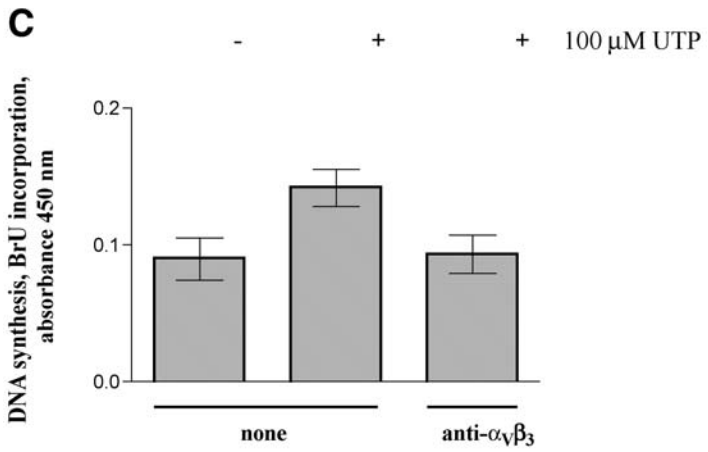

Fig. 1. *(Continued)*

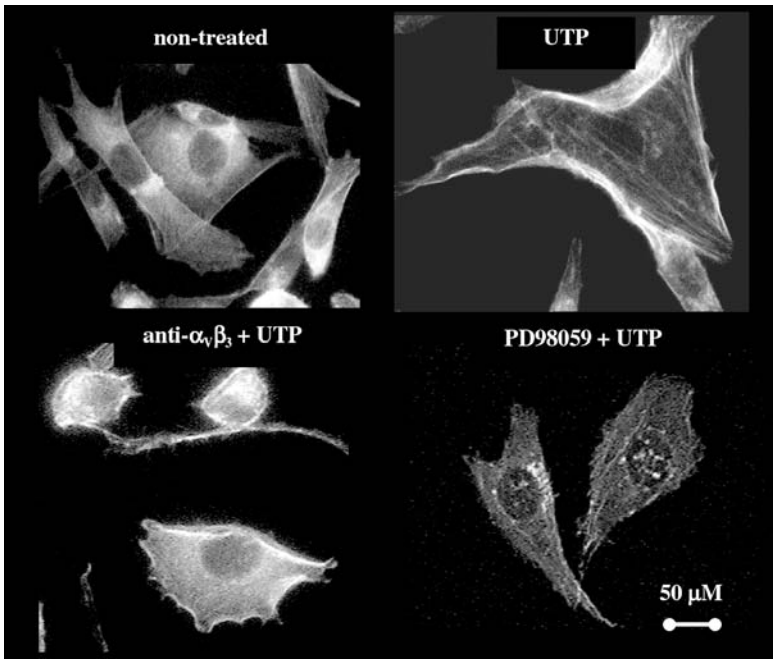

Fig. 2. Cytoskeletal rearrangement induced by $P2Y_2$ receptor agonist. Human 1321N1-$P2Y_2$ cells were plated on tissue plastic slides (Nalge Nunc International, Naperville, IL) at a density of 1×10^5 cells/mL and incubated at 37°C in serum-free DMEM supplemented with 0.5% BSA, in the presence or absence of 10 µg/mL anti-$\alpha_v\beta_3$ antibodies for 18 h or 20 µ*M* PD98059, a MEK1/2 inhibitor (Calbiochem, San Diego, CA) for 1 h, as indicated. Cells were then treated for 30 min at 37°C in the presence or absence of 100 µ*M* UTP. Cells were fixed in 4% paraformaldehyde for 7 min, washed with phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature, followed by incubation for 20 min with Alexa-fluor 488-conjugated phalloidin (25 U/mL in PBS) to visualize F-actin (Molecular Probes, Eugene, OR). Images were acquired using a fluorescence microscope, Olympus 8160-PixCell II LCM System (Arcturus, Mountain View, CA).

MEK1/2 and its downstream component ERK1/2 in $P2Y_2$ receptor-mediated cytoskeleton rearrangement (Fig. 2). Interfering the interaction between $P2Y_2$ receptor and $\alpha_v\beta_3$ integrin represents a novel approach for the modulation of responses mediated by $P2Y_2$ receptors.

Conclusion

We still lack P2 receptor-subtype-specific antagonists that can be used to selectively inhibit signaling responses contributed by a single receptor. Current research advances should provide a fuller understanding of the properties of individual P2 receptor subtypes, which should lead to the emergence of novel molecular tools for inhibition of selective nucleotide receptors. These reagents should open up new avenues for research into the physiological roles of P2 receptors and their therapeutic potential.

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