

**REGULATION AND FUNCTION OF
P2Y₂ AND P2X₇ NUCLEOTIDE RECEPTORS
IN THE CENTRAL NERVOUS SYSTEM**

A Dissertation

Presented to

the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by

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AUGUST 2007

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RECEPTORS IN THE CENTRAL NERVOUS SYSTEM**

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ACKNOWLEDGEMENTS

These projects would not have been possible without the support of many people. I would like to express my deepest gratitude to my advisor, Dr. Gary A. Weisman for his excellent guidance in both research and life, for providing me with an active laboratory atmosphere and encouraging me to work independently on the research projects with great patience, and for the great care during the long recovery term after an auto accident in 2002. I also want to thank Dr. Cheikh I. Seye, Dr. Laurie Erb, and Jean M. Camden in Weisman's lab, and Drs. Grace Y. Sun and Agnes Simonyi in Sun's lab for their tremendous support on my research projects.

I extend my gratitude to members of my doctoral committee: Drs. Laurie Erb, Grace Y. Sun, Mark Kirk, Dennis Lubahn and Cathy Krull, for their insightful comments and continuous guidance on my work.

I want to thank Jun Liu and Zhongji Liao, my best friends at MU, who have shared happiness with me and supported me to overcome my difficulties. I would also like to express my appreciation to many other friends at MU: Yu Ningpu, Wang Min, Qun Wang, Jennifer Lynn Hamilton, Sue Yu, Xiaolin Zhang, Brent Krugh, Olga Baker, Antje Heese, Troy Scott Peterson, Ann Schrader, Nishant Jain, Shivaji Rikka, Tixieanna Dissmore, Fernand Gendron, Sriparna Bagchi, Julie Hayes, Jessica Newton, Korey Griffin, and Richard Garrad.

Finally, I would like to thank my parents, my sister, and my boyfriend, Xin He, for their continuous love and support.

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LIST OF ABBREVIATIONS

A β	amyloid- β peptide
AD	Alzheimer's disease
ADAM	a disintegrin and metalloprotease
AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
APP	amyloid- β precursor protein
ATP	adenosine 5'-triphosphate
BACE	β -site APP cleaving enzyme
BCAO	bilateral carotid arteries occlusion
BG0136	1-naphthol-3, 6-disulfonic acid, disodium salt
[Ca ²⁺] _i	concentration of cytoplasmic free calcium
CCAs	common carotid arteries
CD39	ecto-nucleoside triphosphate diphosphohydrolase, NTPDase
C83	α -secretase generated membrane bound APP fragment
CE	cerebellum
CNS	central nervous system
CO	cerebral cortex
COS7	African Green Monkey kidney fibroblast cell line
DAG	diacylglycerol

DIV	days after <i>in vitro</i> culture
DND	delayed neuronal death
EC ₅₀	half maximal effective concentration
ERK1/2	extracellular signal-related kinase 1/2
FAK	focal adhesion kinase
GFR	growth factor receptor
GPCR	G protein-coupled receptor
HEK	human embryonic kidney cells
HI	hippocampus
IL-1	interleukin-1
IP	inositol phosphate
IP ₃	Inositol 1, 4, 5-trisphosphate
JNK	Jun N-terminal kinase
KPI	Kunitz Protease Inhibitor
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
MDC9	meltrin-gamma/ADAM9, a disintegrin and metalloprotease-9
MEK	MAPK kinase
2-MeSATP	2-methylthioadenosine 5'-triphosphate
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor κB
NGF	nerve growth factor

OPN	osteopontin
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLA2	phospholipases A2
PLC	phospholipases C
P3	extracellular APP fragment released from C83 following γ cleavage
PMA	phorbol 12-myristate 13-acetate
rCBF	regional cerebral blood flow
RGD	arginine-glycine-aspartic acid
RGE	arginine-glycine-glutamic acid
rPCN	rat primary cortical neuron
RT	reverse transcription
sAPP α	secreted amyloid precursor protein α
sAPP β	secreted amyloid precursor protein β
SH3	Src homology domain 3
SMC	smooth muscle cell
TACE	tumor necrosis factor alpha converting enzyme
TNF- α	tumor necrosis factor α
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling

UDP uridine 5'-diphosphate
UTP uridine 5'-triphosphate
1321N1 cells human 1321N1 astrocytoma cells

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ABSTRACT

It is well known that ATP acts as a classic neurotransmitter in the central nervous system. It is stored in molar concentrations in synaptic vesicles, released upon neuronal excitation, and activates both ion channel receptors (P2X) and metabotropic receptors (P2Y) on post-synaptic cells. Activation of these nucleotide receptors then alters a variety of brain activities, such as the formation of long term memory and the perception of pain. In addition to its role as a neurotransmitter, ATP and other nucleotides are known to be released from injured or ischemic tissue and have been reported to affect inflammatory events in the brain and many other tissues, however, these inflammatory events as well as the subtype(s) of nucleotide receptor(s) influencing these events have not been well defined. This dissertation examines the expression patterns of P2X and P2Y nucleotide receptors in rat primary cortical neurons and adult rodent brain tissue. Based on these studies, particular emphasis was placed on delineating the role of the P2Y₂ receptor subtype in neuronal inflammation and the P2X₇ receptor subtype in neuronal apoptosis.

The P2Y₂ receptor is distinguished from other nucleotide receptor subtypes by an equal-potency for activation by ATP and UTP and its ability to be up-regulated in response to tissue injury or stress. Studies mentored by Dr. Cheikh Seye used a

collared rabbit carotid artery model to indicate for the first time a role for the P2Y₂R in development of intimal hyperplasia associated with atherosclerosis and restenosis (Appendix-1). *In vitro* studies with various cell lines (1321N1, HEK, COS7, and DITNC) in collaboration with others investigated signaling events regulated by the P2Y₂R, including activation of PI3K, Src, calcium mobilization, Akt, and ERK1/2 and the importance of $\alpha_v\beta_3/\beta_5$ integrin interaction with the P2Y₂ receptor in selective (Go but not Gq) signaling and the antagonist role of BG0136 on P2Y₂R activity (Appendix-2, 3).

In a gerbil transient global ischemia model, we analyzed the effect of ischemia on the *in vivo* expression level of P2Y₂R mRNA in the rodent brain by *in situ* hybridization and RT-PCR (Chapter 4). P2Y₂R mRNA was expressed at low levels in normal rodent (rat, mouse and gerbil) brain. Transient ischemia (5 min occlusion) did not affect P2Y₂R mRNA levels in any region of gerbil brain after various reperfusion times (4 h, 16 h and 48 h), indicating that the P2Y₂R was not regulated at the transcriptional level in the gerbil transient ischemia model.

The functional expression of the P2Y₂R was also tested in rat primary cortical neurons (rPCNs). In rPCNs after *in vitro* culture for 7-10 days, P2Y₂R mRNA and receptor activity were virtually absent in unstimulated cells, whereas overnight treatment with IL-1 β caused up-regulation of both P2Y₂R mRNA and receptor activity. Furthermore, activation of P2Y₂R in rPCNs treated with IL-1 β or transfected with P2Y₂R cDNA enhanced the release of sAPP α , a secretory amyloid protein that protects neurons as an injury-induced neurotrophic factor. P2Y₂R-mediated sAPP α

release from rPCNs was dependent on ADAM10/17 and PI3K activity and was partially dependent on ERK1/2 activity, but did not require activation of PKC. These results suggest that under inflammatory conditions, IL-1 β evokes functional up-regulation of P2Y₂R in neurons and subsequent activation of the P2Y₂R signaling cascade results in an increase of sAPP α release through activation of the α , γ -secretase pathway (Chapter 3).

In contrast to the neuroprotective role of extracellular nucleotides just described, ATP has also been reported to cause apoptosis in a neuronal cell line by activating the P2X₇ subtype of nucleotide receptors (P2X₇R). Here, we extended the examination of P2X₇R-mediated apoptosis in rPCNs. Unlike the P2Y₂R, P2X₇R was functionally expressed in unstimulated rPCNs (indicated by RT-PCR and ATP-induced intracellular calcium mobilization). Activation of the P2X₇R via extracellular nucleotides in rPCNs caused biochemical (*i.e.*, caspase activation) and morphological (*i.e.*, nuclear condensation and DNA fragmentation) changes characteristic of apoptotic cell death through an intrinsic JNK1- and ERK-dependent caspase-8/9/3 activation pathway (Chapter 2).

Together, these data describe the expression patterns of P2 nucleotide receptors in rat primary cultures of cortical neurons, in adult rodent brains, in cerebral ischemia and in collared rabbit carotid arteries and demonstrate that the P2Y₂R is up-regulated in response to cytokine stimulation in rPCNs and after collar transplantation around rabbit carotid arteries. Moreover, activation of the P2Y₂R and P2X₇R in rPCNs caused α -secretase-dependent APP processing and caspase8/9/3-dependent apoptosis,

respectively, whereas *in vivo* studies in the collared carotid artery model indicate that P2Y₂R also plays an important role in the development of intimal hyperplasia.

CHAPTER 1

INTRODUCTION

Adenosine 5'-triphosphate release

Nucleotides can be released from various sources in the nervous system include excitatory neurons, injured cells, cells undergoing mechanical or oxidative stress, aggregating platelets, degranulating macrophages, and astrocytes (Bergfeld *et al.*, 1992; Ciccarelli *et al.*, 1999; Ahmed *et al.*, 2000; Ostrom *et al.*, 2001). In addition to acting as energy sources and neurotransmitters, purines such as adenosine 5'-triphosphate (ATP) are trophic factors in both development/growth and regeneration/proliferation and neurotoxic factors in neuronal cell death of different cell types under normal physiological and developmental conditions (Burnstock and Wood, 1996; Neary *et al.*, 1996; Vitolo *et al.*, 1998).

P2 nucleotide receptor classification

Extracellular nucleotides bind to and activate cell surface P2 receptors. To date, these receptors have been classified into two main families: the G protein-coupled receptors (GPCRs; P2YR) and the ligand-gated ion channels (P2XR). Nine P2Y receptors have been cloned and identified as GPCRs with seven transmembrane domains coupled via G proteins (Gq/11 or Gi/o) to phospholipase C (PLC) and/or adenylate cyclase, including P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, P2Y₁₄, and P2Y₁₅ (Sak and Webb, 2002; Inbe *et al.*, 2004), although the P2Y₁₅ receptor has not been conclusively established to be a P2 nucleotide receptor (Abbracchio *et al.*,

2004). Seven P2X receptors have been cloned and identified as ligand-gated ion channels comprised of homo- or hetero-oligomers, including P2X₁, P2X₂, P2X₃, P2X₄, P2X₅, P2X₆, and P2X₇ (Burnstock 2000). Activation of these P2 receptor subtypes in neurons and glial cells under normal and pathological conditions can mediate cell apoptosis, proliferation, migration, inflammation, ion transport, and neurotransmission (Burnstock 2000; Ciccarrelli *et al.*, 2001). 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 expression and regulation of the P2Y₂R and its signaling pathways associated with α -secretase-dependent amyloid precursor protein (APP) processing, and on P2X₇R-mediated apoptosis in neurons.

Expression and regulation of P2 nucleotide receptors in the brain

In the rat brain, P2Y₁ receptor mRNA expression was detected and regulated at different embryonic and postnatal stages; P2Y₂ receptor expression was detected at P8-12 (postnatal 8-12 days; Zhu and Kimelberg, 2004) and in adult rat brain (Loesch and Glass, 2006). P2Y₄ receptor expression was detected at E14 (embryonic 14 days) and P8 (postnatal 8 days) while P2Y₆ receptor expression was detected at E14 (Webb *et al.*, 1998; Cheung *et al.*, 2003; Zhu and Kimelberg, 2001 and 2004; Bennett *et al.*, 2003). P2Y₁₂ receptor expression was low on E13 and E17, but it gradually increased during development from postnatal day 0 to 6 weeks (Sasaki *et al.*, 2003). P2X₃ receptor mRNA was detected at E11, then declined in P7 rats, and was hardly detectable in P14 animals (Kidd *et al.*, 1998; Cheung & Burnstock, 2002). Other P2X

receptor subtypes were also detected at certain developmental stages. P2X₁R expression was detected at P7, P2X₂R expression was detected at E11 and P2X₇ receptor expression was detected at E19 (Kidd *et al.*, 1995; Collo *et al.*, 1997).

In primary cultures of rat cortical astrocytes obtained at different postnatal periods, P2Y₁, P2Y₂, P2Y₄ and P2X₁-P2X₄, P2X₆, and P2X₇ receptor subtypes were expressed in P1 pups (Lenz *et al.*, 2000; Jacques-Silva *et al.*, 2004); P2X₃, P2X₅-P2X₇ and P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptor expression was detectable in P2-P3 pups (postnatal 2-3 days; Figure 1A; Weisman *et al.*, 2005); and all P2 receptor subtypes except P2X₆ were detected in P7 pups (Fumagalli *et al.*, 2003). In rat primary cortical neurons obtained at E17-19 days, mRNA for P2Y₁, P2Y₄, P2Y₆, P2X₃, P2X₅, and P2X₇ receptor subtypes were present after 7-10 days in culture, whereas very low levels of P2Y₂, P2X₂ and P2X₆ receptor mRNA expression could be detected after 35 cycles of polymerase chain reaction (Figure 1B-C; Weisman *et al.*, 2005; Kong *et al.*, 2005). ATP and NGF have been shown to promote sustained up-regulation of various P2 nucleotide receptor subtypes including P2X₂-P2X₄ and P2Y₂ in PC12 cells under neurite regenerating (serum-containing) conditions (Rathbone *et al.*, 1999; D'Ambrosi *et al.*, 2001).

P2X₇ nucleotide receptors and apoptosis

As a mediator of cell-to-cell communication, ATP can trigger a variety of biological responses after being rapidly released in large amounts from various sources, including nerve terminals, activated glial cells, platelets, endothelial cells, antigen-stimulated T cells, and other cell types following hypoxia, stress, and tissue

damage. (Bergfeld *et al.*, 1992; Ciccarelli *et al.*, 1999; Ahmed *et al.*, 2000; Ostrom *et al.*, 2001) For example, in human umbilical cord vein endothelial cells (HUVECs), substantial release of ATP (and UTP) is induced by shear stress (Burnstock 1999), which may lead to alterations in the balance between proliferation and apoptosis regulated by P1 adenosine and P2 (particularly P2X₇) nucleotide receptors (Kaiser *et al.*, 1997). P2X₇ and P1 receptors have been previously linked to apoptosis in other cell types, including immune cells, astrocytes, and thymocytes (Zheng *et al.*, 1991; Di Virgilio *et al.*, 1995; Jacobson *et al.*, 1999). The P2X₇R also has been shown to mediate ATP/UTP-induced cell death in human embryonic kidney cells (Wen *et al.*, 2003), human cervical epithelial cells (Wang *et al.*, 2004), and primary rat cortical neurons (Chapter 2; Kong *et al.*, 2005).

In human fibroblasts, P2X₇ receptors were identified as the main nucleotide receptor involved in high glucose concentration-dependent responses modulated by ATP, including morphological changes, enhanced apoptosis, caspase-3 activation, and IL-6 release (Solini *et al.*, 2000). In the immune system, ATP also plays important roles through nucleotide receptors in leukocyte functions. P2X₇ receptor-mediated cell death has been demonstrated in various cell types, including a lymphocytic cell line, murine thymocytes, murine peritoneal macrophages, human macrophages, mesangial cells, dendritic cells, and microglial cells (Zheng *et al.*, 1991; Zanovello *et al.*, 1990; Lammas *et al.*, 1997; Schulze-Lohoff *et al.*, 1998; Coutinho-Silva *et al.*, 1999; Ferrari *et al.*, 1999). It also has been reported that the P2X₇ receptor modulates macrophage production of TNF- α , IL-1 β and NO following LPS exposure (Hu *et al.*, 1998),

consistent with a role for the P2X₇ receptor in inflammation. These studies have provided compelling evidence suggesting a role for P2 receptors (especially P2X₇R) in cell apoptosis in the central nervous system; however, further studies are needed to determine the precise pathways involved and to accumulate direct evidence that these pathways contribute significantly to neurodegenerative diseases and brain injury. Studies in Chapter 2 have investigated P2X₇ receptor-mediated apoptosis in rat primary cortical neurons and the signaling pathways involved (Kong *et al.*, 2005).

P2Y₂ nucleotide receptors in Alzheimer's disease

Alzheimer's disease is an age-related neurodegenerative disorder that is characterized by progressive memory loss and deterioration of higher cognitive functions. The hallmarks of Alzheimer's disease (AD) include extracellular plaques of aggregated β -amyloid protein (A β) and intracellular neurofibrillary tangles that contain hyperphosphorylated tau protein in brain neurons (Figure 2; reviewed by Drouet *et al.*, 2000). The senile plaques contain a compact deposition of β -amyloid peptides surrounded by dystrophic neuritis, activated microglia and reactive astrocytes. The major amyloid peptides in the plaques are A β ₁₋₄₂ and A β ₁₋₄₀, the 4-kDa peptides derived by proteolytic cleavage of amyloid precursor protein (APP). APP can be cleaved within the A β domain by the combined action of α - and γ -secretases to generate neurotrophic and neuroprotective sAPP α and a P3 fragment (Li *et al.*, 1997; Luo *et al.*, 2001), or undergo cleavage by β - and γ -secretases to form neurodegenerative β -amyloid peptides (A β ₁₋₄₂ and A β ₁₋₄₀) (Younkin, 1998). Under normal conditions, about 90% of secreted A β peptides are A β ₁₋₄₀, a more soluble form

than $A\beta_{1-42}$ that can be eliminated from brain and can only slowly be converted to the plaque-forming β -sheet configuration. In the AD brain, $A\beta_{1-42}$ is first deposited in plaques, whereas $A\beta_{1-40}$ is deposited later as the disease progresses (Iwatsubo *et al.*, 1994). A major goal to date in the development of treatments for AD include approaches that decrease $A\beta$ production by blocking γ -secretase activity. However, this may result in defective haematopoiesis or other side effects (*e.g.*, inhibition of the notch signaling pathway) due to loss of γ -secretase function (Rochette and Murphy, 2002). Clearly, further studies are needed to better understand the regulation of the different APP processing enzymes (*i.e.*, α -, β - and γ -secretases) to design effective treatments for AD.

To date, the research on the functional involvement of P2 nucleotide receptors in AD is very limited. $P2X_7$ receptors mediate superoxide production in primary microglia and are up-regulated in a transgenic mouse model of Alzheimer's disease (Parvathenani *et al.*, 2003). Chronic neuroinflammation is a contributor to the pathophysiology of Alzheimer's disease (AD; Eikelenboom *et al.*, 2000; Hauss-Wegrzyniak *et al.*, 1998). Oxidative stress and oligomeric β -amyloid ($A\beta$) peptide production in AD brain can increase extracellular levels of cytokines and nucleotides that activate receptors in glial cells (*i.e.*, astrocytes and microglia) to stimulate intracellular signaling pathways and promote reactive gliosis, a universal response of the brain to injury (Mehlhorn *et al.*, 2000). In the CNS, we reported that $P2Y_2$ receptors mediate astrocyte migration through interactions with α_v integrins in rat primary cortical astrocytes, which suggests a role for $P2Y_2$ R-mediated signaling in

regulating astrogliosis in brain disorders (Wang et al., 2005). Moreover, previous studies by our group showed that UTP enhances α - and γ -secretase-mediated APP processing in P2Y₂R-transfected 1321N1 astrocytoma cells (Camden *et al.*, 2005). However, relatively little is known about the contribution of P2Y₂Rs to neuronal functions or their co-regulation with cytokine receptors of activities related to AD. Therefore, we have investigated the effect of cytokines on functional P2Y₂R expression in primary cortical neurons from E18 rat embryos, as described in Chapter 3. The role of P2Y₂ receptors in APP processing and the signaling molecules involved was also investigated in rat primary cortical neurons (see Chapter 3).

P2 nucleotide receptors in brain ischemia

Acute ischemic stroke is the leading cause of adult disability and also the most frequent pathogenic mechanism of brain lesion in infancy (Guzzetta *et al.*, 2000). Interestingly, basic and clinical evidence shows that impaired cerebral perfusion originating in the microvasculature triggered most of the key pathologic events associated with Alzheimer's disease (AD); thus the presence of a condition that lowers cerebral perfusion together with advanced aging are proposed as two factors necessary to induce a neurodegenerative process in the AD brain (de la Torre, 2000).

The cerebral circulation becomes inadequate under the following conditions that often lead to global ischemia, rather than focal ischemia: 1) the perfusion pressure is reduced; 2) the vascular resistance becomes too high; or 3) the oxygen or glucose supply is reduced (Garcia, 1975). Transient global ischemia caused by a 5-minute occlusion of gerbil bilateral carotid arteries (BCAOs) leads to selective and delayed

neuronal death (DND) of CA1 pyramidal neurons (Figure 3; Kirino, 1982), associated with activation of surrounding astrocytes in the hippocampus (DeLeo *et al.*, 1987). Transient focal ischemia for 60 min consistently resulted in a large infarct confined to the right MCA cortex in rats (Figure 3C). However, ischemic stroke cannot be mimicked satisfactorily *in vitro* due to its complex pathophysiology involving the interplay of many different cells and tissues including neurons, glia, endothelium, and the immune system. Thus, several animal models of global and focal brain ischemia have been developed for stroke research including the gerbil/mouse model of transient global ischemia by occlusion of both common carotid arteries (CCAs) and the rat/mouse model of focal ischemia model by middle cerebral artery occlusion (MCAO) (Traystman, 2003).

After transient global ischemia, up-regulation of P2X₂ and P2X₄ receptors was observed in neurons and microglia of gerbil hippocampus, respectively (Table 1; Cavaliere *et al.*, 2003). P2X₇ mRNA was also up-regulated in cultured cerebellar granule neurons of organotypic hippocampal cultures *in vitro* (Cavaliere *et al.*, 2002, 2004) and in activated microglia surrounding necrotic regions *in vivo* (Table 1; Collo *et al.*, 1997), suggesting a role for P2X₇Rs in the mechanisms of cellular damage induced by hypoxia/ischemia. Up-regulation of the P2Y₂R subtype also has been found with stress and injury (Seye *et al.*, 2002; Koshiba *et al.*, 1997 and Turner *et al.*, 1997). For example, P2Y₂R up-regulation is induced by a collar placed around a rabbit carotid artery (Seye *et al.*, 2002) and arterial P2Y₂R up-regulation is associated with enhanced neointimal hyperplasia, macrophage infiltration and osteopontin

expression in response to UTP (Seye *et al.*, 1997, 2003, 2004 & 2006). These findings indicate for the first time a role for the P2Y₂R, in development of intimal hyperplasia associated with atherosclerosis and restenosis (Seye *et al.*, 2002; Appendix-1). In Chapter 4, the expression of P2Y₂ receptors was identified in normal adult rodent brains and the relationship of P2Y₂ receptor expression to ischemia-induced injury was determined in a gerbil transient ischemia model (Weisman *et al.*, 2005; Chapter 4).

Outline of the dissertation

This dissertation contains five parts concerning the *Regulation and Function of P2Y₂ and P2X₇ Nucleotide Receptors in the Central Nervous System* from (*i.e.*, Chapters 2-4 and the Appendix).

In Chapter 2, we examined P2X₇R-mediated apoptosis in rat primary cortical neurons (rPCNs). P2X₇Rs were functionally expressed in the rPCNs and their activation by extracellular nucleotides was shown to cause apoptotic cell death through an intrinsic JNK1- and ERK-dependent caspase-8/9/3 activation pathway.

Chapter 3 extends the examination of nucleotide receptors in rPCNs to the P2Y₂ receptor subtype. P2Y₂R mRNA and receptor activity were virtually absent in unstimulated rPCNs but was dramatically up-regulated upon overnight treatment with IL-1 β . Furthermore, activation of P2Y₂R in rPCNs enhanced the release of sAPP α , a putative neuroprotective secretory amyloid protein, by an ADAM10/17- and PI3K/ERK-dependent mechanism.

In Chapter 4, the expression pattern of P2Y₂ receptor was studied in adult rodent

brain slices. Moreover, the effect of ischemia on the expression level of P2Y₂Rs in the rodent brain was analyzed by *in situ* hybridization and RT-PCR in a gerbil transient global ischemia model.

This dissertation concludes with Chapter 5, which addresses future directions and the scientific significance of the completed research followed by an Appendix that presents data from other projects undertaken.

Table 1. Regulation of P2 nucleotide receptor expression after ischemia and in Alzheimer's Disease (AD)

P2R	Injury	Cell type	Change
P2X₂	Ischemia	Neurons (hippocampus, organotypic culture, gerbil)	↑
P2X₄	Ischemia	Microglia (hippocampus, organotypic culture, gerbil)	↑
P2X₇	Ischemia	Microglia (cortex, rat)	↑
	Ischemia	Neurons (hippocampus, organotypic culture)	↑
	Ischemia	Astrocytes, neurons, microglia (cortex, rat)	↑
	AD	Around plaques (hippocampus, Tg2576 mice)	↑
P2Y₁	AD	Neurofibrillary tangles, neuritic plaques, neuropil threads (human hippocampus) Cellular distribution pattern was altered in AD brains.	↓

(Collo *et al.*, 1997; Moore *et al.*, 2000; Cavaliere *et al.*, 2003 & 2004; Parvathani *et al.*, 2003 ; Franke *et al.*, 2004 ; modified from review in Franke and Illes, 2006b)

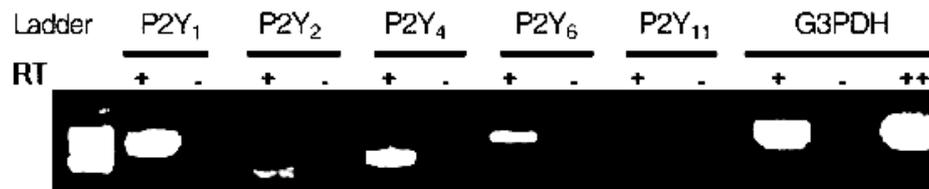
Figure 1. Expression of P2 receptors in rat cortical astrocytes and neurons. Total RNA was isolated from primary rat cortical (A) astrocytes or (B, C) neurons and RT-PCR was used to amplify mRNAs to P2Y and P2X receptors, as previously described (Weisman *et al.*, 2005; Kong *et al.*, 2005). The amplified PCR products were resolved by gel electrophoresis, and data shown are representative of results from three independent experiments performed 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).

A

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



B



C

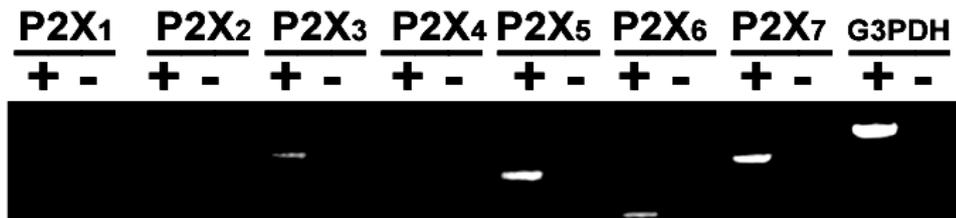
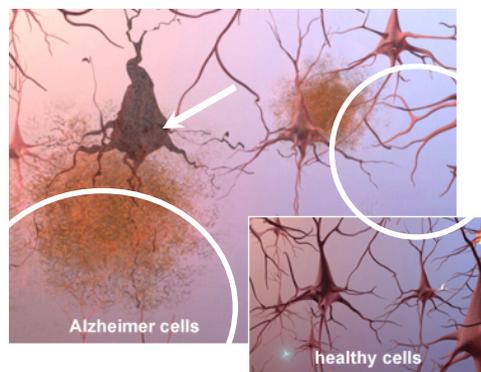
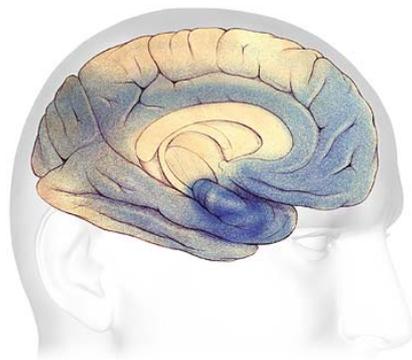


Figure 2. Nerve cells, synapses and brain tissue in Alzheimer's disease. A. Alzheimer's disease tissue has fewer nerve cells and synapses than healthy brain tissue and there are abnormal clusters of phosphorylated tau protein in dead and dying neurons (white arrow) surrounded with A β plaques (white circles) in the extracellular space. B. In advanced Alzheimer's disease, most of the cortex is seriously damaged. The brain shrinks dramatically due to widespread cell death. Pictures are cited from: www.alz.org.

A

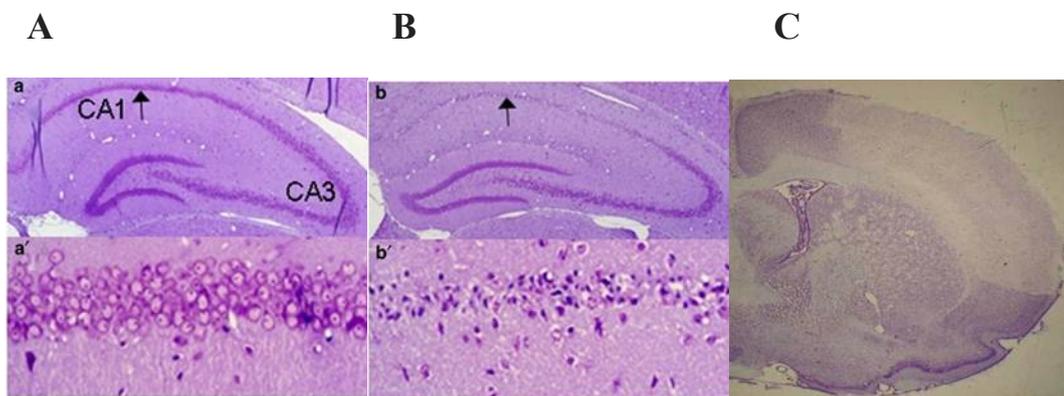


B



(Cited from www.alz.org)

Figure 3. Neuronal cell death in rodent ischemia models. A, B: transient gerbil global cerebral ischemia, Toshiki *et al.*, 2005; and C: rat focal cerebral ischemia, web.psych.ualberta.ca/~fcolbour/research.html. Neuronal densities in the CA1 region of the gerbil hippocampus (B) dramatically decreased 5 days after transient cerebral ischemia compared with the sham-operated gerbil brain (A). Arrows show location of changes. Focal cerebral ischemia–reperfusion injury induced by temporary middle cerebral artery occlusion (MCAO) for 60 min followed by reperfusion causes a large infarct (C; top of right cortex) compared with the sham-operated right MCA cortex.



(A-B: Toshiki *et al.*, 2005; C: web.psych.ualberta.ca/~fcolbour/research.html)

CHAPTER 2

P2X₇ NUCLEOTIDE RECEPTORS MEDIATE CASPASE-8/9/3-DEPENDENT

APOPTOSIS IN RAT PRIMARY CORTICAL NEURONS

Kong, Q. *et al.*, (2005) *Purinergic Signalling* 1:337-347

ABSTRACT

Apoptosis is a major cause of cell death in the nervous system. It plays a role in embryonic and early postnatal brain development and contributes to the pathology of neurodegenerative diseases. Here, we report that activation of the P2X₇ nucleotide receptor (P2X₇R) in rat primary cortical neurons (rPCNs) causes biochemical (*i.e.*, caspase activation) and morphological (*i.e.*, nuclear condensation and DNA fragmentation) changes characteristic of apoptotic cell death. Caspase-3 activation and DNA fragmentation in rPCNs induced by the P2X₇R agonist BzATP were inhibited by the P2X₇R antagonist oxidized ATP (oATP) or by pretreatment of cells with P2X₇R antisense oligonucleotide indicating a direct involvement of the P2X₇R in nucleotide-induced neuronal cell death. Moreover, Z-DEVD-FMK, a specific and irreversible cell permeable inhibitor of caspase-3, prevented BzATP-induced apoptosis in rPCNs. In addition, a specific caspase-8 inhibitor, Ac-IETD-CHO, significantly attenuated BzATP-induced caspase-9 and caspase-3 activation, suggesting that P2X₇R-mediated apoptosis in rPCNs occurs primarily through an intrinsic caspase-8/9/3 activation pathway. BzATP also induced the activation of JNK1 and ERK1/2 in rPCNs, and pharmacological inhibition of either JNK1 or ERK1/2 significantly reduced caspase activation by BzATP. Taken together, these data indicate that extracellular nucleotides mediate neuronal apoptosis through activation of P2X₇Rs and their downstream signaling pathways involving JNK1, ERK and caspases 8/9/3.

INTRODUCTION

ATP is an important extracellular messenger generated from various sources including release from presynaptic vesicles upon nerve stimulation in the peripheral and central nervous systems (Burnstock *et al.*, 1978). Extracellular ATP functions as a neuromodulator by activation of cell surface P2 nucleotide receptors that are widely expressed in the nervous system (Burnstock 1980; Inoue 2002). Based on their distinct structure, P2 nucleotide receptors are classified into two families: G protein-coupled P2Y receptors (P2YR_{1, 2, 4, 6, 11-14}; Abbracchio *et al.*, 2003) and ligand-gated ion channels (P2XR₁₋₇; Burnstock 2000). Functional responses to activation of these P2 receptor subtypes in cells of the nervous system under normal and pathological conditions include cell apoptosis, proliferation and migration, inflammation, ion transport, and neurotransmission (Burnstock 2000; Norenberg & Illes 2000; von Kugelgen & Wetter 2000; Khakh 2001 and Illes *et al.*, 2004). Therefore, P2 receptors in the nervous system may prove to be useful pharmaceutical targets in the treatment of neurological disorders.

The seven subtypes of the ionotropic ATP-gated P2XR family, ranging from 379 to 595 amino acids in length, regulate the intracellular calcium concentration through ligand-stimulated increases in the cell membrane permeability to extracellular Ca²⁺ ions (Benham 1990; Di Virgilio *et al.*, 2001a). In contrast to other P2XRs, the P2X₇R subtype functions exclusively as a homomeric receptor (Chessell *et al.*, 1998; North & Surprenant 2000). The P2X₇R is a 595-amino acid polypeptide containing two membrane-spanning domains, a large extracellular loop, and intracellular N- and

C-terminal domains including a C-terminal tail that is the longest among the cloned P2XRs (Surprenant *et al.*, 1996; Rassendren *et al.*, 1997). P2X₇Rs have been reported in microglial cells (Collo *et al.*, 1997) where they play a role in cytokine release (Ferrari *et al.*, 1997). These receptors are also found in cultured astrocytes and Schwann cells (Duan *et al.*, 2003; Grafe *et al.*, 1999), spinal cord neurons and in other regions of the central nervous system (Deuchars *et al.*, 2001; Atkinson *et al.*, 2004). Brief stimulation of the P2X₇R with ATP results in formation of a non-selective cationic channel that promotes the influx of Ca²⁺ and the equilibration of the transmembrane sodium and potassium gradients leading to membrane depolarization (Surprenant *et al.*, 1996; Rassendren *et al.*, 1997; Weisman *et al.*, 1984; Virginio *et al.*, 1999). Subsequent responses to P2X₇R activation include the formation of a nonselective pore for molecules up to 900 Da (Weisman *et al.*, 1984; Virginio *et al.*, 1999), extensive membrane blebbing, and eventual cell death (Virginio *et al.*, 1999). Activation of the P2X₇R was reported to elicit Ca²⁺ influx in cerebrocortical nerve terminals (Lundy *et al.*, 2002) and hippocampal glutamate release (Sperlagh *et al.*, 2002), which has been confirmed by studies with P2X₇R knockout mice (Papp *et al.*, 2004). In an *ex vivo* model of organotypic hippocampal cultures, P2X₇R activation was shown to directly participate in cell damage induced by oxygen/glucose deprivation (Cavaliere *et al.*, 2004). It has been shown that a Pro-451 to Leu polymorphism within the C-terminal tail of the P2X₇R interferes with the ability of the receptor to induce cell death in murine thymocytes (Le Stunff *et al.*, 2004). The P2X₇R also has been shown to mediate ATP-induced cell death in human embryonic

kidney cells (Wen *et al.*, 2003) and human cervical epithelial cells (Wang *et al.*, 2004). A recent study demonstrated that spinal cord injury was associated with prolonged P2X₇R activation of spinal cord neurons by extracellular ATP, which resulted in high-frequency spiking, increases in cytosolic calcium levels and neuronal degeneration, responses that were reversed by P2X₇R inhibition (Wang *et al.*, 2004).

In the present study, we demonstrate that the P2X₇R agonists BzATP or ATP induce nuclear condensation and DNA fragmentation in rat primary cortical neurons (rPCNs). These apoptotic responses in rPCNs were dependent on the functional expression of P2X₇Rs that mediate the ERK1/2- and JNK1-dependent activation of caspases-8/9/3.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM), Neurobasal Medium, penicillin (100 units/ml), streptomycin (100 units/ml) and B27-AO (B27 without cortex antioxidants) were obtained from Gibco-BRL (Carlsbad, CA, USA). Rabbit anti-rat ERK1/2, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and HRP-conjugated goat anti-mouse IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rat P2X₇R antibody was obtained from Alomone (Jerusalem, Israel). Rabbit anti-rat caspase-8 antibody was obtained from Biovision Inc. (Mountain View, CA,

USA). Mouse anti-NeuN antibody was obtained from Chemicon International Inc. (Temecula, CA, USA). Alexa Fluor™ 488 goat anti-mouse IgG antibody, Alexa Fluor™ 594 goat anti-rabbit IgG antibody, YO-PRO-1, TO-PRO-3 and the Prolong anti-fade kit were obtained from Molecular Probes Inc. (Eugene, OR, USA). All other antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Caspase-3 and caspase-9 inhibitors were obtained from R&D (Minneapolis, MN, USA). Caspase-8 inhibitor was obtained from BioSource International Inc. (Camarillo, CA, USA). The Precision Plus Protein Standards and nitrocellulose membranes (0.45 mm) were obtained from Bio-Rad (Hercules, CA, USA). LumiGLO chemiluminescent substrates were obtained from New England Biolabs (Beverly, MA, USA). The RNeasy Mini Kit was obtained from Qiagen (Chatsworth, CA, USA). The Apoptotic DNA-ladder kit, the First Strand cDNA Synthesis kit and the TUNEL In Situ Cell Death Detection kit were obtained from Roche (Indianapolis, IN, USA). Nucleotides and all other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Primary cell culture of cortical neurons

Experimental procedures for cell culture of rat primary cortical neurons (rPCNs) were carried out essentially as described by Suen *et al.* (Suen *et al.*, 2003). Briefly, cerebral cortices from 18-day-old embryos of Sprague-Dawley rats were removed and the meninges discarded. The brain tissue was mechanically dissociated in HGGMEM comprised of 1.78 g glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin, 7.5 µg/ml fungizone, 2 mM glutamine in 88 ml of MEM and 10% (v/v) horse serum. The tissue

clumps were dispersed with a 10 ml pipette and suspended in 6 ml of 0.25% (w/v) trypsin at 37 °C for 10 min. After incubation, 2 ml of heat-inactivated horse serum were added to block trypsin activity. Cells were centrifuged at 4000 g for 5 min and the pellet was suspended in HGGMEM with 10% horse serum. The cell suspension was filtered through a sterilized 75 mm cell strainer (nylon membrane; Becton Dickinson, Franklin Lakes, NJ) and cells were counted and seeded into plastic culture plates that were precoated with poly-D-lysine (0.1 µg/ml). After 16 h and every 3 days thereafter, the medium was replaced with B27-AO Neurobasal Medium (2 mM glutamine, 8.9 g glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin, 7.5 µg/ml fungizone, 10 ml of B27 minus AO, and Neurobasal Medium (Gibco-BRL) to 500 ml) and the neurons were ready for use after 7 days in culture.

RT-PCR analysis of P2XR mRNA expression

Total RNA was isolated from rPCNs using the RNeasy Mini Kit (Qiagen). cDNA was synthesized from the purified RNA using the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche). Five percent of the synthesized cDNA was used as a template in PCR reactions with the Expand High Fidelity PCR System (Roche). Specific oligonucleotide primers were designed to selectively amplify cDNA for individual P2XR subtypes, as shown in Table 2. The amplification was performed using 35 cycles of denaturation at 95 °C for 30 s, with annealing at 60 °C for 30 s and extension at 72 °C for 1 min. The resulting PCR products were resolved on a 1% (w/v) agarose gel containing 10 mg/ml ethidium bromide and photographed under UV illumination.

Single cell calcium assay

The $[Ca^{2+}]_i$ was quantified in single cells with the Ca^{2+} -sensitive fluorescent dye fura-2, using an InCyt Dual-Wavelength Fluorescence Imaging System (Intracellular Imaging, Cincinnati, OH). Rat PCNs cultured on a poly-D-lysine-coated coverslip were incubated with 2.5 μ M fura-2-acetoxymethylester at 37 °C for 30 min in physiological salt solution (PSS) containing (mM) NaCl 138, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7.4, and washed with PSS. The coverslip with fura-2-loaded cells was positioned on the stage of an inverted epifluorescence microscope (Nikon; model TMD) and stimulated with agonists at 37 °C, as described in the figure legends. Cells were exposed to 340/380 nm light and fluorescence emission at 505 nm was converted to $[Ca^{2+}]_i$ using a standard curve created with solutions containing known concentrations of Ca^{2+} . Increases in $[Ca^{2+}]_i$ were measured by subtracting basal $[Ca^{2+}]_i$ from the maximum agonist-induced increase in $[Ca^{2+}]_i$. The percentage of cells that responded to ATP/BzATP was also determined. Viable cells were identified by responsiveness to carbachol and non-responding cells were eliminated.

Detection of nuclear condensation

Rat PCNs were cultured in B27-AO Neurobasal medium on poly-D-lysine-coated coverslips in 12-well plates until 50-70% confluence. After nucleotide treatments, medium was removed and cells were washed once and incubated with DAPI-methanol staining solution (1 mg/ml) for 15 min at 37 °C. The staining solution was removed and the cells were washed with methanol. The inverted coverslip was

placed onto a microscope slide using glycerol as the mounting agent. Nuclear morphology was observed under a fluorescence microscope (Nikon, Eclipse TE300) with a 340/380 nm excitation filter and a LP 430 nm barrier filter. DAPI has an absorbance maximum at 340 nm and an emission maximum at 488 nm in aqueous solution (Kapus'cin'ski *et al.*, 1978).

DNA laddering assay

Rat PCNs were cultured in B27-AO Neurobasal medium in 100 mm dishes to 70% confluence, and incubated in serum-free medium overnight with or without BzATP, or treated for 2 h with 500 μ M H₂O₂ and DNA was isolated using the Apoptotic DNA-ladder kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. DNA also was isolated from lyophilized apoptotic U937 cell lysate (provided with the DNA-ladder assay kit) for use as a positive control. Briefly, the medium was replaced with 200 μ l of PBS and 200 μ l of binding buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, pH 4.4, 20% (v/v) Triton X-100). After 10 min at 15-25 °C, 100 ml of isopropanol were added and the samples were transferred to a filter tube and centrifuged for 1 min at 10,000 g (Spectrafuge Labnet, Edison, NJ). The fluid in the collection tube was discarded and the upper filter column was washed with 500 μ l of washing buffer (20 mM NaCl and 2 mM Tris-HCl, pH 7.5, 40% (v/v) ethanol) followed by centrifugation twice for 1 min at 13,000 g. After the residual washing buffer was removed, the DNA was eluted with 200 μ l of pre-warmed (70 °C) elution buffer (10 mM Tris, pH 8.5). The concentration and purity of the eluted DNA was determined by measuring the optical density at 260 and

280 nm. DNA was electrophoresed in a 2% (w/v) agarose gel at 80 V for 1 h in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTANa₂-salt, pH 8.3) and visualized under UV light.

TUNEL assay

After treatment with nucleotides or H₂O₂, rPCNs were fixed with fresh 4% (v/v) paraformaldehyde in PBS and treated with 3% (v/v) H₂O₂ in methanol to inhibit endogenous peroxidase activity. Then, DNA strand breaks in fixed cells were detected with a TUNEL *In Situ* Cell Death Detection kit (Roche) according to the manufacturer's instructions. The number of TUNEL-positive cells and the total cell number were determined by fluorescence (Nikon; Eclipse TE300) using an excitation wavelength in the range of 450-500 nm and a detection wavelength in the range of 515-565 nm (green) and light microscopy with cell counting, respectively, at 200x magnification.

Application of P2X₇ antisense oligonucleotide

Antisense oligonucleotide treatment was performed as previously described (Seye *et al.*, 2002). Phosphorothioate-modified oligonucleotides to rat P2X₇ were synthesized and purified by Integrated DNA Technologies (ADT, Coralville, IA, USA). Sequences were designed as follows: sense 5'-AGAGCGTGAATTACGGCA CATCAA-3', antisense 5'-TTGATGGTGCCGTAATTCACGCTCT-3'. Sequences were checked for uniqueness with the National Center for Biotechnology Information's Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/BLAST>). The rPCNs were incubated with 0.1 or 1 μmol/L P2X₇ sense or

antisense S-oligonucleotide for 6 h in serum-free medium containing 1.4% (v/v) DOTAP liposomal reagent (Roche Diagnostics). Fresh serum-free medium was added with or without BzATP, and cells were cultured for an additional 18 h.

Western blot analysis

Rat PCNs cultured in 6-well plates were treated with 300 μ M BzATP in the presence or absence of inhibitors as indicated. The cells were washed twice with ice-cold PBS, and lysed for 5 min in 200 μ l of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 2% (w/v) sodium dodecyl sulfate (SDS), and 1 mM sodium orthovanadate). Lysate (30 mg of protein) was added to Laemmli sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% (w/v) SDS, 1.8% (v/v) β -mercaptoethanol and 0.003% (w/v) bromophenol blue). Samples were heated for 5 min at 96-100 $^{\circ}$ C, and subjected to 7.5% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for protein immunoblotting. After overnight blocking at 4 $^{\circ}$ C with 5% (w/v) fat-free milk in TBS-T (10 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 0.1% (v/v) Tween-20), membranes were incubated with either 1:1000 dilution of anti-ERK1/2, anti-phospho-ERK1/2, anti-phospho-JNK, or anti-P2X₇R antibodies for 2 h at room temperature, or 1:1000 dilution of anti-cleaved caspase-3, anti-caspase-3, anti-cleaved caspase-9, anti-caspase-9, or anti-caspase-8 antibodies overnight at 4 $^{\circ}$ C followed by incubation with HRP-conjugated anti-rabbit or anti-mouse IgG antibodies (1:1000 dilution in TBS-T containing 5% (w/v) fat-free milk) for 1 h at room temperature. For normalization of protein loading, the membranes were stripped of antibodies by incubation for 30 min at 60 $^{\circ}$ C in stripping buffer (62.5 mM Tris-HCl,

pH 6.8, 100 mM 2-mercaptoethanol, and 2% (w/v) SDS), and re-probed with 1:2000 dilution of rabbit polyclonal anti-actin antibody (2 h at room temperature; Cytoskeleton, Denver, CO) followed by 1:2000 dilution of HRP-conjugated anti-rabbit IgG antibody (1 h at room temperature). Protein immunoreactivity was visualized on autoradiographic film using the LumiGlo Chemiluminescence System (New England BioLabs), according to the manufacturer's instructions. The protein bands detected on X-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad, Hercules, CA). The activation levels of kinases or caspases were calculated as a percentage of control (*i.e.*, total kinase, caspase, or actin).

Immunofluorescence

After fixation and permeabilization, rPCNs cultured on coverslips in 6-well plates were incubated in 2.5% (w/v) BSA and 2.5% (v/v) goat serum in PBS for 1 h at room temperature followed by incubation overnight with mouse anti-NeuN antibody (1:100 dilution; Chemicon) and rabbit anti-cleaved caspase-3 antibody (1:100 dilution; Cell Signaling) in 0.25% BSA and 0.25% goat serum in PBS. Cells were washed three times with PBS and then incubated with 1:200 dilution of goat anti-mouse Alexa Fluor 488- or goat anti-rabbit Alexa Fluor 594-conjugated IgG antibody (Molecular Probes, Eugene, OR) in 0.25% BSA and 0.25% goat serum in PBS for 1 h at 37 °C. Nuclei were stained with TO-PRO-3 (Molecular Probes), cells were rinsed with PBS and mounted on glass slides in ProLong antifade reagent (Molecular Probes). Images were obtained on a Bio-Rad MRC 1024 laser scanning confocal imaging system

coupled to an Olympus IX70 inverted microscope. The imaging system was controlled by Laser Sharp software (version 3.2; Bio-Rad, Hercules, CA). Alexa 488-labeled slices were excited by 488 nm laser light, and images were acquired with a 522 nm emission filter with a bandwidth of 35 nm, whereas Alexa 594-labeled slices were excited by 568 nm light and images acquired with a 585 nm longpass emission filter. Settings of the confocal microscope were optimized for imaging in an initial experiment and then equivalently applied to all sections. Laser power and photomultiplier tube (PMT) gain were adjusted to ensure that even the brightest fluorescence was below saturation levels. Neurons were randomly chosen and scanned at 512 x 512 pixel resolution.

Statistical analysis

Results are expressed as the means \pm S.E.M. Statistical analysis of data was performed using Graph Pad Prism version 4.0. Statistical significance was determined by student t-test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Purity of rat cortical neurons in primary culture

The purity of rPCNs was determined by monitoring morphology with phase contrast microscopy and by immunofluorescence assay with the neuron-specific marker NeuN and the nuclear stain, TO-PRO-3 (Figure 1A). NeuN staining was found primarily in the nucleus of the neurons with lighter staining in the cytoplasm. About 95% of the cells were determined to be cortical neurons based on the ratio of NeuN

positive cells to total cells counted by TO-PRO nuclear staining (data not shown).

Functional P2X₇Rs are expressed in rat PCNs

Rat PCNs express P2X₇R mRNA, as well as mRNAs for P2X₂, P2X₃, P2X₅, and P2X₆ receptors (Figure 1B). Furthermore, immunoblot analysis indicated that rPCNs express P2X₇R protein (Figure 1C). The P2X₇R agonists ATP and BzATP (Erb *et al.*, 1990) caused a dose-dependent increase in [Ca²⁺]_i in rPCNs (Figure 2), suggesting that calcium influx is stimulated by activation of the endogenously expressed P2X₇R protein. Calcium responses were also observed by stimulation of rPCNs with 2-methylthio-ADP (data not shown), an agonist of the P2Y₁R, indicating that other P2R subtypes besides the P2X₇R can regulate increases in [Ca²⁺]_i.

ATP and BzATP induce nuclear condensation and DNA fragmentation in rPCNs

Treatment of rPCNs with either BzATP (300 μM) or ATP (100 μM) overnight caused nuclear condensation as indicated by increased DAPI staining of nuclei (Figure 3). BzATP-induced nuclear condensation was inhibited by the P2X₇R antagonist oATP, consistent with the involvement of P2X₇Rs in this process. Moreover, BzATP treatment of rPCNs caused DNA fragmentation, suggestive of apoptotic cell death (Figure 4). BzATP or ATP also caused DNA strand breakage in rPCNs, another indicator of apoptosis, detected as an increase in the number of TUNEL-positive cells (Figure 5). These data suggest that BzATP or ATP can cause apoptosis in rPCNs through a pathway involving P2X₇R activation.

P2X₇R-mediated caspase-3 and caspase-9 activation

Caspase-3 is an essential component of apoptotic pathways in many cell types, including neurons (Salvesen and Dixit, 1997), and is activated by the sequential release of cytochrome c from mitochondria and the activation of caspase-9 (Li *et al.*, 1997). Consistent with a role for caspase-3 in nucleotide-induced neuronal apoptosis, nuclear condensation caused by BzATP was diminished by treatment of rPCNs with the caspase-3 inhibitor Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-FMK (Z-DEVD-FMK; Figure 3C). Results also indicated that treatment of rPCNs with either ATP or BzATP significantly increased the cleavage of caspase-3 in a time- and dose-dependent manner (Figure 6A). BzATP-induced caspase-3 cleavage was inhibited by oATP (Figure 6B), the caspase-3 inhibitor Z-DEVD-FMK (Figure 6B), or P2X₇ antisense oligonucleotide (Figure 6C). Although recent studies indicate that oATP might act independently from P2 receptor inhibition (Beigi *et al.*, 2003), inhibition of BzATP-induced caspase-3 cleavage by P2X₇R antisense oligonucleotide confirms the involvement of the P2X₇R. Since no preparation of primary neurons is devoid of glial cells, dual immunofluorescence analysis for neuron-specific NeuN and cleaved caspase-3 was performed to evaluate whether the apoptotic cells were neurons. The majority of cells treated with BzATP were positive for cleaved caspase-3 and NeuN (*e.g.*, pink and white arrows in Figure 6D), indicating that a large percentage of cells undergoing apoptosis were neurons. NeuN immunoreactivity has been reported to decrease under several pathological conditions (Unal-Cevik *et al.*, 2004), and therefore NeuN detection may decrease with caspase-3 activation in apoptotic neurons, suggesting that some apoptotic neurons may go undetected. BzATP also caused the

cleavage of caspase-9 (Figure 7A), whereas activation of caspase-9 and caspase-3 were significantly inhibited by the caspase-8 inhibitor Ac-IETD-CHO (Figure 7B) or the caspase-9 inhibitor Z-LEHD-FMK (data not shown), indicating that caspase-8 also plays a role in activating downstream caspase family members (e.g., caspase-9/3) of the P2X₇R-mediated apoptosis pathway. We also detected an inhibitory effect of the caspase-9 inhibitor, Z-LEHD-FMK, on caspase-8 cleavage (Figure 7C) suggesting that caspase-8 and caspase-9 may regulate caspase-3 activity in a non-linear fashion.

Caspase-3 activation by BzATP in rPCNs is dependent upon ERK1/2 and JNK1 phosphorylation

P2X₇R-mediated activation of extracellular signal-regulated kinases (ERK1/2) in rat primary astrocytes (Panenka *et al.*, 2001) and SAPK/JNK in human and rodent macrophages occur independently of the activation of caspase-1- or caspase-3-like proteases (Humphreys *et al.*, 2000). Here, we tested whether ERK1/2 or JNK1 activities were involved in BzATP-induced apoptosis in rPCNs. As indicated in Figure 8, both ERK1/2 and JNK1 were rapidly phosphorylated in response to BzATP or ATP in a dose- and time-dependent manner. Inhibition of ERK1/2 or JNK1 phosphorylation in rPCNs with either the MEK inhibitor UO126 or the JNK inhibitor SP600125, respectively, attenuated caspase-3 activation, indicating the involvement of ERK1/2 and JNK1 in BzATP-induced neuronal cell death (Figure 9).

DISCUSSION

The results obtained with rat PCNs indicate that activation of the P2X₇R by its

agonists BzATP or ATP promotes the cleavage of caspases-8/9/3 and the appearance of cellular markers indicative of apoptosis including DNA degradation and nuclear condensation and fragmentation. Since BzATP can activate other P2X receptor subtypes, albeit at higher concentrations than the P2X₇R (Bianchi *et al.*, 1999), we explored the role of the P2X₇R in the apoptotic effects of BzATP in rPCNs using P2X₇ antisense oligonucleotide to downregulate P2X₇R expression and the P2X₇R antagonist oATP. Results indicate that inhibition of P2X₇R activity significantly inhibited BzATP-induced nuclear condensation (by P2X₇R antagonist oATP; Figure 3) and caspase-3 cleavage (by both oATP and P2X₇R antisense oligonucleotide; Figure 6), apoptotic responses that were diminished by incubation of rPCNs with the caspase-3 inhibitor, Z-DEVD-FMK (Figures 3C and 6B). These data strongly suggest that P2X₇Rs mediate BzATP-induced apoptosis in rPCNs, although this does not rule out a potential role for other P2 nucleotide receptors.

P2X₇R-mediated neuronal apoptosis *in vivo* requires that ATP be released from neurons or non-neuronal cells to activate cell surface receptors on neighboring neurons. Neuronal P2X₇ receptors are targeted to presynaptic terminals in the central and peripheral nervous systems and activation of the P2X₇R has been indicated to promote release of vesicular contents from presynaptic terminals (Deuchars *et al.*, 2001). ATP is present at high concentrations in synaptic vesicles and is released as a co-transmitter with noradrenaline, acetylcholine and other neurotransmitters (Burnstock *et al.*, 1978). ATP release also has been demonstrated in a model of spinal cord injury (Sim *et al.*, 2004). Thus, ATP release from excited neurons or damaged

cells is an assumed consequence of any brain injury or disease (*e.g.*, ischemia, spinal cord injury, or neurodegenerative diseases), suggesting that pro-apoptotic P2X₇Rs are likely activated under pathological conditions *in vivo*. Recent research indicates a lack of P2X₇R expression in adult rat hippocampal neurons (Khera *et al.*, 2004), whereas our studies demonstrate functional P2X₇R expression in rPCNs from 18-day old embryos. We have detected P2X₇R mRNA in freshly isolated rPCNs (data not shown) and 7-10 day-old cultures (Figure 1), suggesting that the *in vitro* cell culture procedure alone was not required for P2X₇R expression.

Caspase activation is known to play a central role in the execution of apoptosis by causing nuclear condensation, DNA fragmentation and other apoptotic changes. In the mitochondrial-initiated pathway of apoptosis, caspase activation is triggered by the formation of a multimeric Apaf-1/cytochrome c complex that is fully functional in recruiting and activating procaspase-9 (Li *et al.*, 1997). Activated caspase-9 will then cleave and activate downstream caspases such as caspase-3, -6, and -7. In rPCNs, it was not surprising that activation of caspase-3 was significantly inhibited by the caspase-9 inhibitor Z-LEHD-FMK (data not shown), since caspase-9 is known to activate caspase-3. We also found that in rat PCNs, the caspase-8 inhibitor Ac-IETD-CHO partially inhibited caspase-9 and caspase-3 activation (Figure 7B) indicating that caspase-8 cleavage may be an early event in this neuronal apoptosis pathway. Caspase-8 has been reported to play a role in cytochrome c-dependent apoptosis (Viswanath *et al.*, 2001) and caspase-8 activation is associated with Fas receptor-induced apoptosis (Zhuang *et al.*, 1999) and can result in the cleavage and

activation of Bid, a proapoptotic member of the Bcl-2 family (Li *et al.*, 1998). Truncated Bid translocates from the cytoplasm to the mitochondria, where it appears to antagonize the actions of anti-apoptotic Bcl-2, thereby causing an efflux of cytochrome c from the mitochondria and downstream caspase-9/3 activation (Li *et al.*, 1998; Kuwana *et al.*, 1998; Luo *et al.*, 1998; Schendel *et al.*, 1999; Wei *et al.*, 2001). In human embryonic kidney HEK-293 cells expressing P2X₇Rs, extracellular ATP induced apoptosis associated with activation of poly (ADP-ribose) polymerase (PARP) and a decrease in Bax protein expression (Wen *et al.*, 2003). We examined the effect of ATP/BzATP on the expression of Bcl-2 family members in rPCNs but failed to detect a significant change in Bax and Bcl-2 protein expression (data not shown). Evidence from cell-free and *in vitro* expression systems has indicated that caspase-3 can induce cleavage and activation of the upstream initiator caspase-8 (Slee *et al.*, 1999; Wolf and Green, 1999; Tang *et al.*, 2000). Our data indicated that inhibition of caspase-9 cleavage decreased caspase-8 cleavage (Figure 7C), consistent with the ability of downstream caspases to regulate the cleavage of initiator caspases.

We have demonstrated that activation of P2X₇Rs in rPCNs increases calcium influx (Figure 2) and stimulates phosphorylation of the intracellular kinases ERK1/2 and JNK1 (Figure 8). Furthermore, inhibition of ERK1/2 or JNK1 phosphorylation decreased BzATP-induced caspase-3 cleavage (Figure 9). Our previous studies demonstrating that P2X₇R-mediated ERK1/2 activation was dependent on extracellular Ca²⁺ (Gendron *et al.*, 2003) suggest a similar role for BzATP-stimulated calcium influx in rPCNs (Figure 2). Recent studies indicate that activation of ERK

and phosphatidylinositol 3-kinase/Akt signaling pathways caused inhibition of caspase-3 and cell apoptosis (Khreiss *et al.*, 2002). It also has been reported that inhibition of MEK/ERK signaling is required for the induction of apoptosis by Sulindac metabolites (Rice *et al.*, 2004). Nonetheless, our data support the hypothesis that ERK1/2 and JNK1 activation can promote P2X₇R-mediated apoptosis in rPCNs, although the mechanism involved requires further investigation. P2X₇Rs can promote neuronal cell death by mediating the release of glutamate and inflammatory factors from cortical neurons, RAW 264.7 macrophages, and astrocytes (Duan *et al.*, 2003; Hu *et al.*, 1998; Guerra *et al.*, 2003). Moreover, glutamate can induce ATP release from astrocytes (Queiroz *et al.*, 1999), suggesting that ATP and glutamate have reciprocal roles that contribute to the propagation of an apoptotic signal. It also has been reported that the P2X₇R modulates macrophage production of TNF- α , IL-1 β and nitric oxide (NO) following LPS exposure (Hu *et al.*, 1998), consistent with a role for the P2X₇R in inflammation (Di Virgilio *et al.*, 2001b). In addition to these factors, structural features within the P2X₇R indicate that the mechanism whereby it regulates apoptosis is likely to be complex. There are several functional domains located in the P2X₇R C-terminal tail including an LPS-binding domain, a SH3-binding domain, and an overlapping Fcelldeath domain, which appear to be important for receptor trafficking and various physiological functions (Denlinger *et al.*, 2001), and the role of these domains in P2X₇R-mediated apoptosis of neurons warrants further investigation. Our studies with the membrane impermeant fluorescent dye YO-PRO-1 (molecular weight: 375 Da) indicate that BzATP causes pore formation (YO-PRO-1

uptake) in rPCNs (data not shown), consistent with the report that apoptosis in the retinal microvasculature is associated with P2X₇ receptor-mediated pore formation (Sugiyama *et al.*, 2004).

A recent study indicated that spinal cord neurons expressed an abundance of P2X₇Rs, and exposure of freshly prepared spinal cord slices to ATP or BzATP evoked high frequency firing and irreversible increases in the cytosolic Ca²⁺ concentration (Wang *et al.*, 2004). Significant cell death occurred 24 h after spinal cord injury in areas of high ATP release surrounding the epicenter of the injury, while local injection of oATP in the peritraumatic zone strongly reduced both apoptotic cell number and the severity of histological injury, suggesting the involvement of a P2X₇R. Other studies indicate that the P2X₇R is up-regulated in a mouse model of Alzheimer's disease (Parvathenani *et al.*, 2003). Thus, the P2X₇R may represent a promising therapeutic target to inhibit neurodegeneration due to injury or disease.

In summary, the data presented indicate that BzATP and ATP can stimulate P2X₇Rs in embryonic rat primary cortical neurons leading to the activation of the caspases-8/9/3 and the induction of DNA degradation and nuclear condensation and fragmentation, apoptotic responses that appear to be regulated by ERK1/2 and JNK1. Since neuronal apoptosis occurs in Alzheimer's disease, Parkinson's disease and other neurodegenerative disorders, a better understanding of the mechanisms of P2X₇R-mediated apoptosis may lead to novel therapies to prevent apoptosis-related brain injuries.

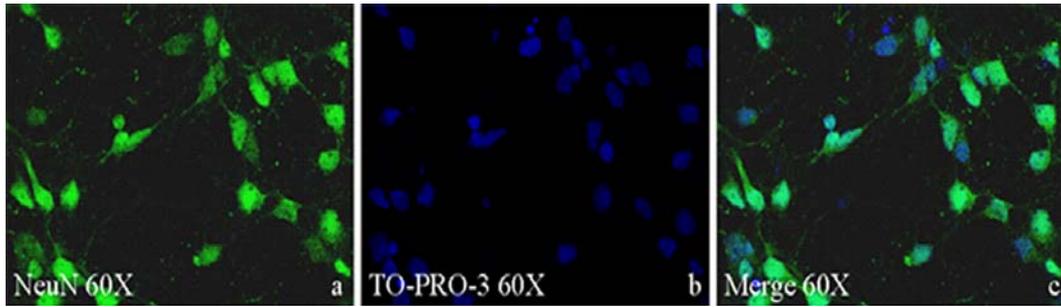
Table 2. Sequence-specific oligonucleotide primers used for RT-PCR studies.

Receptor Subtypes		Oligonucleotide Sequences
P2X ₁	Forward	5'-AAC AGC ATC AGC TTT CCA CG-3'
	Reverse	5'-TGT AGT AGT GCC TCT TAG GC-3'
P2X ₂	Forward	5'-ACC TGC CTC TCC GAC GCC GA-3'
	Reverse	5'-GAA GTC AGA GCT GTG GCC AG-3'
P2X ₃	Forward	5'-CAA CTT CAG GTT TGC CAA A-3'
	Reverse	5'-TGA ACA GTG AGG GCC TAG AT-3'
P2X ₄	Forward	5'-TAC GAC ACG CCG CGC ATC-3'
	Reverse	5'-TGC ACG ATT TGA GGT AGG ACG-3'
P2X ₅	Forward	5'-CAA AGT CCA TGC CAA CGG AT-3'
	Reverse	5'-ACG GAA CTC TAC CCC ATT AG-3'
P2X ₆	Forward	5'-GTA GTG CTG TGC CCA GGA AA-3'
	Reverse	5'-GGA CTC CAC GCC TGA GGC TG-3'
P2X ₇	Forward	5'-ACA ATG TTG AGA AAC GGA CTC TGA-3'
	Reverse	5'-CCG GCT GTT GGT GGA ATC CAC ATC-3'
G3PDH	Forward	5'-TGA AGG TCG GTG TCA ACG GAT TTG GC-3'
	Reverse	5'-CAT GTA GGC CAT GAG GTC CAC CAC-3'

Figure 1. Purity of rat primary cortical neurons (rPCNs) and P2X₇R expression.

(A) Rat PCNs were cultured on coverslips in 6-well plates and neurons were labeled with mouse NeuN monoclonal antibody (Panel a) and cell nuclei with TO-PRO-3 (Panel b); merged picture shown in Panel c. (B) RNA was isolated from rPCNs maintained in culture for 7-10 days and RT-PCR was used to amplify mRNAs to P2X₁₋₇Rs, as described in the “Materials and Methods”. The amplified PCR products were resolved by gel electrophoresis, and data shown are representative of results from three independent experiments. Results are shown of PCRs performed in the presence (+) or absence (-) of reverse transcriptase (*RT*). G3PDH primers were used to amplify G3PDH mRNA as a positive control. (C) P2X₇R protein expression was detected by Western blot analysis, as described in the “Materials and Methods”. Human 1321N1 cells expressing the recombinant human P2X₇R or the empty expression vector pLXSN were used as positive (+) or negative (-) controls, respectively. Precision Plus Protein Standards are indicated as ‘75KD’ and ‘100KD’.

A



B



C

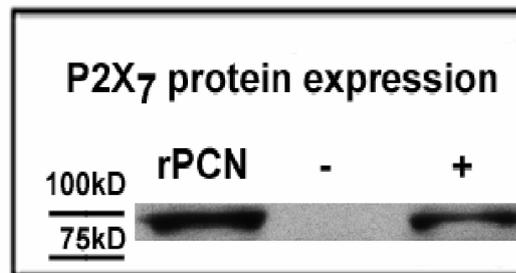
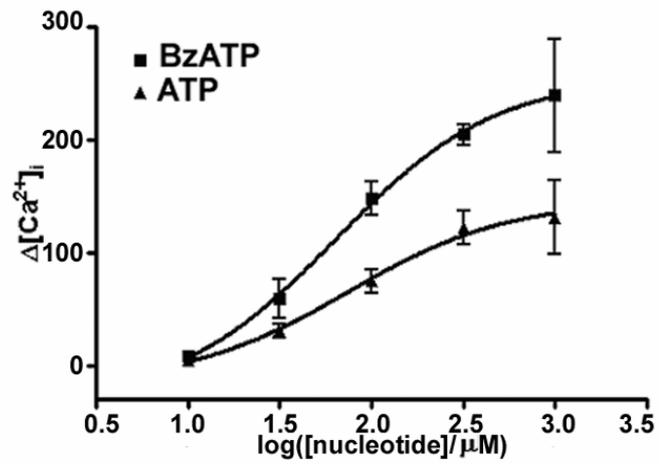


Figure 2. ATP- and BzATP-induced increases in $[Ca^{2+}]_i$ in rat PCNs. (A) Single cell calcium assays were performed on rPCNs that were cultured for 7-10 days on poly-D-lysine-coated coverslips. Cells were incubated in PSS with the indicated concentration of BzATP or ATP and the maximum increase in $[Ca^{2+}]_i$ was determined, as described in the “Materials and Methods”. (B) The percentage of cells responding to BzATP or ATP is shown as the mean \pm S.E.M. of results from three experiments.

A



B

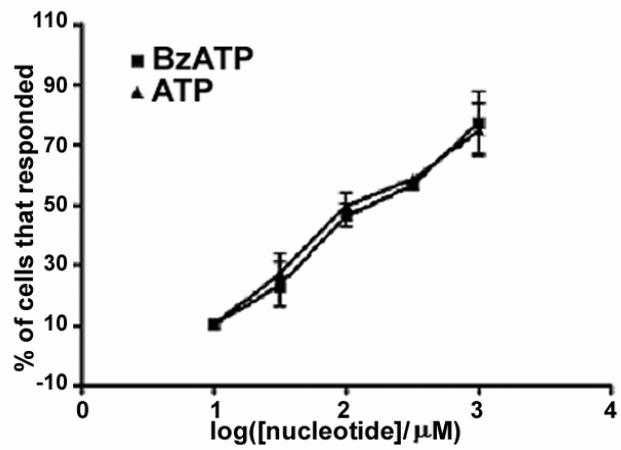
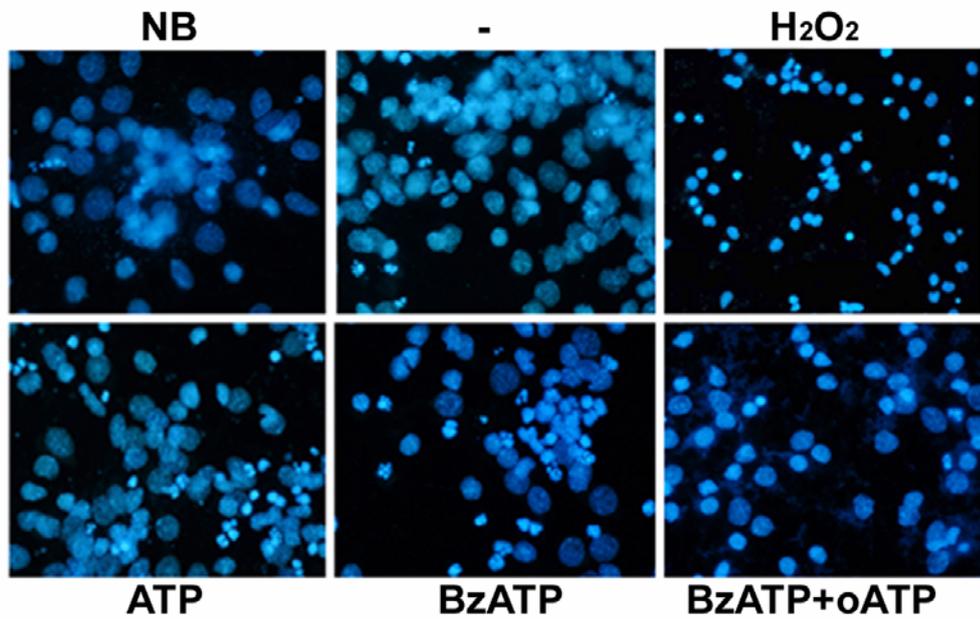
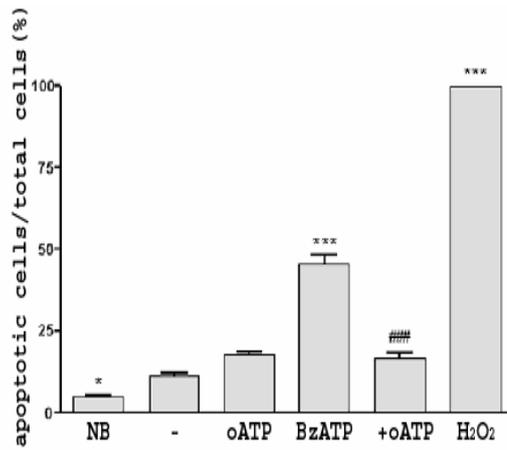


Figure 3. ATP/BzATP-induced nuclear condensation mediated by the P2X₇R is dependent on caspase-3 activation. (A) Rat PCNs were cultured for 7-10 days, incubated in serum-free HGGMEM for 6 h and stimulated with BzATP (300 μ M) or ATP (100 μ M) for 16 h or with H₂O₂ (1 mM) for 2 h. When indicated, 500 μ M oATP was added 2 h prior to addition of BzATP. Cells cultured in B27-AO Neurobasal medium (NB) or in serum-free HGGMEM (-) overnight were used as controls. Then, nuclear condensation was determined by DAPI staining and detected by fluorescence microscopy, as described in the “Materials and Methods”. (B) Cells were treated as in (A) except that the data were expressed as a percentage of cells that exhibited DAPI stained nuclei. Data are the means \pm S.E.M. of results from at least four experiments, where * p < 0.05, and *** p < 0.001 indicate significant differences from the serum-starved control (-), and where ### p < 0.001 indicates a significant difference from BzATP treatment. (C) rPCNs were treated as in (A) except that 10 μ M Z-DEVD-FMK, a caspase-3 inhibitor, was added for 1 h prior to BzATP, when indicated.

A



B



C

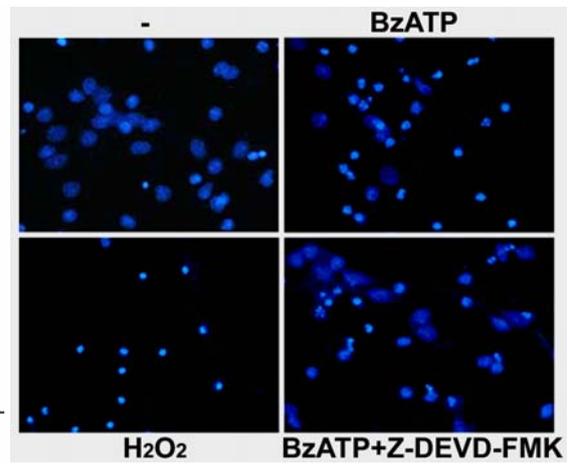


Figure 4. BzATP stimulates DNA fragmentation in rat PCNs. Rat PCNs were incubated in serum-free HGGMEM for 6 h and then stimulated with BzATP (300 μ M) for 16 h or H₂O₂ (1 mM) for 2 h. Cells cultured in serum-free HGGMEM (-) overnight were used as the control. Then, cells were lysed, total DNA was purified, the DNA concentration was determined, and 3 μ g of purified DNA was loaded onto each lane of a 2% (w/v) agarose gel and electrophoresed, as described in the “Materials and Methods”. DNA fragmentation was visualized after electrophoresis of DNA. Apoptotic U937 cells (+++; provided in the Apoptotic DNA-ladder kit) were used as a positive control and “M” indicates the 100 bp DNA ladder marker.

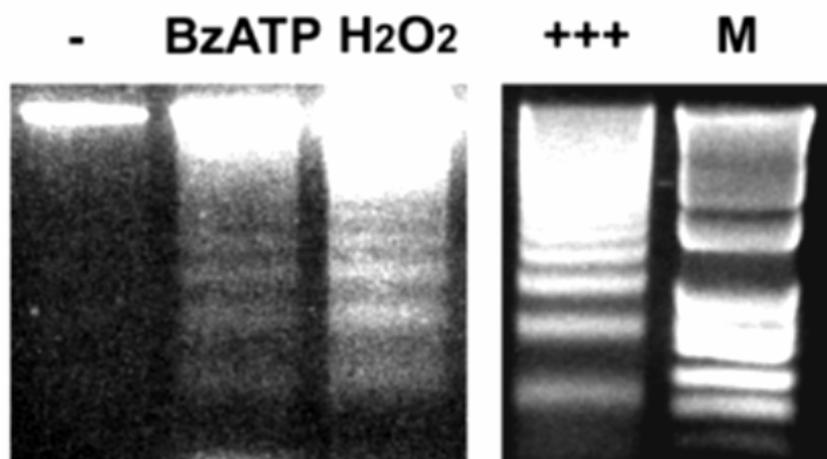


Figure 5. BzATP or ATP induces DNA strand breakage in rat PCNs. Rat PCNs were cultured for 7-10 days, incubated in serum-free HGGMEM for 6 h, and stimulated with BzATP (300 μ M) or ATP (100 μ M) for 16 h or with H₂O₂ for 2 h. Cells cultured in serum-free HGGMEM (-) overnight were used as the control. Then, cells were fixed and DNA strand breakage was determined by TUNEL assay, as described in the “Materials and Methods”. DNA strand breakage was observed as green fluorescence using fluorescence microscopy.

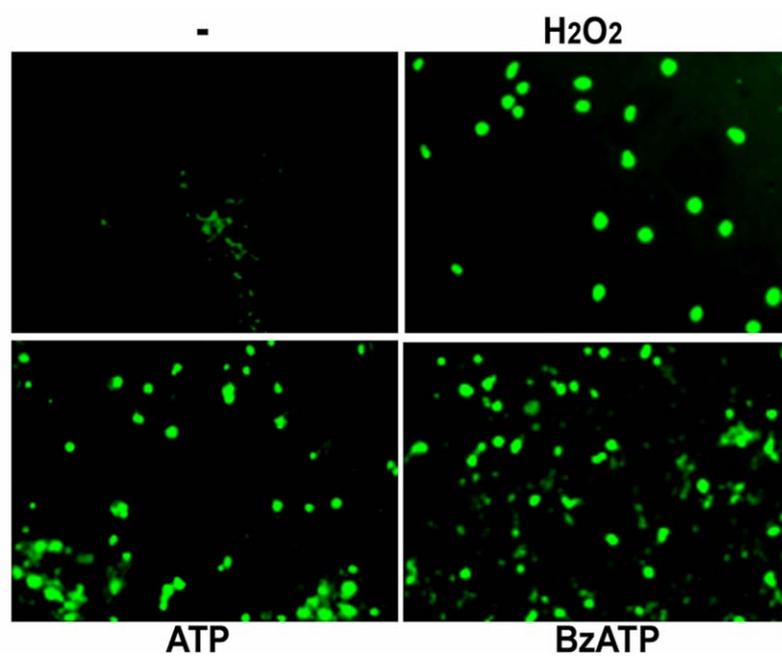
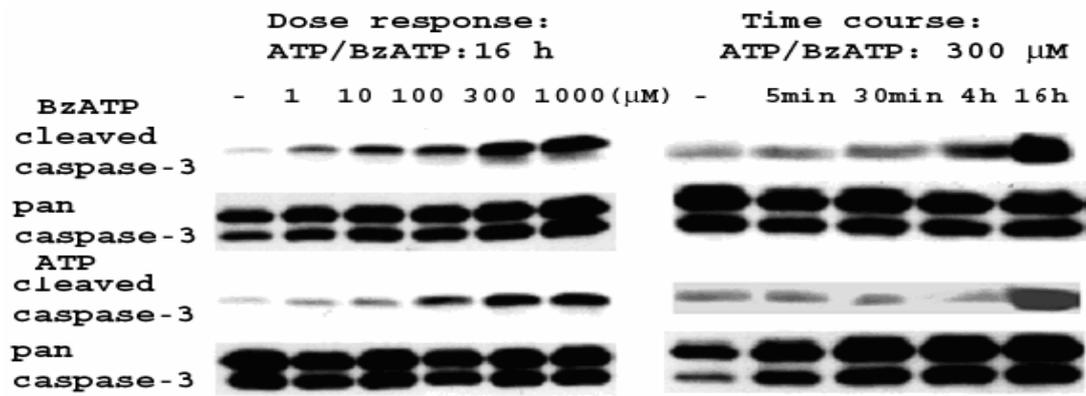
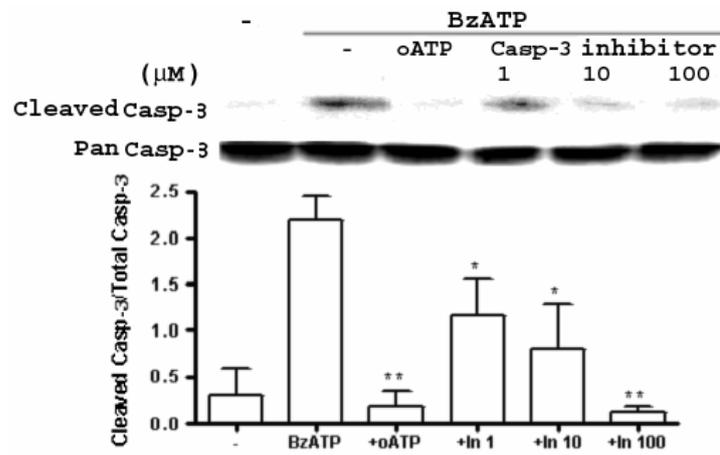


Figure 6. P2X₇Rs mediate caspase-3 activation in rat PCNs. (A) Rat PCNs were incubated in serum-free medium for 6 h and then stimulated with the indicated concentration of ATP or BzATP for 16 h or the time indicated. Cells cultured in B27-AO Neurobasal medium (NB) or in serum-free HGGMEM (-) overnight were used as controls. (B) Cells were treated as in (A) except that 500 μ M oATP was added for 2 h or the indicated concentration (1, 10 or 100 μ M) of Z-DEVD-FMK was added for 1 h prior to addition of 300 μ M BzATP for 16 h. (C) Cells were treated as in (A) except that P2X₇ antisense or sense oligonucleotide was added at the indicated concentration in μ g/ml for 8 h prior to addition of 300 μ M BzATP for 16 h or 1 mM H₂O₂ for 2 h. Western analysis was performed to determine the relative amounts of caspase-3, cleaved caspase-3 or P2X₇R protein. Data are the means \pm S.E.M. of results from at least three experiments, where * p < 0.05, ** p < 0.01, and *** p < 0.001 indicate significant differences from either serum-starved (A) or BzATP-treated (B, C) cells. (D) Rat PCNs were serum-starved overnight in the presence (Panels B, D and F) or absence (Panels A, C, and E) of 300 μ M BzATP and then immunofluorescence of the neuron-specific marker NeuN (green; Panels A, B), cleaved caspase-3 (red; Panels C and D), and the nuclear stain TO-PRO (blue; Panels E and F) were detected as described in the “Materials and Methods”. Arrows (pink or white) indicate apoptotic neurons that are both caspase-3 and NeuN positive, whereas yellow arrows indicate apoptosis in a NeuN-negative cell. Similar results were obtained in three individually isolated groups of primary cultures.

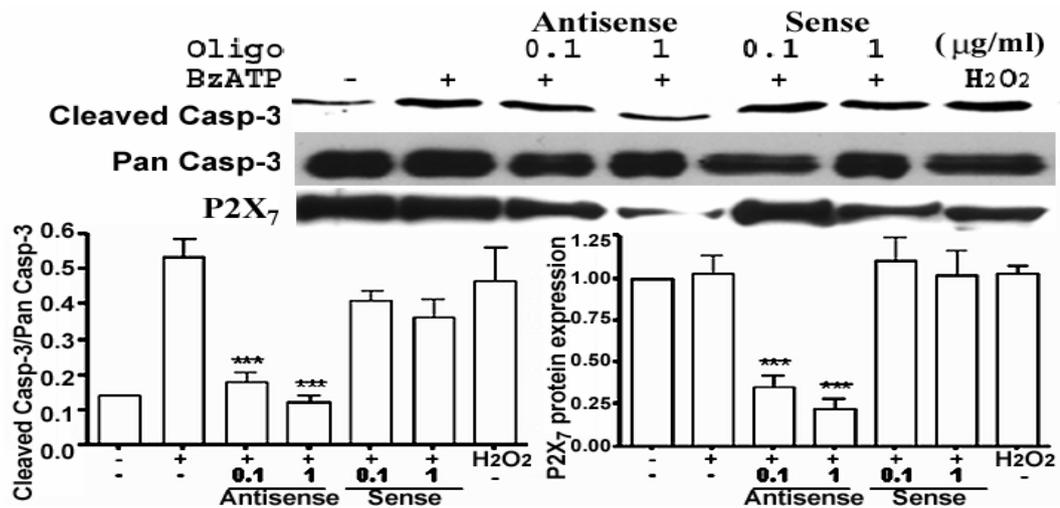
A



B



C



D

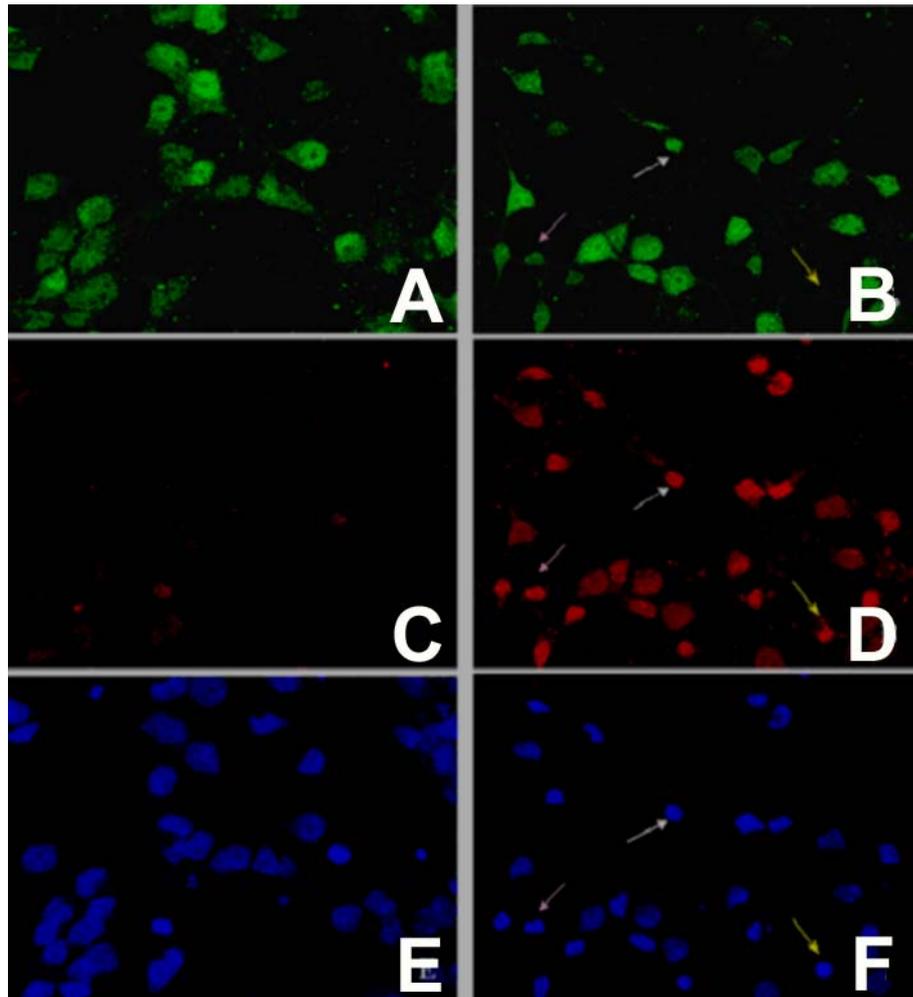
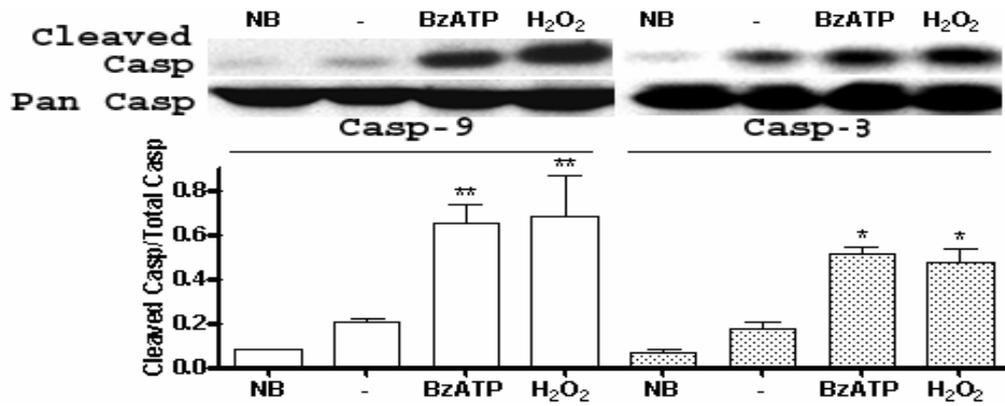


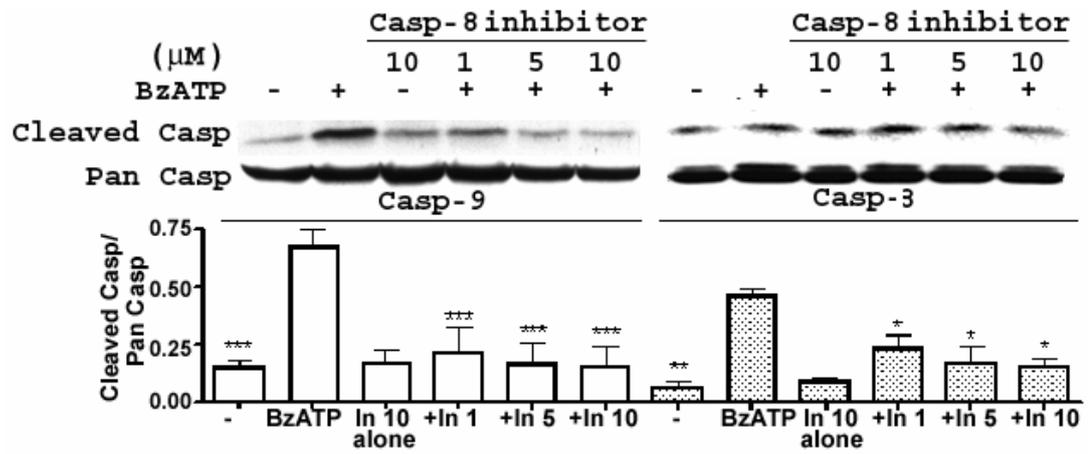
Figure 7. Role of caspase-8 and caspase-9 in P2X₇R-mediated apoptosis in rPCNs.

(A) Rat PCNs were incubated in serum-free HGGMEM and BzATP (300 μ M) was added for 16 h. Cells cultured in B27-AO Neurobasal medium (NB) or in serum-free HGGMEM (-) overnight were used as controls. (B) Cells were treated as in (A) except that the indicated concentration (μ M) of a specific caspase-8 inhibitor, Ac-IETD-CHO was added for 1 h prior to addition of 300 μ M BzATP. (C) Cells were treated as in (B) except that the indicated concentration of a specific caspase-9 inhibitor, Z-LEHD-FMK, was added. “Pan” (full-length) and cleaved caspase-8 were detected by Western analysis, as described in the “Materials and Methods”. Data are the means \pm S.E.M. of results from at least three experiments, where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate significant differences from either serum-starved (A) or BzATP-treated (B, C) cells.

A



B



C

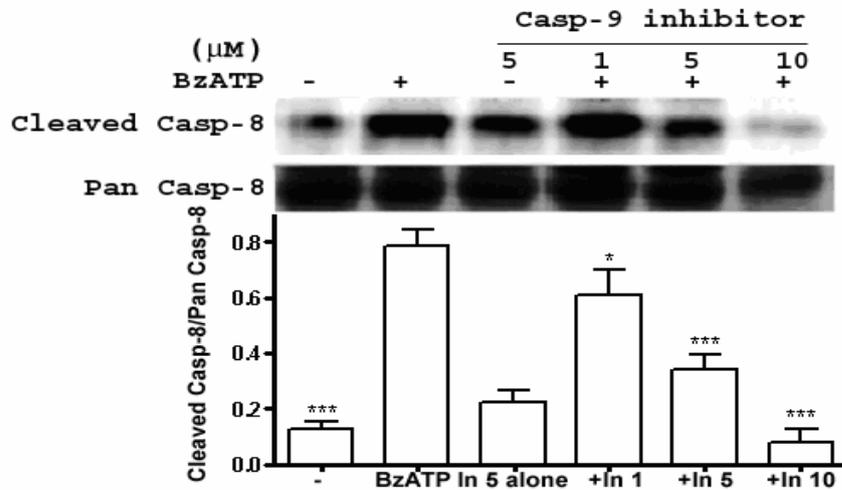
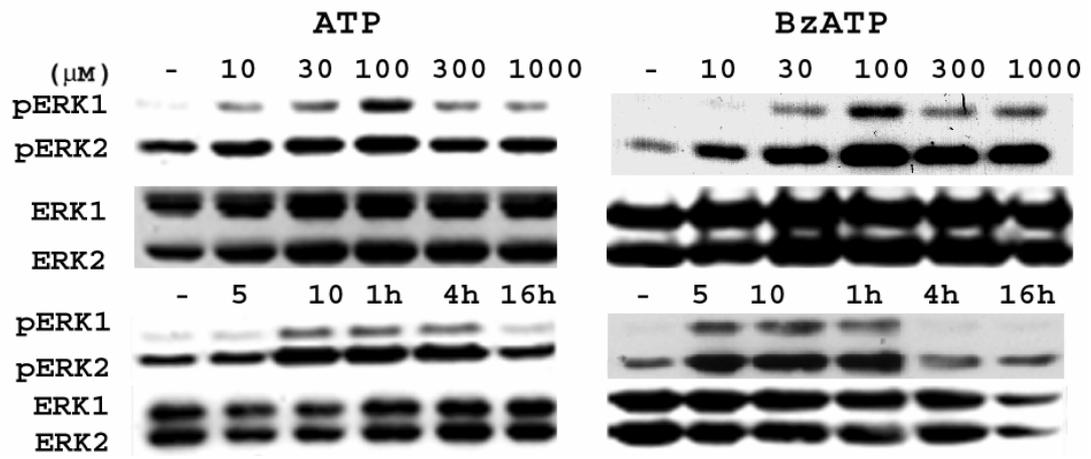


Figure 8. BzATP or ATP induces ERK1/2 and JNK1 phosphorylation in rPCNs.

Rat PCNs were incubated in serum-free HGGMEM for 6 h and treated with the indicated concentration (μM) of BzATP or ATP for 5 min or with 100 μM ATP or 300 μM BzATP for the indicated time period. Cells cultured in serum-free HGGMEM (-) overnight were used as the control. Then, cell lysates were prepared and (A) ERK1/2 or (B) JNK1 phosphorylation was detected by Western analysis, as described in the “Materials and Methods”. Total ERK1/2 and actin were used as protein loading controls for (A) and (B), respectively.

A



B

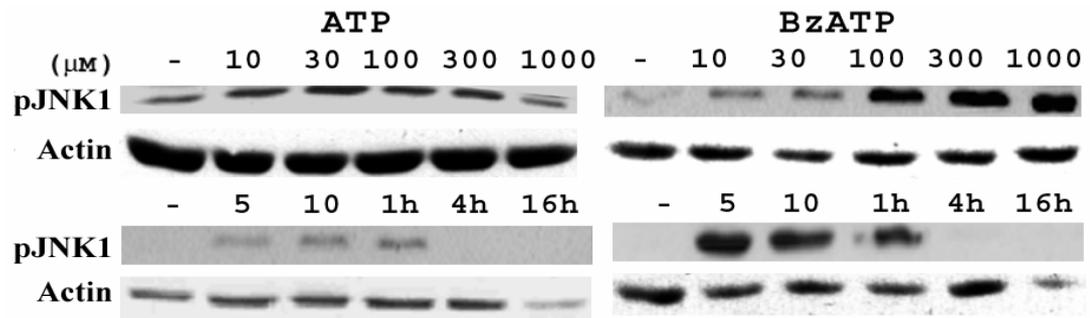
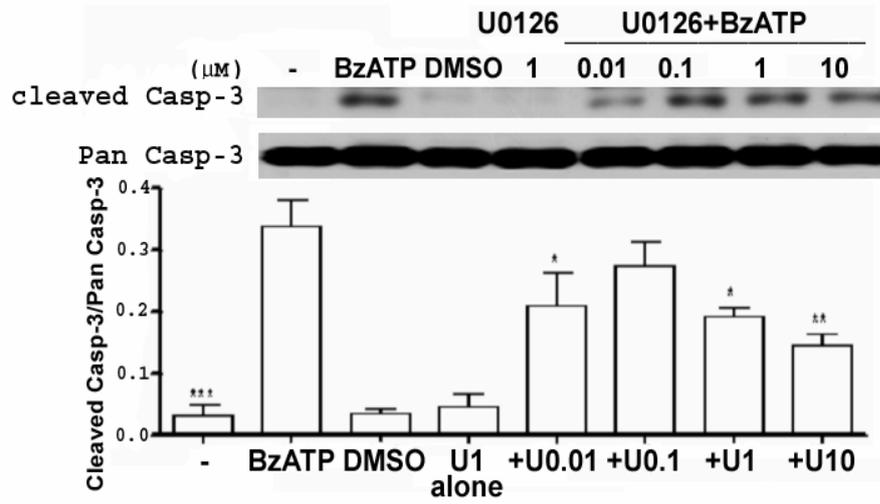
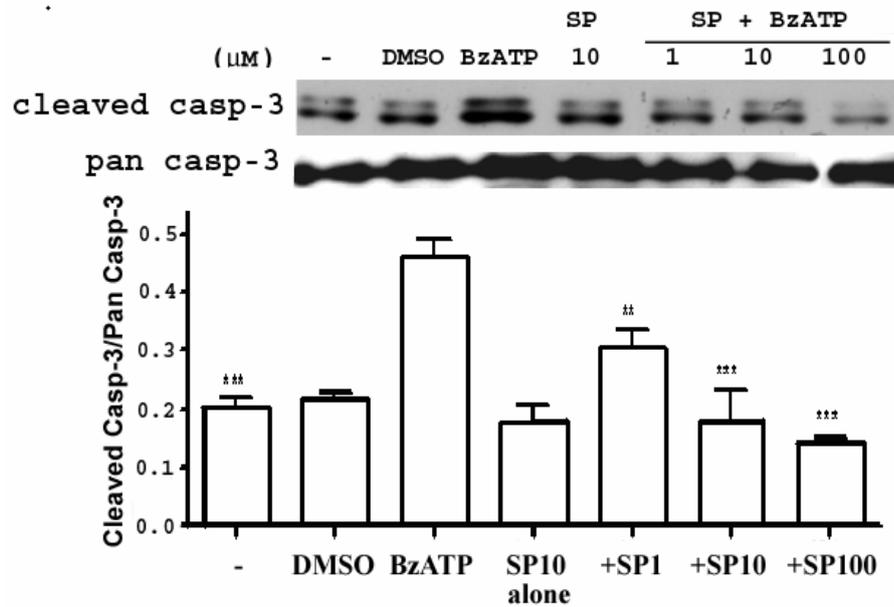


Figure 9. Inhibitors of MEK and JNK decrease BzATP-induced caspase-3 cleavage in rat PCNs. Rat PCNs were incubated in serum-free HGGMEM for 6 h and treated for 30 min with the indicated concentration (μM) of (A) the MEK inhibitor U0126, (B) the JNK inhibitor SP600125 or (A, B) 0.1% (v/v) DMSO as a vehicle control. Then, the cells were incubated with 300 μM BzATP for 16 h, and Western analysis was used to detect expression of cleaved or pan caspase-3, as described in the “Materials and Methods”. Cells cultured in serum-free HGGMEM (-) overnight were used as the control. Data are the means \pm S.E.M. of results from at least three experiments, where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate significant differences from BzATP-treated cells.

A



B



CHAPTER 3

IL-1 β ENHANCES NUCLEOTIDE-INDUCED α -SECRETASE-DEPENDENT APP PROCESSING IN RAT PRIMARY CORTICAL NEURONS VIA UPREGULATION OF THE P2Y₂ RECEPTOR

Kong, Q. et al., to be submitted

ABSTRACT

The P2Y₂ nucleotide receptor (P2Y₂R) stimulates α -secretase-dependent cleavage of the amyloid precursor protein (APP) in human 1321N1 astrocytoma cells, causing extracellular release of the neuroprotective protein sAPP α . In this study, we analyzed functional upregulation of the P2Y₂R by cytokines and the P2Y₂ receptor-mediated production of sAPP α in rat primary cortical neurons (rPCNs). In rPCNs, P2Y₂R mRNA and receptor activity were virtually absent in unstimulated cells, whereas overnight treatment with the inflammatory cytokine IL-1 β upregulated both P2Y₂R mRNA expression and receptor activity. Furthermore, the P2Y₂R agonist UTP enhanced the release of sAPP α in rPCNs treated with IL-1 β or transfected with P2Y₂R mRNA. UTP-induced release of sAPP α from rPCNs was completely inhibited by pretreatment of the cells with the metalloproteinase inhibitor TAPI-2 or the PI3K inhibitor LY2904, partially inhibited by the MEK inhibitor U0126, and unaffected by the PKC inhibitor GF109203, suggesting that P2Y₂R-mediated release of sAPP α from neurons is dependent on ADAM10/17 and PI3K activity, partially dependent on ERK1/2 activity, and does not require activation of PKC. These results suggest that under inflammatory conditions, IL-1 β evokes the functional upregulation of the P2Y₂R in neurons and implicates the activation of the P2Y₂R in neuroprotection.

INTRODUCTION

Inflammation is established as a central component of several chronic diseases. Various inflammatory mediators, such as cytokines, chemokines, proteases and protease inhibitors, have been detected in the AD brain (Parihar and Hemnani, 2004). Interleukin-1 (IL-1) and tumor necrosis factor α (TNF- α) are rapidly upregulated in a variety of tissues in response to pro-inflammatory stimuli to amplify downstream signaling cascades that mediate inflammation (Cacquevel *et al.* 2004). In Alzheimer's disease, IL-1 directly regulates the expression and processing of β -amyloid precursor protein (β -APP), the phosphorylation of tau and the increased production and activity of acetylcholinesterase (AChE) in neurons. These processes result in neuronal stress and injury, which in turn further enhance microglial activation and IL-1 overexpression in a self propagating cycle. Recent studies, however, indicate that certain aspects of the inflammatory response may have therapeutic potential (Wyss-Coray *et al.*, 2001). Nucleotides co-released with neurotransmitters can activate a widely distributed family of nucleotide receptors in the CNS. P2 nucleotide receptors (*i.e.*, P2X ion channels and G protein-coupled P2Y receptors) have been implicated in the regulation of numerous functions in the mammalian nervous system, including neurotransmission, glial cell migration, cell proliferation, ion transport, neuronal cell apoptosis and survival (Burnstock, 2000; Ciccarelli *et al.*, 2001; Weisman *et al.*, 2005). The P2Y₂ nucleotide receptor is distinguished among other P2 receptors by its ability to be activated equipotently by ATP and UTP and has been shown to be upregulated in response to stress or injury in various cell types, including

activated thymocytes, salivary gland epithelial cells, and models of vascular tissue injury (Koshiba *et al.*, 1997; Turner *et al.*, 1997; Seye *et al.*, 2002). In rat primary cortical astrocytes, the P2Y₂R is functionally expressed and promotes astrocyte migration through receptor interaction with α_v integrins, which suggests an important role for P2Y₂R-mediated signaling pathways in regulating astrogliosis in brain disorders (Wang *et al.*, 2005). However, very little is known about the functional role of the P2Y₂ nucleotide receptor subtype in neurons.

Previously, we demonstrated that the G protein-coupled P2Y₂R mediates the α -secretase-dependent release of sAPP α from human 1321N1 astrocytoma cells (Camden *et al.*, 2005). Cleavage of APP by α -secretase results in the shedding of nearly the entire ectodomain of APP to yield a large soluble derivative called sAPP α , and essentially precludes the generation of neurotoxic A β peptide. Several zinc metalloproteinases, including TNF- α converting enzyme (TACE/ADAM17), ADAM9, ADAM10 and MDC-9, and the aspartyl protease BACE2 can cleave APP at the α -secretase site (Allison *et al.*, 2003). While the proteolytic cleavage of APP is constitutive, it can also be increased by substances such as GPCR agonists that activate protein kinase C (PKC) or other signaling pathways (Nitsch *et al.*, 1997; Kirazov *et al.*, 1997) and may function in the modulation of neuronal excitability, synaptic plasticity, neurite outgrowth, synaptogenesis, and cell survival (Mattson, 1997).

The aim of this study was to determine whether cytokines such as IL-1 β regulate functional expression of the P2Y₂R in rat primary cortical neurons and how P2Y₂R signaling pathways affect APP processing in these cells.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM), Neurobasal Medium, penicillin (100 units/ml), streptomycin (100 units/ml) and B27-AO (B27 without cortex antioxidants) were obtained from Gibco-BRL (Carlsbad, CA). Rabbit anti-rat ERK1/2, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and HRP-conjugated goat anti-mouse IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-rat beta amyloid antibody was obtained from Signet Laboratories (Dedham, MA). All other antibodies were obtained from Cell Signaling Technology (Beverly, MA). The Dual Color Protein Standards and nitrocellulose membranes (0.45 mm) were obtained from Bio-Rad (Hercules, CA). LumiGLO chemiluminescent substrates were obtained from New England Biolabs (Beverly, MA). The RNeasy Mini Kit was obtained from Qiagen (Chatsworth, CA). The First Strand cDNA Synthesis Kit and the PCR Kit were obtained from Roche (Indianapolis, IN). The GenCarrierTM2 Transfection Reagent Kit was obtained from Epoch Biolabs (Sugar Land, TX). Nucleotides and all

other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Primary cell culture of cortical neurons

Experimental procedures for cell culture of rat primary cortical neurons (rPCNs) were carried out as previously described (Kong *et al.*, 2005). Briefly, cerebral cortices from 18-day-old embryos of Sprague-Dawley rats were removed and the meninges were discarded. The brain tissue was mechanically dissociated in HGDMEM comprised of DMEM, 10% (v/v) FBS, 2 mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 7.5 mg/ml fungizone. The tissue clumps were dispersed with a 10 ml pipette and suspended in 6 ml of 0.25% (w/v) trypsin at 37 °C for 10 min. Then, 2 ml of heat-inactivated horse serum were added to neutralize trypsin activity. The cell suspension was centrifuged at 900 g for 5 min and the cell pellet was suspended in HGDMEM. The resulting cell suspension was filtered through a sterilized 75 mm cell strainer (Becton Dickinson, Franklin Lakes, NJ) and the cells were seeded into plastic culture plates precoated with poly-D-lysine (0.1 µg/ml). After 16-18 h and every 3 days thereafter, the medium was replaced with B27-AO Neurobasal Medium (2 mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 7.5 mg/ml fungizone, 10 ml of B27-AO, and Neurobasal Medium (Gibco-BRL) to 500 ml) and 15 µg/ml 5-fluoro-2'-deoxyuridine was added to retard glial cell proliferation. The neurons were used for experiments after 7 days in culture (DIV7).

RT-PCR analysis of P2Y₂R mRNA expression

Total RNA was isolated from rPCNs using the RNeasy Mini Kit (Qiagen). cDNA was synthesized from the purified RNA using the First Strand cDNA Synthesis Kit

for RT-PCR (AMV; Roche). Five percent of the synthesized cDNA was used as a template in PCR reactions with the Expand High Fidelity PCR System (Roche). Specific oligonucleotide primers were designed to selectively amplify cDNA for P2Y₂R and G3PDH, as previously described (Wang *et al.*, 2005). The amplification was performed using 35 cycles for P2Y₂R and 30 cycles for G3PDH of denaturation at 95 °C for 30 s, with annealing at 60 °C for 30 s and extension at 72 °C for 1 min. The resulting PCR products were resolved on a 1% (w/v) agarose gel containing 10 mg/ml ethidium bromide and photographed under UV illumination.

Single cell calcium assay

The [Ca²⁺]_i was quantified in single cells with the Ca²⁺-sensitive fluorescent dye fura-2, using an InCyt Dual-Wavelength Fluorescence Imaging System (Intracellular Imaging, Cincinnati, OH). Briefly, rat PCNs cultured on poly-D-lysine-coated coverslips were incubated with 2.5 mM fura-2-acetoxymethylester at 37 °C for 30 min in physiological salt solution (PSS) containing (mM) NaCl 138, KCl 5, CaCl₂ 2, MgCl₂ 1, HEPES 10, glucose 10, pH 7.4, and washed with PSS. The coverslips with fura-2-loaded cells were positioned on the stage of an inverted epifluorescence microscope (Nikon; model TMD) and stimulated with agonist at 37 °C, as described in the figure legends. The percentage of cells that responded to agonist was determined, as previously described (Kong *et al.*, 2005). Viable cells were identified by responsiveness to carbachol and non-responding cells were eliminated.

Overexpression of P2Y₂ receptors in rPCNs

Rat primary cortical neurons at DIV7 expressing very low levels of endogenous

P2Y₂R were transiently transfected with 1 µg of human P2Y₂ receptor cDNA using GenCarrier™2 (Epoch Biolabs; Sugar Land, TX), according to the manufacturer's instructions. The transiently transfected cells were cultured for an additional 24-48 h in Neurobasal Medium with B27-AO, and used to detect sAPP α release.

Harvesting the cells and preparation of conditioned medium

rPCNs were plated on 6-well plates and grown until DIV7. Cells were serum-starved and incubated in 1 ml serum free DMEM with or without various compounds for specified times as indicated in the figure legends. At the end of the incubation period, the media were collected and centrifuged at 12,000 x g for 5 min to remove cellular debris. The supernatants representing conditioned media were stored at -80 °C for future use. To measure the level of secreted APP α , 200 µl of supernatant was diluted 4:1 with 50 µl of 5X Laemmli sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% (w/v) SDS, 1.8% (v/v) β -mercaptoethanol and 0.003% (w/v) bromophenol blue). In addition, the cells were washed twice with ice-cold PBS, and lysed in 2X Laemmli sample buffer (200 µl/well).

Western blot analysis

Samples of cell lysates and conditioned media obtained above were heated for 5 min at 65 °C, and subjected to 7.5% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for protein immunoblotting. After overnight blocking at 4 °C with 5% (w/v) fat-free milk in TBS-T (10 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 0.1% (v/v) Tween-20), membranes were incubated with either 1:1000 dilution of anti-ERK1/2, anti-phospho-ERK1/2, or

anti-actin antibodies for 2 h at room temperature, or 1:1000 dilution of anti-sAPP α overnight at 4 °C followed by incubation with HRP-conjugated anti-rabbit or anti-mouse IgG antibodies (1:1000 dilution in TBS-T containing 5% (w/v) fat-free milk) for 1 h at room temperature. Protein immunoreactivity was visualized on autoradiographic film using the LumiGlo Chemiluminescence System (New England BioLabs), according to the manufacturer's instructions. The protein bands detected on X-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad, Hercules, CA). The activation levels of kinases or sAPP α release were expressed as a percentage of controls (*i.e.*, total kinase or actin).

Statistical analysis

Results are expressed as the means \pm S.E.M. of data obtained from at least 3 experiments. Statistical analysis of data was performed using Graph Pad Prism version 4.0. Statistical significance was determined by student t test between groups. Differences were considered statistically significant when $p < 0.05$ (*) and not significant when $p > 0.05$ (n.s.).

RESULTS

Upregulation of functional P2Y₂R in rat primary cortical neurons by IL-1 β

Several subtypes of P2 nucleotide receptor mRNA are expressed in rPCNs, including P2Y₁, P2Y₄, P2Y₆, P2X₃, P2X₅, P2X₆ and P2X₇ (Kong *et al.*, 2005; Weisman *et al.*, 2005). However, P2Y₂R mRNA and receptor activity are barely detectable in these cells (Weisman *et al.*, 2005, Kong *et al.*, 2005, Figure 1A). Since

previous studies demonstrated that IL-1 β causes an increase in P2Y₂R mRNA expression in mouse primary striatal astrocytes (Stella *et al.*, 1997), we used RT-PCR analysis to examine whether this cytokine could increase P2Y₂R mRNA expression in rPCNs. After overnight incubation with 0-100 ng/ml IL-1 β , P2Y₂R mRNA (Figure 1A-B), but not P2Y₄R or P2Y₆R mRNA (data not shown), was upregulated in an IL-1 β dose-dependent manner. Stimulation of IL-1 β -treated rPCNs with the P2Y₂R agonist UTP caused an increase in [Ca²⁺]_i (Figure 1C) and activation of the MAPKs ERK1/2 (Figure 2), indicating that functional expression of the P2Y₂R is upregulated by IL-1 β in rPCNs. Co-incubation of rPCNs with TNF- α and IL-1 β did not further enhance the effect of IL-1 β on P2Y₂R-mediated calcium mobilization (data not shown) or ERK1/2 phosphorylation (Figures 2C-D).

P2Y₂R-mediated release of sAPP α from rPCNs treated with IL-1 β

The release of sAPP α from rPCNs was detected by immunoblot analysis of the cell culture media with a rabbit anti-rodent sAPP α antibody that recognizes amino acid residues 3-16 of A β and reacts well with APP (130 kDa) and all isoforms of sAPP α (100-120 kDa). We found that overnight treatment of rPCNs with IL-1 β (1-100 ng/ml) caused a dose-dependent increase in sAPP α release from cells stimulated with 100 μ M UTP (Figure 3). UTP also increased the release of sAPP α from rPCNs transfected with P2Y₂R cDNA, as compared to untransfected rPCNs (Figure 4), confirming the hypothesis that UTP enhances sAPP α release from rPCNs through activation of the P2Y₂R subtype.

P2Y₂R-mediated release of sAPP α is dependent on ADAM10/17

Currently, several zinc metalloproteinases have been identified as potential α -secretases including tumor necrosis factor alpha converting enzyme (TACE or ADAM17 for a disintegrin and metalloprotease 17) that catalyzes the shedding of the ectodomain of APP and other transmembrane proteins (Black *et al.*, 1997; Moss *et al.*, 1997), and ADAM10 and MDC9 that also catalyze sAPP α production (Lammich *et al.*, 1999; Koike *et al.*, 1999). In IL-1 β -treated rPCNs, UTP-induced release of sAPP α was significantly inhibited by TAPI-2 (Figure 5), a competitive inhibitor of ADAM17/TACE and ADAM10 (Kaup *et al.*, 2002). Thus, P2Y₂R-mediated APP processing in rPCNs is likely mediated by ADAM10/17.

P2Y₂R-mediated sAPP α release is independent of PKC

P2Y₂R activation causes the phospholipase C-dependent stimulation of PKC, and the activation of several other GPCRs has been reported to induce sAPP α release through PKC-dependent and -independent pathways (Nitsch *et al.*, 1994 & 1995; Checler, 1995; LeBlanc *et al.*, 1998). Therefore, we determined whether P2Y₂R-mediated sAPP α release is dependent on activation of PKC. Results indicated that the level of UTP-induced sAPP α release decreased when IL-1 β -treated rPCNs were incubated for 30 min with GF109203 (10 μ M), an inhibitor of PKC. However, the inhibition was not significant according to the student t test, although GF109203 significantly inhibited 1 μ M PMA-induced release of sAPP α (Figure 6). Thus, sAPP α release mediated by P2Y₂R activation occurs independently of PKC.

P2Y₂R-mediated sAPP α release is dependent on PI3K activation

The release of sAPP α induced by insulin requires activation of the PI3K/Akt

pathway (Solano *et al.*, 2000). Since P2Y₂Rs couple to PI3K activation in many cell types (Muscella *et al.*, 2003; Kaczmarek *et al.*, 2005; Wang *et al.*, 2005), we determined whether PI3K/Akt regulates P2Y₂R-mediated sAPP α release in IL-1 β -treated rPCNs. Figure 7 shows that incubation of IL-1 β -treated rPCNs with 10 μ M LY29004, a selective inhibitor of PI3K, prevents UTP-induced sAPP α release, but has no effect on constitutive release of sAPP α (Figure 7).

Role of MAPK in P2Y₂R-mediated release of sAPP α

Previous studies in our laboratory have shown that inhibition of MAPK (*i.e.*, ERK1/2) partially inhibits UTP-stimulated sAPP α release in human 1321N1 astrocytoma cells (Camden *et al.*, 2005). Therefore, we investigated the role of the MAPK pathway in UTP-stimulated sAPP α release in IL-1 β -treated rPCNs. U0126, an inhibitor of the MAPK/ERK kinase (MEK), partially inhibited UTP-induced sAPP α release but not the constitutive release of sAPP α in IL-1 β -treated rPCNs (Figure 8). These results suggest that P2Y₂R-mediated sAPP α release in rPCNs is partially dependent on MAPK/ERK1/2 activation.

DISCUSSION

In the present study, we demonstrate that the pro-inflammatory cytokine IL-1 β upregulates expression of the P2Y₂R in rat primary cortical neurons (rPCNs). Furthermore, we report that activation of the P2Y₂R in IL-1 β -treated rPCNs enhances the release of sAPP α , a neuroprotective cleavage product of APP (Li *et al.*, 1997; Luo *et al.*, 2001; Stein *et al.*, 2004), by stimulating α -secretase activity mediated by the

metalloproteinases, ADAM10/17. Inhibitor studies reveal that P2Y₂R-mediated sAPP α release from rPCNs requires PI3K activity and is partially dependent on MAPK/ERK1/2 activation, but independent of PKC activation.

Previous studies have demonstrated that P2Y₂R mRNA and functional activity is upregulated in stressed or injured tissue, including ligated salivary gland ducts (Turner *et al.*, 1998), collared carotid arteries (Seye *et al.*, 2002), and submandibular gland cells from the NOD.B10 mouse model of Sjogren's syndrome (Schrader *et al.*, 2005). It was also reported that IL-1 β induced P2Y₂R mRNA expression in vascular smooth muscle cells (SMCs; Hou *et al.*, 2000). IL-1 binds to a specific plasma membrane IL-1 type-I receptor (IL-1RI) (Sims *et al.*, 1993), which subsequently associates with the IL-1 receptor-accessory protein (IL-1RAcP) to initiate intracellular signals (Wesche *et al.*, 1997). Since IL-1 β release has been associated with the pathophysiology of Alzheimer's disease (Grammas and Ovase, 2001), we speculated that cytokines could upregulate P2Y₂ receptor expression in neuronal cells. We found that endogenous P2Y₂R mRNA expression and receptor activity were very low in untreated rPCNs but were significantly enhanced in response to IL-1 β treatment (Figures 1 and 2; Weisman *et al.*, 2005), suggesting that P2Y₂R expression is regulated by transcription factors coupled to IL-1 receptor activation.

As IL-1 β -induced P2Y₂R mRNA expression in vascular SMCs was dramatically enhanced in combination with TNF- α treatment (Hou *et al.*, 2000), we also tested whether TNF- α could enhance IL-1 β -induced P2Y₂R expression and function. In rPCNs, TNF- α did not significantly enhance IL-1 β -induced P2Y₂R mRNA expression

(data not shown) or receptor activity (Figure 2D).

Although the role of P2Y₂R expression in neuronal cells is poorly understood, it is better established that cytokines, including IL-1 β and their receptors mediate inflammation in Alzheimer's brain (Brugg *et al.*, 1995; Hauss-Wegrzyniak *et al.*, 1998). Therefore, we postulate that IL-1 β -induced P2Y₂R expression may contribute to inflammation. However, other studies suggest that P2Y₂R activity in human 1321N1 astrocytoma cells can promote β -amyloid precursor protein (APP) processing (Camden *et al.*, 2005) and therefore we investigated whether this pathway was manifested upon P2Y₂R upregulation in IL-1 β -treated rPCNs. APP is a transmembrane glycoprotein that can be detected in most tissues but is abundantly expressed in the brain (Anderson *et al.*, 1989). Post-translational processing of APP occurs by the action of least three proteinases called α -, β - and γ -secretases (Esler and Wolfe, 2001). Cleavage of APP by β - and γ -secretases results in the release of several protein fragments, including amyloid β (A β), the main component in senile plaques found in the brains of patients with Alzheimer's disease (Martins *et al.*, 1991). Although sAPP α is constitutively released from cells, secretion of this protein is also regulated by neural activity and can be influenced by agonists of various receptors (Robert *et al.*, 2001; Canet-Aviles *et al.*, 2002; Camden *et al.*, 2005). The studies presented here suggest that IL-1 β -induced upregulation of P2Y₂Rs in rPCNs (Figure 3) or transfection of rPCNs with P2Y₂R cDNA (Figure 4) significantly increases UTP-stimulated sAPP α release. Since sAPP α release is postulated to be neuroprotective in AD (Li *et al.*, 1997; Luo *et al.*, 2001), we suggest that P2Y₂R

expression in neuronal cells may play neuroprotective as well as pro-inflammatory roles in the AD brain (Weisman *et al.*, 2005). P2Y₄ receptors are endogenously expressed in rat neurons and have an agonist potency order of ITP = ATP = UTP = ATP_γS = 2-MeSATP = Ap₄A (Bogdanov *et al.*, 1998). Therefore, it is plausible to suggest that P2Y₄Rs may contribute to the enhanced release of sAPP α evoked by UTP. In rPCNs without IL-1 β treatment, P2Y₄R mRNA is expressed at higher levels than P2Y₂R mRNA (data not shown), and therefore the P2Y₄R may mediate UTP-induced increases in [Ca²⁺]_i seen in 1 out of 10 rPCNs in the absence of IL-1 β stimulation (Figure 1C). However, in the absence of IL-1 β treatment, UTP did not significantly increase sAPP α release from rPCNs (Figure 3), suggesting that the P2Y₄R does not contribute to APP processing in rPCNs.

There are three major isoforms of amyloid precursor protein (APP₇₇₀, APP₇₅₁ and APP₆₉₅; Kitaguchi *et al.*, 1988) due to alternative splicing of APP mRNA. As the predominant isoform in the brain (Tanaka *et al.*, 1989), APP₆₉₅ has received the most attention in research on Alzheimer's disease. The ratio of APP₇₇₀:APP₇₅₁:APP₆₉₅ mRNA is 1:10:20 in the cerebral cortex of the brain (Tanaka *et al.*, 1989). Compared with neurons, which express higher levels of APP₆₉₅, astrocytes synthesize APPs of higher molecular weights, as expected for the Kunitz Protease Inhibitor (KPI)-containing isoforms, APP₇₅₁ and APP₇₇₀. Soluble APP α release from rPCNs into the medium can be detected in the form of a doublet (100-120 kDa for APP₆₉₅ and APP₇₅₁; data not shown), indicating that multiple APP isoforms are present, consistent with previous reports (LeBlanc *et al.*, 1991 & 1997; Lahiri *et al.*, 1994;

Parvathy *et al.*, 1998; Solano *et al.*, 2000; Ma *et al.*, 2005).

The data presented (Figure 5) also suggest that UTP induces APP processing in IL-1 β -treated rPCNs through activation of the metalloproteinases ADAM10/17. However, other members of the adamalysin family may also contribute to P2Y₂R-mediated sAPP α release since micromolar concentrations of TAPI-2 used to inhibit metalloproteinase activity have been reported to inhibit members of the adamalysin family other than ADAM10/17 (Parkin *et al.*, 2002).

Stimulation of GPCRs has been shown to regulate APP processing by PKC-dependent signaling pathways (Nitsch, 1992). However, APP processing can also be regulated by increases in [Ca²⁺]_i, and activation of PLA₂, PKA and tyrosine kinases (reviewed in Mills and Reiner, 1999). In human 1321N1 astrocytoma cells expressing human P2Y₂R cDNA, UTP-induced sAPP α release is independent of P2Y₂R-mediated activation of PKC (Camden *et al.*, 2005). UTP-induced sAPP α release also occurred independently of PKC activation in IL-1 β -treated rPCNs (Figure 6). The MAPK signaling pathway has been implicated in the regulation of both PKC-dependent and PKC-independent APP catabolism (Mills *et al.*, 1997; Desdouits-Magnen *et al.*, 1998). PI3K also regulated α -secretase-dependent APP processing (Solano *et al.*, 2000; Fu *et al.*, 2002). It also has been reported that short-term interleukin-1 β incubation increases the release of sAPP α via ERK1/2-dependent α -secretase activation in Neuroglioma U251 cells whereas PI3K activity is unnecessary (Ma *et al.*, 2005). To test the involvement of ERK/PI3K in UTP-stimulated sAPP α release in the rPCNs, after upregulation of P2Y₂Rs by

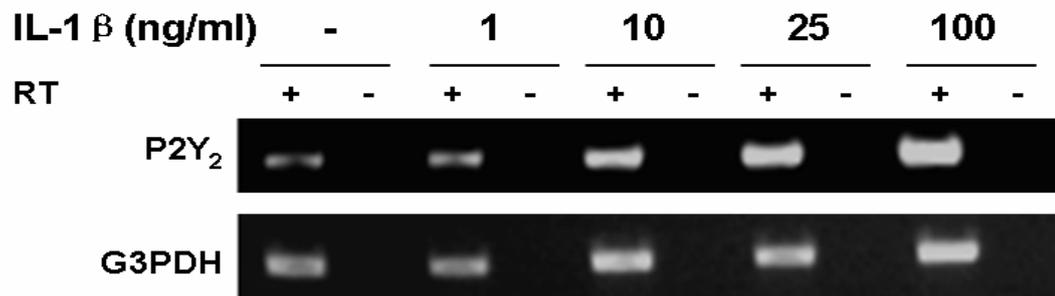
overnight IL-1 β treatment, we incubated cells in serum free DMEM containing specific MEK or PI3K inhibitors followed by UTP stimulation. We found that inhibition of PI3K or MAPK/ERK1/2 activity in IL-1 β -treated rPCNs significantly reduced UTP-induced sAPP α release (Figures 7 and 8). Therefore, unlike short-term effects of IL-1 β , long-term IL-1 β treatment can regulate UTP-induced sAPP α release in a PI3K/ERK-dependent manner, most likely involving transcriptional upregulation of P2Y₂R.

The results of this study strongly suggest that IL-1 β evokes functional upregulation of the P2Y₂R in rPCNs and subsequent activation of the P2Y₂R signaling cascade by UTP results in a significant increase in sAPP α release that is at least partially dependent on PI3K- and ERK1/2-induced activation of the metalloproteinases ADAM10/17. Together, these lines of evidence suggest that under inflammatory conditions in the brain where IL-1 β accumulates, functional P2Y₂R expression in neuronal cells may serve a neuroprotective role in the regulation of α -, γ -secretase-mediated APP processing. Since P2Y₂R expression in glial cells has been postulated to regulate inflammatory responses that may contribute to neurodegeneration in AD (Weisman *et al.*, 2005), future studies are required to evaluate how both neurodegenerative (*i.e.*, glial cell-mediated inflammation) and neuroprotective (*i.e.*, sAPP α release) responses coupled to P2Y₂R activation are coordinately regulated during the progression of Alzheimer's disease.

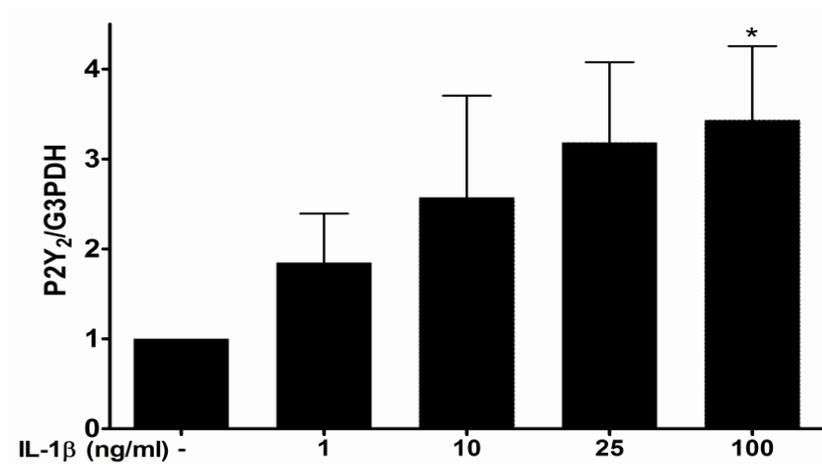
Figure 1. IL-1 β upregulates functional expression of P2Y₂ receptors in rPCNs.

(A-B) Rat primary cortical neurons at DIV7 were treated for 24 h at 37 °C in serum-free DMEM with or without IL-1 β (0 ng/ml, 1 ng/ml, 10 ng/ml, 25 ng/ml, and 100 ng/ml) and cells were harvested for RNA isolation. The effect of IL-1 β treatment on the expression of P2Y₂R mRNA was determined by RT-PCR, as described in "Materials and Methods". Panel B indicates the ratio of P2Y₂R to G3PDH mRNA levels. (C) IL-1 β -treated cells were loaded with fura-2 AM for measurement of [Ca²⁺]_i and positioned on the stage of an inverted epifluorescence microscope (Nikon; model TMD), as described in the "Materials and Methods". Then, rPCNs were incubated in PSS with UTP (100 μ M), and the maximum increase in [Ca²⁺]_i was determined, as described in the "Materials and Methods". Results were expressed as the percentage of rPCNs responding to UTP. Data represent the means \pm S.E.M. of results from three experiments, where * represents significant differences ($p < 0.05$), as compared to rPCNs treated without IL-1 β .

A



B



C

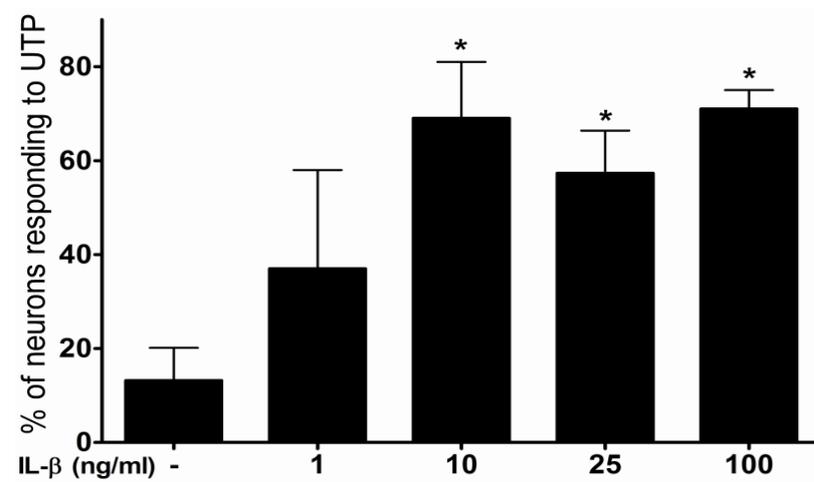
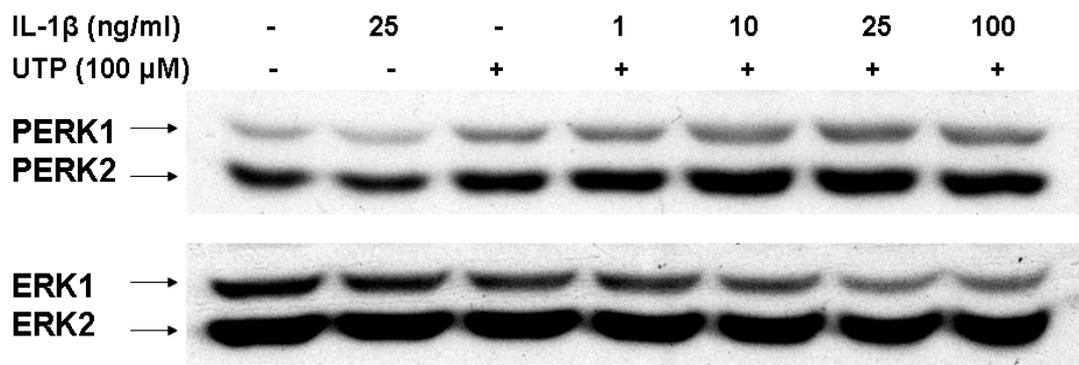
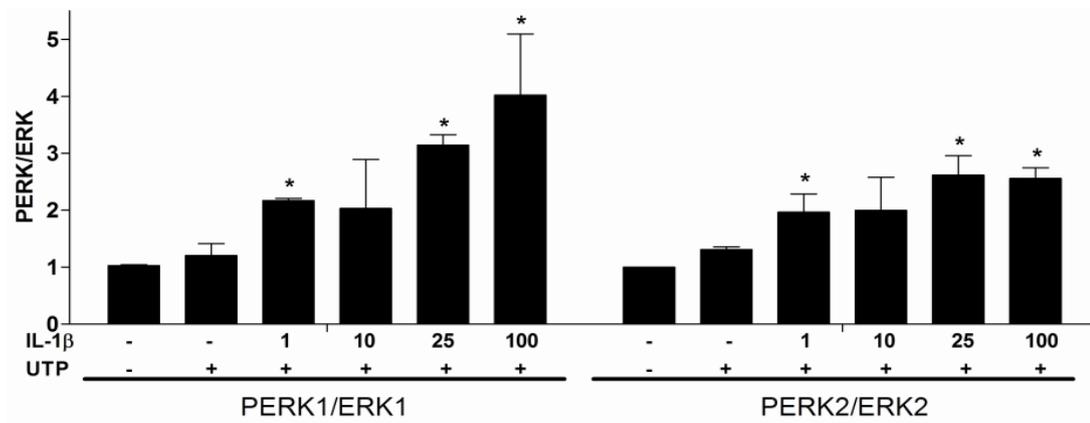


Figure 2. UTP stimulates phosphorylation of ERK1/2 in IL-1 β -treated rPCNs. (A, C) rPCNs were deprived of serum and treated for 24 h at 37 °C in DMEM (A) with or without IL-1 β (0 ng/ml, 1 ng/ml, 10 ng/ml, 25 ng/ml, and 100 ng/ml) or (C) with or without 10 ng/ml TNF- α and/or 25 ng/ml IL-1 β . The next day, the media were removed and cells were incubated for 10 min with serum-free DMEM with or without 100 μ M UTP at 37 °C. Cell lysates were obtained and subjected to Western blot analysis for phosphorylated ERK1/2 and total ERK1/2, as described in "Materials and Methods". (B, D) UTP-induced ERK1/2 phosphorylation was expressed as the ratio of phosphorylated ERK1/2 to total ERK1/2 and the basal level in ERK1/2 phosphorylation in the absence of UTP was subtracted. The increase of ERK1/2 phosphorylation induced by IL-1 β in rPCNs was expressed as the percentage increase over UTP-stimulated rPCNs without IL-1 β -pretreatment. Data represent the means \pm S.E.M. of results from three experiments, where "n.s." represents no significant differences ($p > 0.05$) and where * represents significant differences ($p < 0.05$), as compared to rPCNs treated without cytokines.

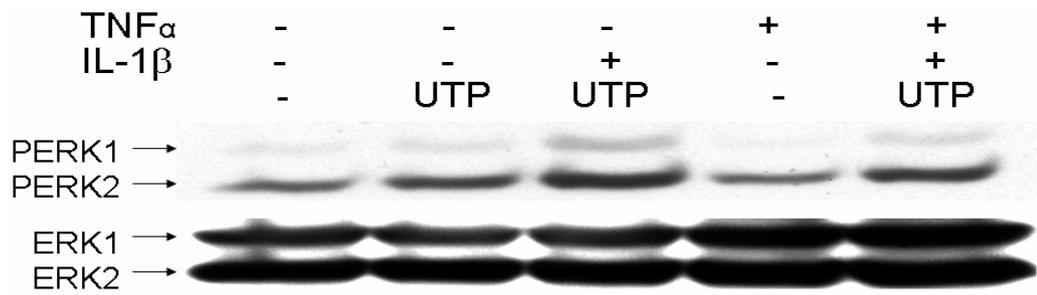
A



B



C



D

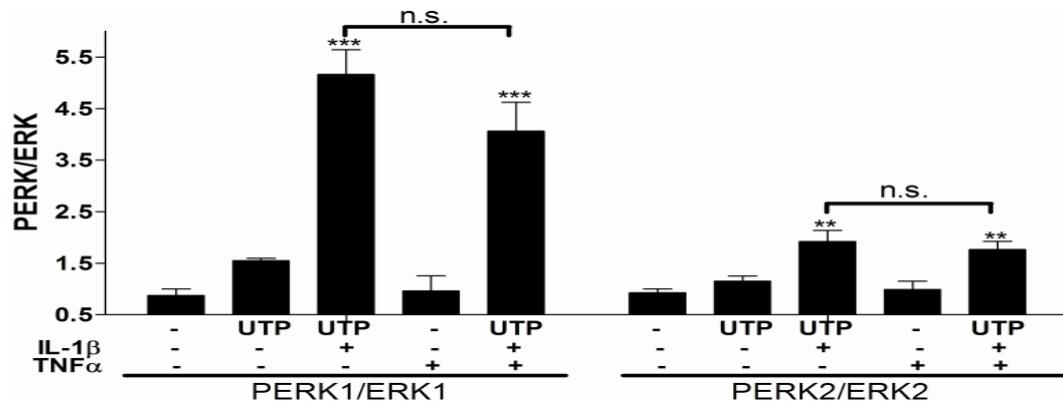
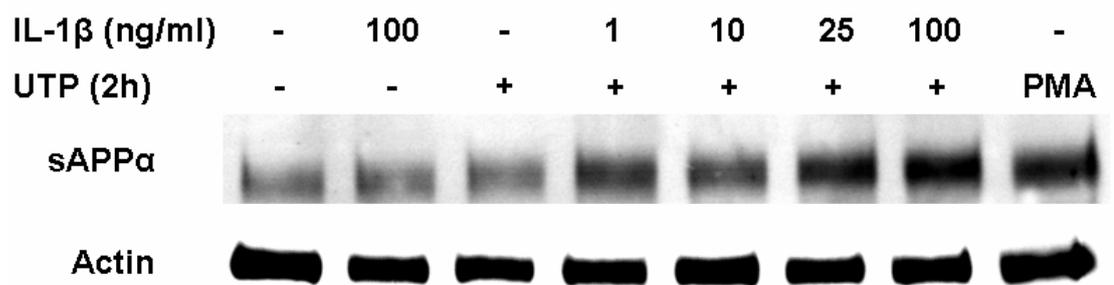


Figure 3. Release of sAPP α is stimulated by UTP in IL-1 β -treated rPCNs. (A) rPCNs were treated with or without IL-1 β (0 ng/ml, 1 ng/ml, 10 ng/ml, 25 ng/ml, and 100 ng/ml) for 24 h in serum free DMEM. The next day, IL-1 β was removed and cells were incubated for 2 h in serum-free DMEM with or without 100 μ M UTP or 1 μ M PMA, a positive control. The medium was removed and assayed for sAPP α release, whereas actin levels were determined in cell lysates, as described in "Materials and Methods". (B) UTP- and PMA-induced sAPP α release in rPCNs with or without IL-1 β pretreatment is expressed relative to actin levels in cell lysates, after subtracting basal sAPP α release in the absence of agonist treatment. Data represent the means \pm S.E.M. of results from at least three experiments, where * represents significant differences ($p < 0.05$), as compared to UTP-treated rPCNs in the absence of IL-1 β pretreatment.

A



B

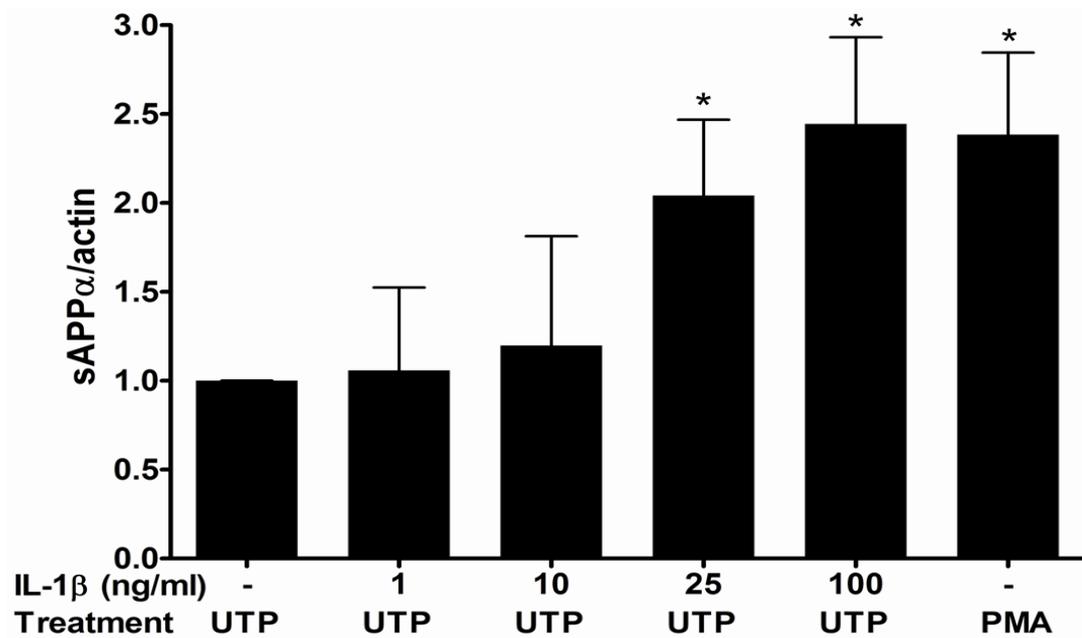
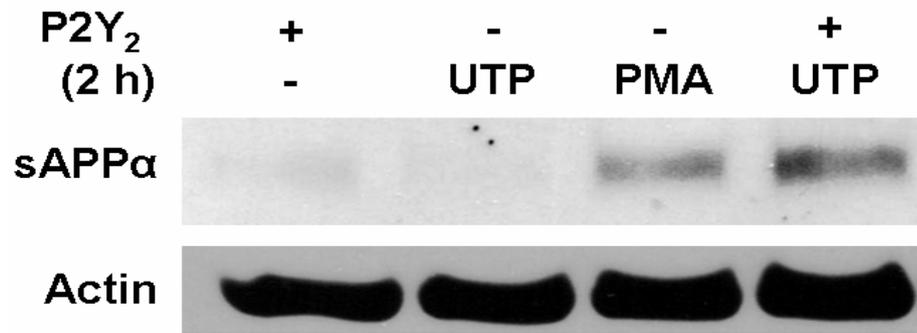


Figure 4. P2Y₂ receptor expression in rPCNs enhances α -secretase-mediated APP processing induced by UTP. (A) rPCNs were transfected with human P2Y₂R cDNA, as described in “Materials and Methods”. Cells expressing P2Y₂R mRNA (data not shown) were incubated for 2 h in serum-free DMEM with or without 100 μ M UTP or 1 μ M PMA, and sAPP α release was determined as described in “Materials and Methods”, and compared to untransfected (-) controls. Actin levels in cell lysates are shown as a loading control. (B) sAPP α release is expressed relative to actin levels in cell lysates. Data represent the means \pm S.E.M. of results from three independent experiments, where * represents significant differences ($p < 0.05$) between the indicated sample groups.

A



B

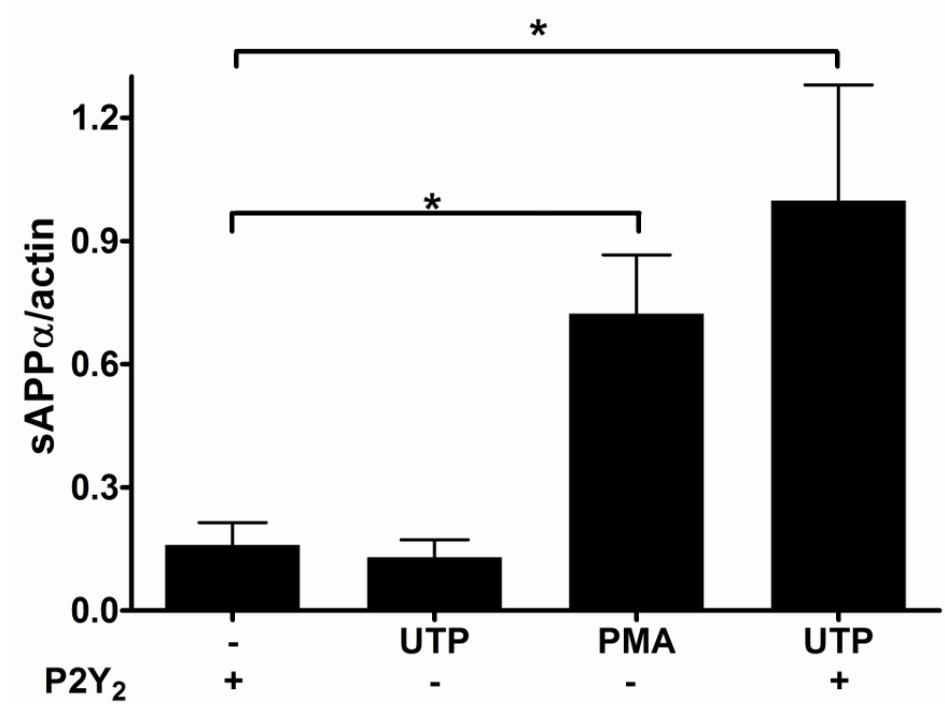
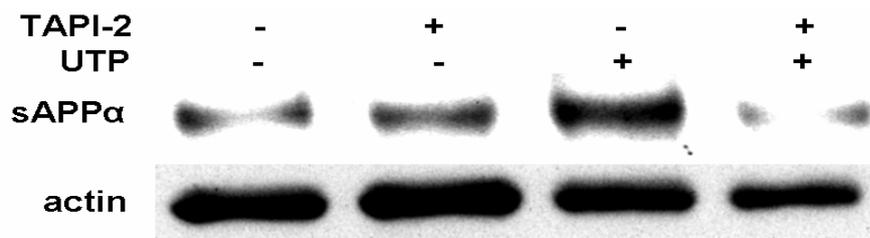


Figure 5. A metalloprotease inhibitor prevents UTP-induced sAPP α release from rPCNs. (A) rPCNs were pretreated overnight with 25 ng/ml IL-1 β and then incubated for 30 min with or without TAPI-2 (10 μ M) followed by incubation with or without UTP (100 μ M) for 2 h in serum-free DMEM. The release of sAPP α into the medium was analyzed, as described in “Materials and Methods”. (B) sAPP α release is expressed relative to actin levels in cell lysates. Data represent the means \pm S.E.M. of results from six experiments, where * represents significant differences ($p < 0.05$) between the indicated sample groups.

A



B

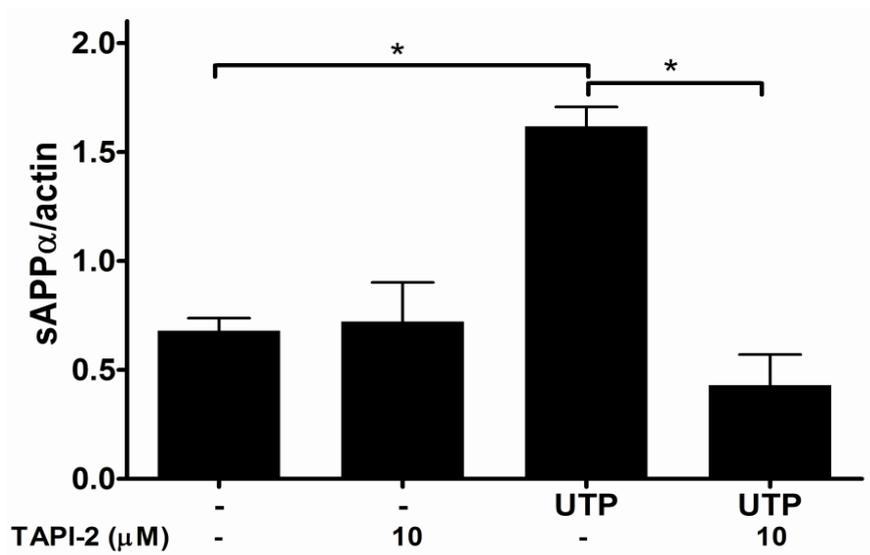
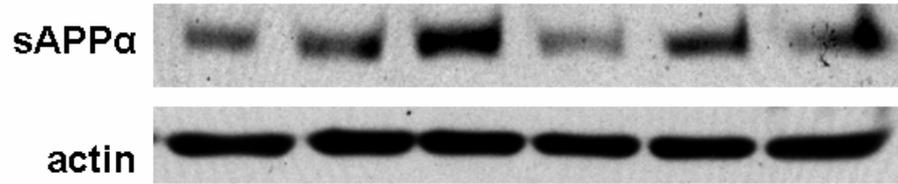


Figure 6. UTP-induced sAPP α release occurs independently of PKC activation in IL-1 β -treated rPCNs. (A) rPCNs were pretreated overnight with 25 ng/ml IL-1 β and then incubated for 30 min with or without GF109203 (10 μ M) followed by incubation with or without UTP (100 μ M) or PMA (1 μ M) for 2 h in serum-free medium. Then, sAPP α release into the medium was analyzed, as described in “Materials and Methods”. (B) sAPP α release is expressed relative to actin levels in cell lysates. Data represent the means \pm S.E.M. of results from three experiments, where “n.s.” represents no significant differences ($p > 0.05$) and where * represents significant differences ($p < 0.05$) between the indicated sample groups.

A

GF109203 (μ M)	-	10	-	10	-	10
	-	-	PMA	PMA	UTP	UTP



B

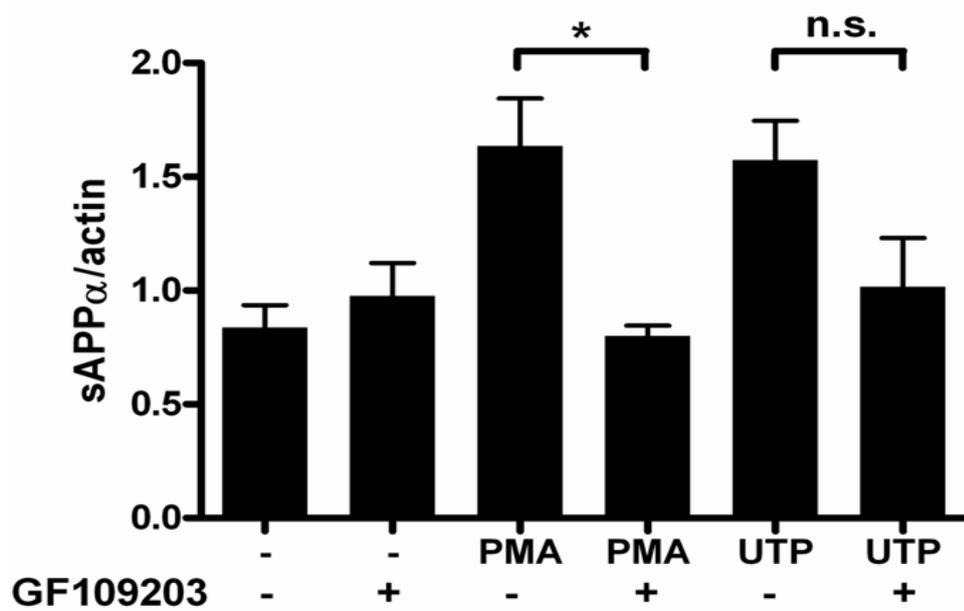
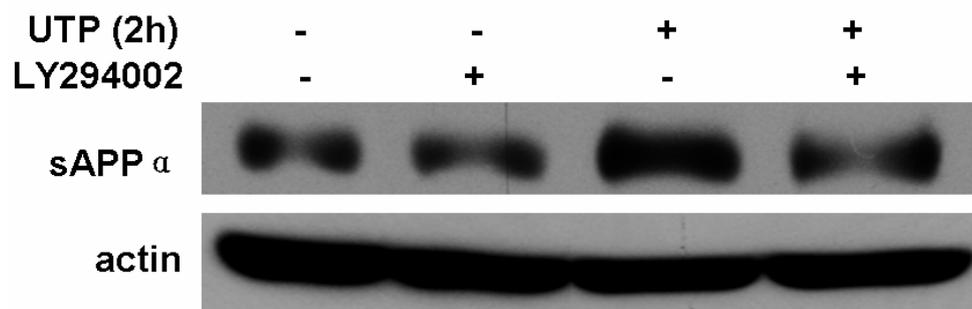


Figure 7. Inhibition of PI3K prevents UTP-induced sAPP α release in IL-1 β -treated rPCNs. (A) rPCNs were pretreated overnight with 25 ng/ml IL-1 β and then incubated for 30 min with or without LY29004 (1 μ M) followed by incubation with or without UTP (100 μ M) for 2 h in serum-free medium. Then, sAPP α release into the medium was analyzed, as described in “Materials and Methods”. (B) sAPP α release is expressed relative to actin levels in cell lysates. Data represent the means \pm S.E.M. of results from three experiments, where * represents significant differences ($p < 0.05$) between the indicated sample groups.

A



B

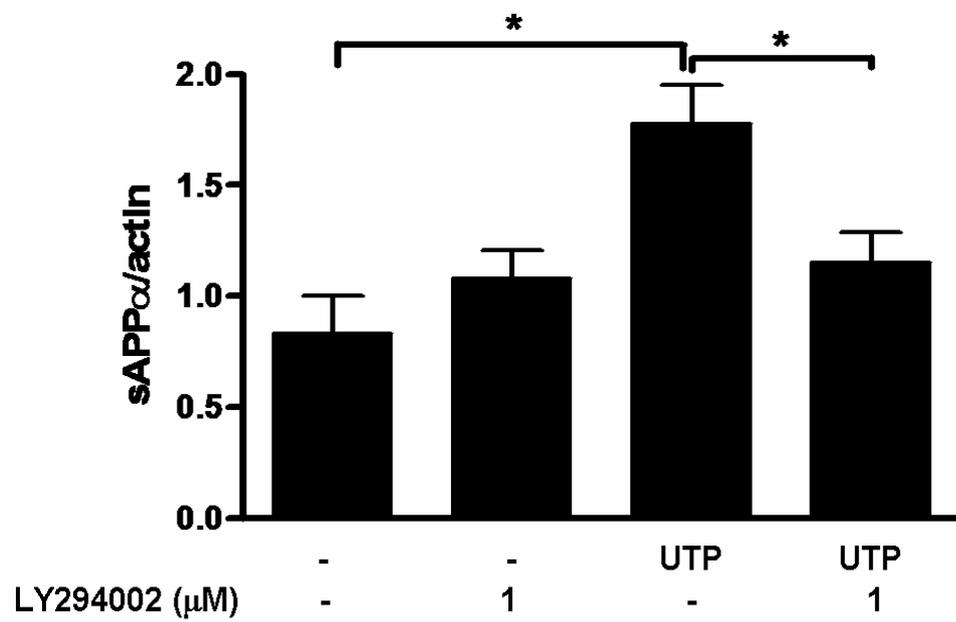
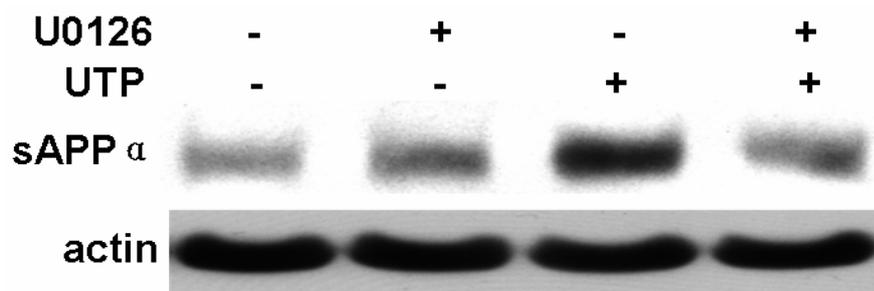
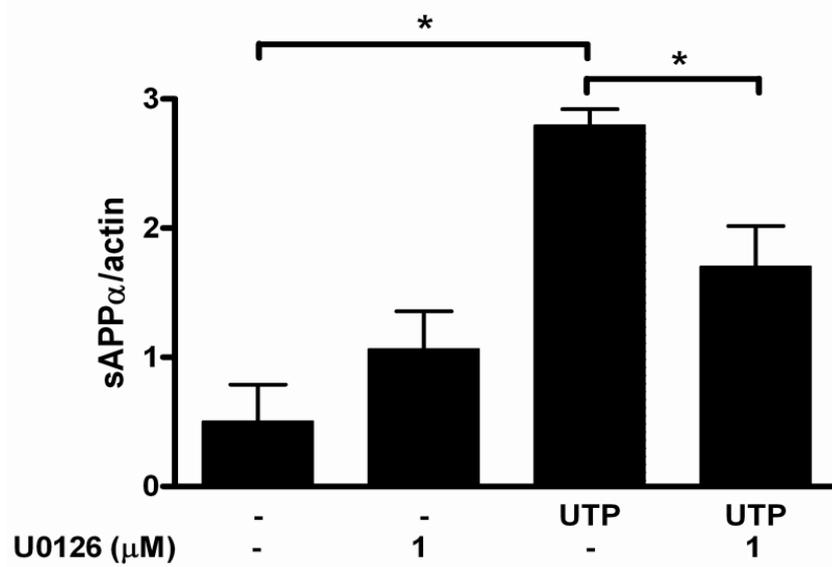


Figure 8. UTP-stimulated sAPP α release in IL-1 β -treated rPCNs is partially dependent on MAPK/ERK1/2 activation. (A) rPCNs were pretreated overnight with 25 ng/ml IL-1 β and then incubated for 30 min with or without U0126 (1 μ M) followed by incubation with or without UTP (100 μ M) for 2 h in serum-free medium. Then, sAPP α release into the medium was analyzed, as described in “Materials and Methods”. (B) sAPP α release is expressed relative to actin levels in cell lysates. Data represent the means \pm S.E.M. of results from three experiments, where * represents significant differences ($p < 0.05$) between the indicated sample groups.

A



B



CHAPTER 4

EXPRESSION AND REGULATION OF P2Y₂ NUCLEOTIDE RECEPTORS IN RODENT BRAINS AND THE EFFECT OF CEREBRAL ISCHEMIA

Unpublished data; Weisman, G.A., *et al.*, *Mol Neurobiol* 31(1-3): 169-183.

ABSTRACT

Large amounts of adenosine 5'-triphosphate (ATP) released from cellular sources under pathological conditions such as ischemia may activate purinoceptors of the P2X and P2Y types. Although signaling pathways for P2Y₂ receptors are well understood in both primary glial cultures and cell lines derived from various tissues, less is known about P2Y₂ receptor distribution in the brain. Therefore, in the present study, *in situ* hybridization and RT-PCR were utilized to examine the regional expression patterns of P2Y₂ receptors in adult rat/mouse/gerbil brain. P2Y₂R mRNA was expressed at relatively low levels in normal rodent brain, although expression levels are similar in rat, mouse and gerbil brains. In general, more abundant P2Y₂R mRNA expression was detected in the hippocampus and cerebellum. In the hippocampus, P2Y₂R mRNA was detected in the dentate gyrus and to a lesser extent in the CA1 and CA3 subregions. Transient global cerebral ischemia (*i.e.*, 5 min occlusion of the CCA) did not affect P2Y₂R mRNA levels in any region of gerbil brain after various reperfusion times (4 h, 16 h and 48 h), indicating that the P2Y₂R is not regulated at the transcriptional level in this ischemia model.

INTRODUCTION

Cerebral ischemia occurs when blood supply to the brain or to regions of the brain is decreased due to blockage or obstruction of cerebral blood vessels. Ischemia can result in rapid increases in the intracellular calcium concentration and release of ATP from pre-synaptic neurons followed by ATP depletion that can lead to neuronal cell death (Golstein and Kroeme, 2006).

ATP release can activate two types of purinergic receptors: ligand-gated ion channel P2X receptors and G protein-coupled P2Y receptors (Burnstock, 1980). Among the known P2 receptor subtypes, P2Y₂ and P2X₇ nucleotide receptors have gained recent attention due to their widespread distribution in neuronal cells and their roles in the regulation of cell proliferation, inflammation, and apoptosis (reviewed in Franke et al., 2006).

Recent studies showed that brain astrocytes express P2Y₂ receptors and that P2Y₂R expression, as opposed to P2Y₁R, is upregulated in freshly isolated astrocytes in hippocampus during development (Zhu and Kimelberg, 2001). Upregulation of P2Y₂Rs also has been shown to occur in many other cell types, tissues and injury models as previously described (Chapter 3; Turner et al., 1997; Erlinge et al., 1998; Hou et al., 1999; Seye et al., 1997 & 2002; Shen et al., 2004; Schrader et al., 2005). Here, *in situ* hybridization and RT-PCR were utilized to examine the regional expression patterns of P2Y₂ receptor subtypes in adult rodent brain and a gerbil transient ischemia model was used to study the regulation of P2Y₂R mRNA expression in response to brain ischemia.

MATERIALS AND METHODS

Materials

The RNeasy Mini Kit was obtained from Qiagen (Chatsworth, CA, USA). The cDNA Synthesis Kit and the PCR Kit were obtained from Invitrogen Life Technology (Carlsbad, California, USA). The Lig'nScribe Reaction Kit was from Ambion (Austin, TX, USA). The [³⁵S]-NTP RNA Labeling Kit (SP6/T7) was from Roche (Indianapolis, IN, USA). [³⁵S]-UTP and [³⁵S]-dATP were obtained from NEN (Boston, MA). All other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

RNA extraction and RT-PCR

Different regions (*i.e.*, hippocampus, cerebellum, cerebral cortex, brain stem and the rest of the brain) were dissected from adult rodent brain. Total RNA was extracted from tissues in four steps: a. tissue was reduced from pieces (10-100 mg) to a powder with a mortar and pestle unit containing liquid nitrogen; b. powdered tissue was dissolved in 1 ml Trizol reagent and large pieces of undissolved tissue were removed; c. 200 µl of chloroform was added to the samples, which were vortexed vigorously and centrifuged for 15 min at 12,000 x g at 4 °C; and d. the upper aqueous phase was collected and one volume of 70% (v/v) ethanol was added, mixed and RNA was purified using the Qiagen Rneasy Mini Kit (Qiagen).

cDNA was synthesized by reverse transcription using purified RNA from mouse tissues (Invitrogen Life Technology). A P2Y₂ receptor cDNA fragment was amplified from the mouse cDNA by PCR using a 35 cycle program (94 °C for 1 min, 60 °C of the annealing temperature for 30 sec, 72 °C for 1 min; *G3PDH* was used as a control.

with 30 cycles and 55 °C for annealing) in the presence of 50 U/ml *Taq* polymerase (ThermoAce™ DNA polymerase from Invitrogen) and two *P2Y₂R* C-terminal primers (1 μmol/l): gerbil *P2Y₂* forward: 5'-TGC TGC CCC TGT CCT ACG GCG TGG-3' (1-24 of AF313448; the sequence of gerbil *P2Y₂R* from <http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=12751144>), gerbil *P2Y₂* reverse: 5'-AGG TTG GTG TAG AAG AGG AAG CGC-3' (241-218 of AF313448). The following primers for human *G3PDH* were used as a control: sense 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3', antisense 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'. PCR products were resolved by 2% agarose gel electrophoresis, and *P2Y₂/G3PDH* ratios were determined by densitometry.

Gerbil global cerebral ischemia model

Adult male Mongolian gerbils (60–80 g body weight) (Charles River, Wilmington, MA) were given free access to water and lab chow and maintained at 22 ± 2 °C with a constant humidity under a 12:12 h light:dark cycle. Gerbils were randomly divided into a sham control group, an ischemic group and an ischemic group with resveratrol. For some animals in each group, resveratrol (Sigma, St. Louis, MO) was dissolved in corn oil and injected intraperitoneally (30 mg/kg body weight) immediately after and 24 h after induction of ischemia by occlusion of both common carotid arteries (CCAs), as previously described (Wang et al., 2002). Sham controls also were injected with the same volume of corn oil and underwent similar surgical procedures without occlusion of the CCAs. The animal protocol was approved by the University of Missouri-Columbia Animal Care and Use Committee

(Protocol #1741). Experiments were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. To eliminate gerbils that have communicating arteries, regional cerebral blood flow (rCBF) was monitored before and after clamping the bilateral CCA using a laser Doppler flowmeter (MBF3D, Moor Instruments, Devon, UK). Gerbils showing a decrease in rCBF of less than 80% upon occlusion of CCAs were excluded from subsequent analyses (Wang et al., 2002).

Tissue sections

Normal/sham/ischemic rodent brains were removed, wrapped in parafilm and aluminum foil and placed in tightly capped 50 ml conical tubes, unless the brains were sectioned immediately. Stored tissue was cut into 12 μ m-thick sections, thaw-mounted onto microscope slides, dried briefly on a warm plate (32 °C; 1 min), and kept in 100-place slide boxes at -80°C until *in situ* hybridization.

Probes

To generate P2Y₂R specific riboprobes for *in situ* hybridization, a T7 promoter adapter was ligated to the 247-bp P2Y₂R fragment amplified by reverse transcription-polymerase chain reaction (RT-PCR) with the Lig'nScribe Reaction Kit (Ambion). Antisense and sense riboprobes were obtained by *in vitro* transcription with [³⁵S]-UTP using the [³⁵S]-NTP RNA Labeling Kit (SP6/T7) (Roche). P2Y₂R oligoprobes were prepared by labeling the following oligonucleotides with [³⁵S]-dATP at the free 3' hydroxyl end using terminal deoxynucleotidyl transferase: 1. 5'-GAT GGC GTT GAG GGT GTG GCA ACT GAG GTC AAG TGA TCG GAA GGA-3';

and 2. 5'-CGT TCC AGG TCT TGA GGC GGC ACA GGA AGA TGT AGA)-3'.

In situ hybridization

Hybridization was performed overnight at 55 °C for the riboprobes and 37 °C for the oligoprobes in a humidified chamber, as described previously (Simonyi *et al.*, 2002). Briefly, normal rodent brain or ischemic hippocampal sections were fixed *in situ* by 4% (v/v) paraformaldehyde for 5 min, then rinsed with 2 × SSC. Next, sections were dehydrated and delipidated with the following graded series of ethanol (EtOH) dilutions and chloroform: 1 min in 50% EtOH, 1 min in 70% EtOH, 1 min in 80% EtOH, 2 min in 95% EtOH, 1 min in 100% EtOH, 5 min in Chloroform, 1 min in 100% EtOH, and 1 min in 95% EtOH. The sections then were air-dried. Sections to be used as negative controls were treated with RNase A solution (10 mg RNase/50 ml, 0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH = 8) at 37 °C for 30 min, then washed in 1 X SSC three times for 1 min each, dehydrated twice with 70% EtOH for 2 min, twice with 80% EtOH for 2 min, and twice with 90% EtOH for 2 min. Next, sections on slides were hybridized under coverslips in a humidified chamber at 55 °C overnight in 25 µl hybridization buffer containing approximately 1-2 × 10⁶ dpm radioactive probe (antisense or sense) and 1 µl nonradioactive probe. After sections were cooled to room temperature, coverslips were removed in 4 × SSC, washed 4 times in 4 × SSC for 15 min each at room temperature, and incubated in RNase buffer for 30 min at 37 °C to reduce nonspecific background. Then, sections were washed, dehydrated, and exposed to a radiosensitive film (Kodak Biomax MR-1).

Image analysis and data analysis

Using standard autoradiographic techniques, the sections on slides were apposed to radiosensitive film (Kodak Biomax MR-1) with [¹⁴C]-labeled calibration standards (American Radiolabeled Chemicals, St. Louis, MO) for 1 week and then developed. The standards comprised of 16 [¹⁴C]-labeled standards on glass slides with 0.002 to 3.58 μCi/g radioactivity were used to construct linear calibration curves of tissue-equivalent radioactivity (μCi/g) relative to the film optical density. Based on the standard curve, microdensitometry of film autoradiograms was performed on the signal over different hippocampal subregions using the BIOQUANT True Color Windows 95 software version 2.50, as previously described (Shelat *et al.*, 2006). The amount of [³⁵S]-labeled probe radioactivity (μCi/g) within various hippocampal subregions was quantitated, from which background radioactivity/optical density was subtracted. The average density measured from various hippocampal subregions fell within the linear range of the standards.

Data are expressed as means ± S.E.M. The number of animals in each group varied between 15 and 23 except for the resveratrol-treated groups which contained 7 animals. Statistical differences between sample groups were evaluated by ANOVA and Student-Newman-Keuls test, where $p < 0.05$ was considered to be significant..

RESULTS

P2Y₂R mRNA expression in rodent brains

P2Y₂ receptor riboprobes were designed from a cloned 247 base pair sequence of

gerbil P2Y₂R cDNA that has high similarity to the rat gene sequence. Two oligoprobes complementary to different regions of the cDNA were prepared. These probes and the antisense riboprobe produced identical patterns of hybridization when tested on adjacent sections (data not shown). Sense riboprobe and RNase treatment were used as two negative controls for nonspecific binding. Both the RNase-treated tissue labeled with oligoprobes (or antisense probe) and the untreated ischemic tissue labeled with sense riboprobe had clean backgrounds, which indicated weak nonspecific binding. P2Y₂ receptor mRNA was expressed at relatively low levels in various brain regions, but was most abundant in hippocampus and cerebellum in rat, mouse and gerbil brains (Figure 1). Similar *P2Y₂R* expression patterns in adult rat brains were demonstrated by RT-PCR (Figure 2A). P2X₇ receptor mRNA was detected in all brain regions (*i.e.*, cerebral cortex, cerebellum, brain stem, hippocampus and the rest of the brain), but expression was highest in hippocampus, cerebellum and cortex (Figure 2B). Other P2Y receptor mRNAs were expressed in rat hippocampus (P2Y₁) and cortex (P2Y₁ and low levels of P2Y₆) (Figure 2C).

P2Y₂ mRNA expression in the gerbil hippocampus after transient ischemia

Transient ischemia induced by a 5-minute occlusion of CCAs in the gerbil causes selective and delayed neuronal death (DND) of CA1 pyramidal neurons associated with activation of surrounding astrocytes in the hippocampus (Kirino, 1982). Regulation of P2Y₂R mRNA expression in response to ischemia was studied in hippocampal subregions of gerbil brain after a 5 min CCA occlusion followed by various reperfusion times (Figure 3). In control hippocampus, the hybridization signal

was relatively high in dentate gyrus as compared to the CA1 and CA3 regions (Figure 4).

Since increased expression of P2Y₂ receptors has been associated with inflammation, tissue damage and disease (Weisman *et al.*, 2005; Seye *et al.*, 2006), P2Y₂ mRNA expression was quantified in gerbil hippocampus after ischemic insult/reperfusion. Statistical analysis showed that there was no significant change of P2Y₂ mRNA expression in the CA1 region during early stages of transient ischemic injury (5 min occlusion + 4 h/16 h/48 h reperfusion; Figure 3 A-C). P2Y₂ receptor mRNA levels were not significantly changed in other hippocampal subregions in response to CCA occlusion followed by 4-48 h reperfusion (Figure 4). Neurons in the hippocampal CA1 region were depleted 3 days after occlusion/reperfusion, whereas microglia and astrocytes proliferated in this brain region. Although P2Y₂R mRNA was hard to detect in the CA1 region (Figure 3D), results appear to indicate that P2Y₂R mRNA expression is not transcriptionally regulated by ischemia in this animal model.

Resveratrol administration does not affect P2Y₂R mRNA levels in normal or ischemic gerbil brain

Increased oxidative stress has been implicated in the mechanisms of DND following cerebral ischemic insult (Kirino, 2000). As a polyphenolic compound enriched in grape and red wine, resveratrol has drawn wide attention lately as an effective anti-oxidative agent (Ray *et al.*, 1999; Hung *et al.*, 2000; Huang *et al.*, 2001; Giovannini *et al.*, 2001; Fremont, 2003). Resveratrol has been shown to protect

against global cerebral ischemic injury in gerbils (Wang *et al.*, 2002) and neurotoxicity induced by kainic acid (Wang *et al.*, 2004). Therefore, we tested whether resveratrol, a polyphenolic antioxidant enriched in grape, has an effect on the P2Y₂R mRNA expression and/or promote neuroprotective signaling through activation of P2Y₂R in gerbil ischemic brain. Mongolian gerbils were divided into three groups: a sham control group, an ischemic group and ischemic group treated with resveratrol. Results indicated that injection of resveratrol significantly prevented DND (data not shown), but had no effect on the P2Y₂R mRNA expression levels in ischemic animals (Figure 5).

DISCUSSION

This study demonstrated similar expression patterns for P2Y₂ receptor mRNA in the brains of adult rats, mice and gerbils. P2Y₂ receptor mRNA was expressed at relatively low levels in various brain regions, but was most abundant in hippocampus and cerebellum. P2X₇ receptor mRNA was detected in all brain regions, but was expressed at highest levels in hippocampus, cerebellum and cortex.

Rapid release of ATP from pre-synaptic vesicles in the early stages of ischemic injury can activate P2 nucleotide receptors and is likely to be an important signal in the ischemic process (Franke and Illes, 2006b). The P2Y₂ receptor subtype can mediate cellular responses typically associated with cell proliferation and inflammation that may contribute to ischemic responses in cells (Erb *et al.*, 2006). Moreover, upregulation of the P2Y₂R subtype has been found in cells and tissue sexposed to stress and injury (Koshiba *et al.*, 1997; Turner *et al.*, 1997 and Seye *et al.*,

2002). Thus, it is important to investigate how P2Y₂Rs are regulated in neuronal cells in response to brain ischemia.

Extensive evidence demonstrates that ischemia and reperfusion causes the upregulation of P2X (P2X_{2, 4, 7}) and P2Y (P2Y₁) receptor subtypes in neurons and glial cells in specific brain regions (Franke and Illes, 2006b) and suggests a direct role for these receptors in the pathophysiology of cerebral ischemia *in vitro* and *in vivo* (Cavaliere et al., 2002, 2003, 2004; Franke et al., 2004a). In contrast, the present studies show that P2Y₂ receptor mRNA expression is not significantly altered in gerbil brain hippocampus at different ischemic stages (*i.e.*, 4-48 h of reperfusion after a 5 min CCA occlusion).

Resveratrol is a polyphenolic antioxidant enriched in grapes and red wine that has been found to reduce ischemia/reperfusion injury in both non-neural organs such as rat hearts and kidney (Ray et al., 1999; Hung et al., 2000; Giovannini et al., 2001) and in the rat focal cerebral ischemia and gerbil transient global cerebral ischemia models (Huang et al., 2001; Wang et al., 2002). The present studies indicate that resveratrol injection did not alter P2Y₂R mRNA levels in ischemic gerbil brain. Therefore, resveratrol mediates neuroprotective signaling independent of the transcriptional activation of *P2Y₂R* in gerbil ischemic brain, although further studies are warranted to examine the mechanisms underlying transcriptional regulation of P2Y₂R expression and the effect of antioxidants.

Figure 1. P2Y₂ receptor mRNA expression in the adult rodent brain. The expression pattern of P2Y₂ nucleotide receptor mRNA in gerbil/rat/mouse brains was identified by *in situ* hybridization with a [³⁵S]-labeled riboprobe, as described in “Materials and Methods”. Rnase A-treated sections were used as a negative control. HI, hippocampus; CE, cerebellum; CO, cerebral cortex.

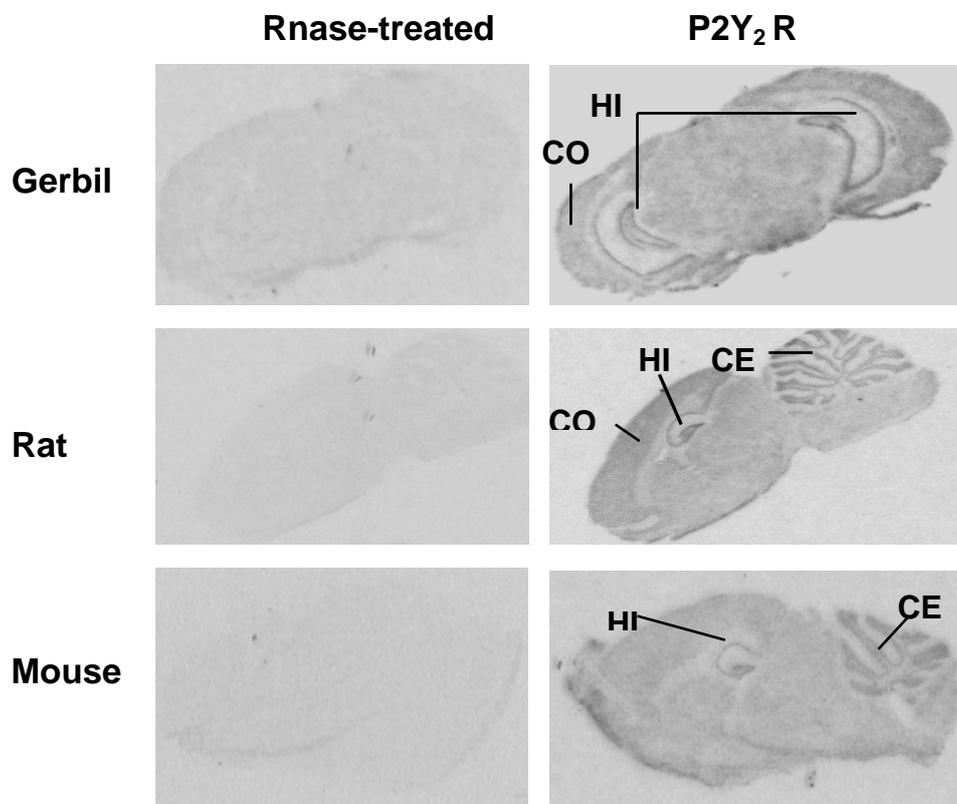
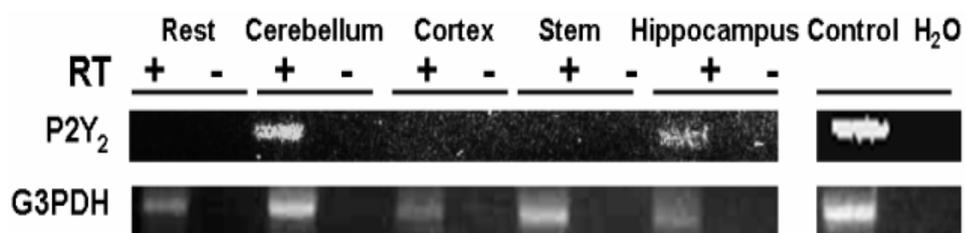
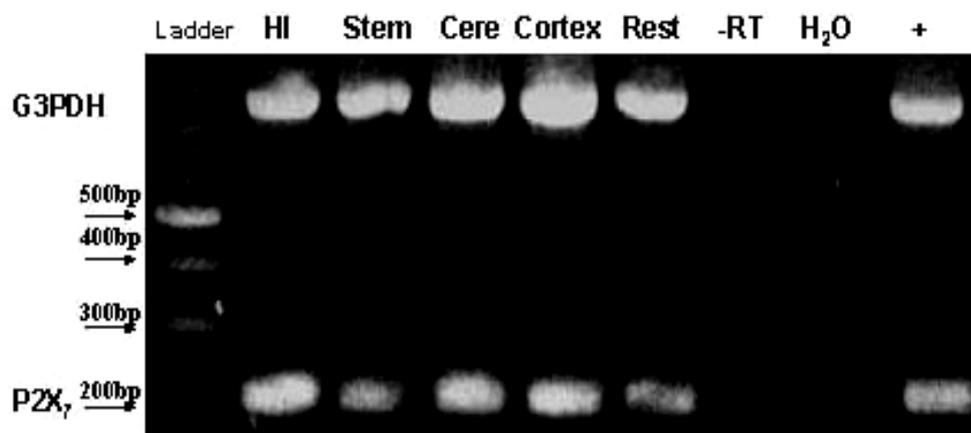


Figure 2. Expression of mRNA for P2Y₂, P2X₇, P2Y₁, P2Y₄ and P2Y₆ nucleotide receptors in adult rat brain. (A) P2Y₂, (B) P2X₇, and (C) P2Y₁, P2Y₄ and P2Y₆ receptor mRNA expression levels in different regions of adult rat brain were analyzed by RT-PCR. Total RNA was prepared from hippocampus (HI), cerebral cortex (CT), cerebellum, brain stem and the rest of the male Sprague-Dawley rat brain. Human 1321N1 astrocytoma cells expressing specific rat P2Y receptor subtypes were used as a positive control for each PCR reaction. Expression levels of the housekeeping gene *G3PDH* were measured as a control. RT-PCR was performed in the presence (+) or absence (-) of reverse transcriptase.

A



B



C

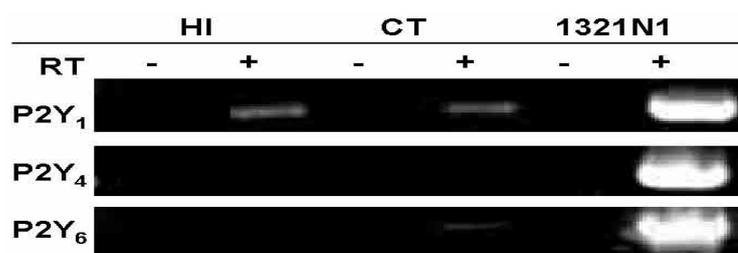
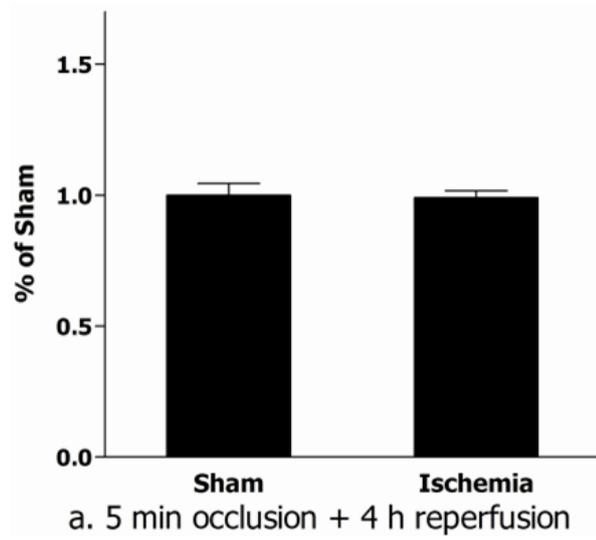
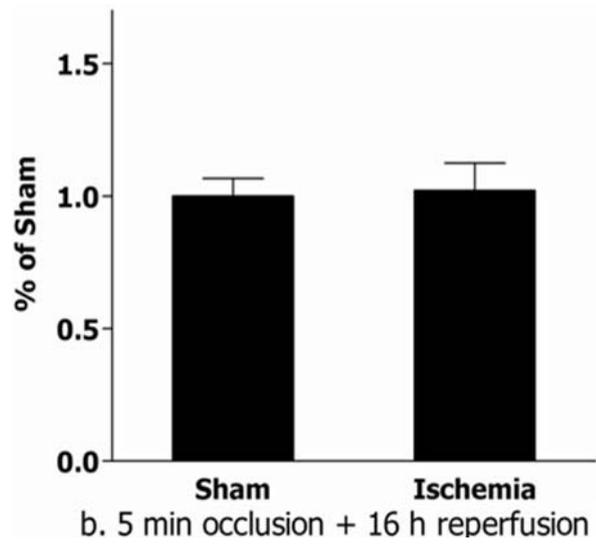


Figure 3. P2Y₂R mRNA levels in the gerbil hippocampal CA1 region after ischemic injury. P2Y₂R mRNA expression in the gerbil hippocampal CA1 region was quantified at different stages of transient cerebral ischemia/reperfusion, as described in “Materials and Methods”. (A) 5 min occlusion + 4 h reperfusion; (B) 5 min occlusion + 16 h reperfusion; and (C) 5 min occlusion + 48 h reperfusion; and (D) gerbil hippocampal sections are shown after a 5 min CCA occlusion + 4-72 h reperfusion.

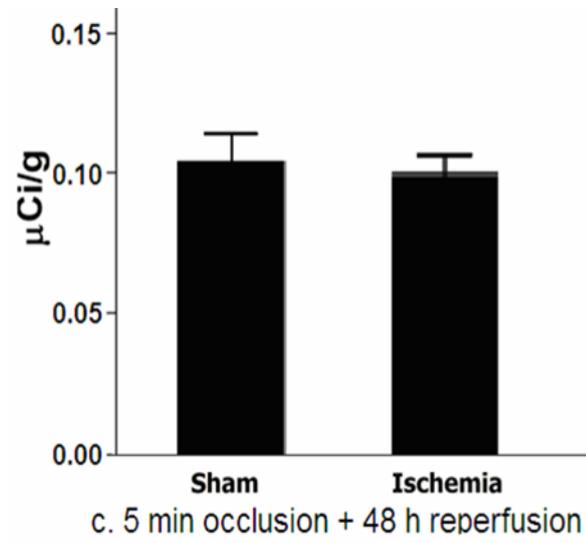
A



B



C



D



5 min occlusion + 4/16/48 h reperfusion
A. P2Y₂ mRNA is expressed in gerbil hippocampus



5 min occlusion + 3 days reperfusion
B. P2Y₂ mRNA is hard to detect in CA1 region

Figure 4. Expression of P2Y₂R mRNA in hippocampal subregions after global cerebral ischemia. P2Y₂R mRNA levels were quantified in different hippocampal regions of sham-operated gerbils (control) and gerbils after a 5 min CCA occlusion followed by a 16 h reperfusion. CA3: hippocampal CA3 subregion; hilus: also called CA4 but considered part of the dentate gyrus; DG: dentate gyrus, which contains upper blade part, lower blade part and the hilus.

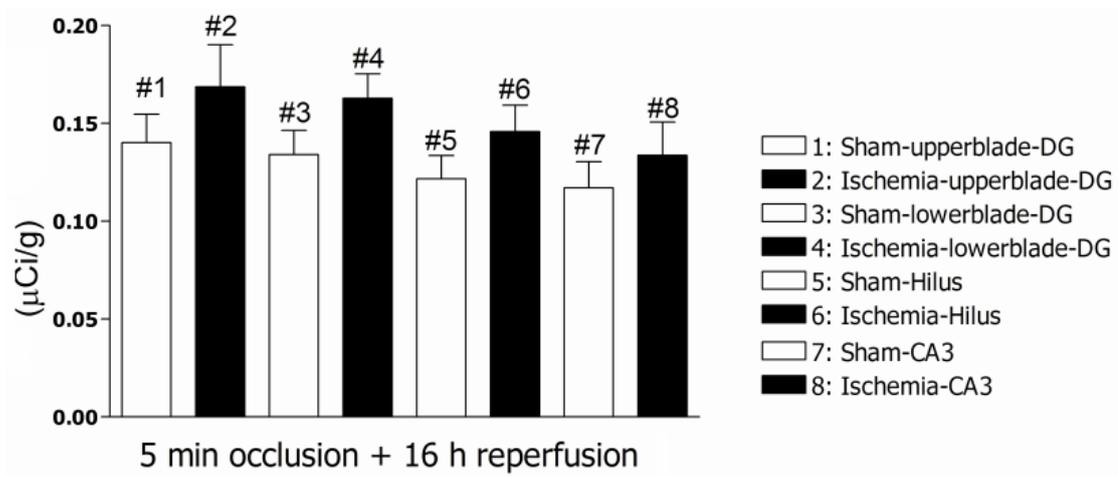
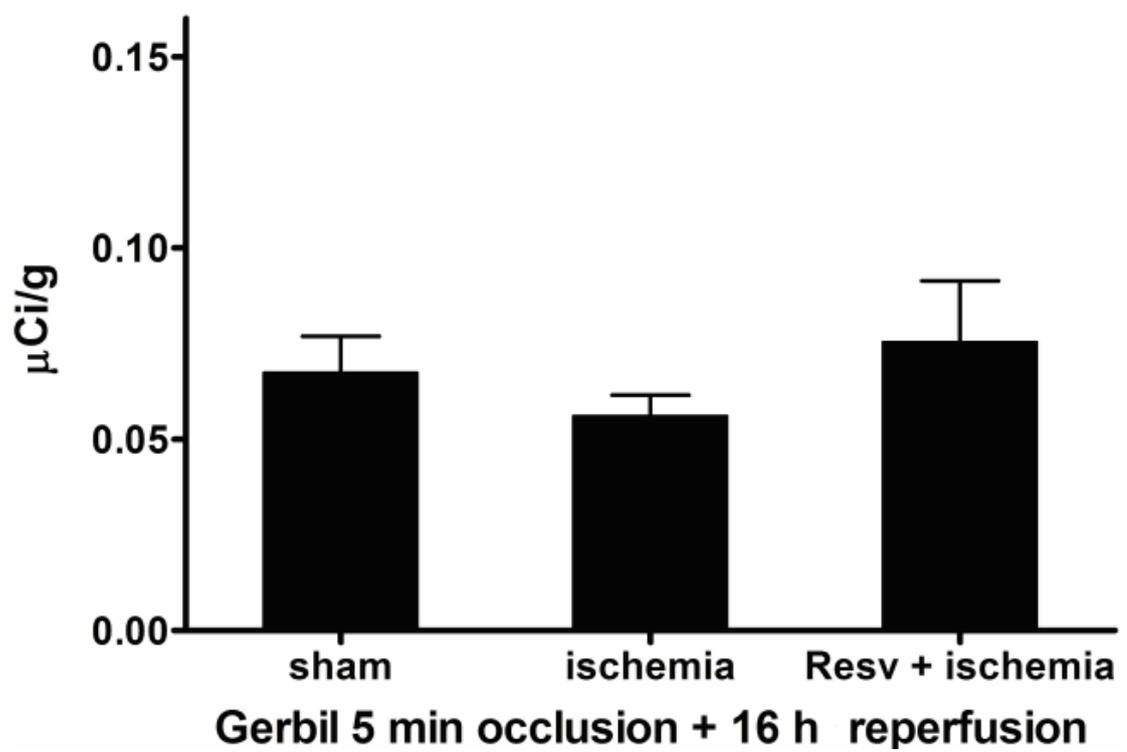


Figure 5. Effect of resveratrol on P2Y₂R mRNA expression in the CA1 region of gerbil brain after transient global cerebral ischemia. Mongolian gerbils were administered resveratrol by injection (Resv; 30 mg/kg body weight) immediately after and 24 h after a 5 min CCA occlusion followed by reperfusion (ischemia). A sham-operated group served as a control. Injection of resveratrol did not significantly affect P2Y₂ receptor mRNA levels in the CA1 region under ischemic conditions (n = 7 animals).



CHAPTER 5

GENERAL DISCUSSION

ATP and other extracellular nucleotides are released from a variety of cell types in the central nervous system including neurons, glia, endothelium, and blood cells (Bergfeld *et al.*, 1992; Ciccarelli *et al.*, 1999; Ahmed *et al.*, 2000; Ostrom *et al.*, 2001). Nucleotides have been suggested to act as a neuroprotective growth factor by regulating cell differentiation, proliferation, and survival, as well as a neurotoxic signal in promoting neuronal degeneration and cell death (Franke and Illes, 2006). Extracellular nucleotides activate two classes of P2 nucleotide receptors (P2X₁₋₇ ligand-gated ion channels and P2Y_{1,2,4,6,11-14} G protein-coupled receptors; Sak and Webb, 2002; Inbe *et al.*, 2004). P2X/Y receptor families are widely distributed and developmentally expressed in the central nervous system (CNS), as previously described (reviewed in Franke and Illes, 2006b), which suggests a role for purinergic signaling in the development of brain structures. Further studies show that P2 nucleotide receptors play important physiological and pathophysiological roles in a variety of biological processes including neuronal growth, survival and brain injury (Franke *et al.*, 2006a & b).

The upregulation of P2 nucleotide receptor expression has been reported after ATP/NGF/cytokine stimulation (Rathbone *et al.*, 1999; D'Ambrosi *et al.*, 2001), and in tissue injury and disease models of the CNS and other tissues (Collo *et al.*, 1997; Moore *et al.*, 2000; Seye *et al.*, 2002; Cavaliere *et al.*, 2003 & 2004; Parvathenani *et*

al., 2003; Franke *et al.*, 2004). In Alzheimer's disease and brain ischemia, *in vivo* and *in vitro* studies have shown that P2X₂ (in ischemia), P2X₄ (in ischemia), P2X₇ (in ischemia and AD) and P2Y₁ (in AD) receptor expression is upregulated in specific cell types, as described in Table 1.

The P2Y₂R can be equipotently activated by ATP and UTP to stimulate G protein-coupled signaling pathways, and to transactivate growth factor receptors (Liu *et al.*, 2004) and integrins, *e.g.*, $\alpha_v\beta_3/\beta_5$ (Erb *et al.*, 2001; Wang *et al.*, 2005; Liao *et al.*, 2007). Studies also indicate that upregulation of the P2Y₂R occurs in a variety of cell and tissue types after injury (Seye *et al.*, 2002; Schrader *et al.*, 2005; Kunzli *et al.*, 2007). The P2X₇R is distinguished from other P2XR subtypes due to its inability to form heteromeric channels, the presence of an extended C-terminal tail; the high concentrations of agonist required for activation, and the ability to form pores that enable the passage of large molecular weight molecules (Sperlagh *et al.*, 2006). P2X₇R functions in the CNS include the regulation of synaptic transmission and neuronal cell death (Sperlagh *et al.*, 2006).

This dissertation describes the functional expression and regulation of P2Y₂ receptor activity by IL-1 β in primary neurons and the upregulation of the P2Y₂R in tissue injury models. Results obtained demonstrate that P2Y₂Rs mediate the release of neuroprotective sAPP α , whereas P2X₇R mediate apoptosis in rat primary cortical neurons. However, further studies are needed to better understand the roles of P2Y₂ and P2X₇ nucleotide receptors in neurodegeneration, such as the mechanism whereby IL-1 β induces upregulation of P2Y₂R expression, the role of P2Y₂R in APP

translocation/phosphorylation and A β production induced by caspase-dependent APP processing, and the relationship of P2X₇R-mediated neuronal apoptosis to the AD phenotype.

Major contributions and significance

The scientific significance of this dissertation work to the field is summarized below.

1) **P2Y₂ receptor expression was identified:** a) in rat primary cortical neurons (Kong *et al.*, 2005 & manuscript in preparation); b) in adult rodent brains; and c) in brain hippocampus at different stages of cerebral ischemia (unpublished data; Weisman *et al.*, 2005; Chapter 4). 2) **Upregulation of the P2Y₂R occurs after IL-1 β treatment in rPCNs and after collar placement around rabbit carotid arteries.** In rat primary cortical neurons, IL-1 β treatment increased P2Y₂R mRNA expression and receptor activity in a dose-dependent manner (Kong *et al.*, manuscript in preparation; Chapter 3). Other studies demonstrate the upregulation of P2Y₂Rs in endothelial and smooth muscle cells of collared rabbit carotid arteries (Appendix-1; Seye *et al.*, 2002 & 2003; reviewed in Seye *et al.*, 2005). 3) **P2Y₂R activation enhances α -secretase-dependent amyloid precursor protein processing in rPCNs.** Previous studies from our lab with the human 1321N1 astrocytoma cell line that does not express endogenous P2Y₂Rs, indicated that transfection with human P2Y₂R cDNA enables UTP to induce α -secretase-dependent APP processing (Camden *et al.*, 2005). The current studies (Chapter 3) confirmed that P2Y₂Rs upregulated by IL-1 β treatment of primary cultures of rat cortical neurons mediate neuroprotective APP

processing, and suggest that P2Y₂R expression in neurons could be targeted to suppress neurodegenerative A β deposition in Alzheimer's disease (Kong *et al.*, manuscript in preparation). 4) **P2X₇Rs mediate apoptosis of rPCNs.** In rat PCNs, functional expression of P2X₇Rs was detected and activation of the P2X₇R was found to mediate caspase-dependent apoptosis and studies presented identified the signaling pathways involved (Kong *et al.*, 2005; Chapter 2). 5) **P2Y₂Rs mediate UTP-induced intimal hyperplasia in collared rabbit carotid arteries.** Our studies demonstrate for the first time that functional P2Y₂R upregulation leads to the development of neointimal hyperplasia in collared rabbit carotid arteries, a response associated with atherosclerosis and restenosis after angioplasty (Seye *et al.*, 2002; Appendix-1). Further studies showed that the P2Y₂ receptor also mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells that promotes the binding of monocytes leading to vascular inflammation (Seye *et al.*, 2003). 6) **Studies in collaboration with other lab members identified P2Y₂ receptor signaling pathways involving integrin interactions and demonstrated that antagonists of CD39 ectonucleotide triphosphate diphosphohydrolase (E-NTPDase) activity also inhibit P2Y₂R functions** (Erb *et al.*, 2001; Gendron *et al.*, 2002; Wang *et al.*, 2005). Studies with the E-NTPDase inhibitor BG0136 showed that this compound displays inhibitory activity towards the P2Y₂R expressed in human 1321N1 cells, which suggests that BG0136 may represent a novel P2 nucleotide receptor antagonist (Gendron *et al.*, 2002; Appendix-2). Other studies demonstrate that the P2Y₂ nucleotide receptor interacts with α_v integrins through an RGD domain

in the first extracellular loop of the P2Y₂R. These P2Y₂R/ α_v integrin interactions were shown to be required for G_o-mediated signal transduction that regulates astrocyte migration (Erb *et al.*, 2001; Appendix-3).

APPENDIX

BRIEF REVIEW OF OTHER RESEARCH PROJECTS

**A-1. FUNCTIONAL P2Y₂ NUCLEOTIDE RECEPTORS MEDIATE URIDINE
5'-TRIPHOSPHATE-INDUCED INTIMAL HYPERPLASIA IN COLLARED
RABBIT CAROTID ARTERIES**

Seye *et al.*, (2002) *Circulation* 106(21):2720-2726.

ABSTRACT

Extracellular UTP induces mitogenic activation of smooth muscle cells (SMCs) through binding to P2Y₂ nucleotide receptors. Previous reports also show that P2Y₂ receptor mRNA is upregulated in intimal lesions of rat aorta, but it is unclear how P2Y₂ receptors contribute to development of intimal hyperplasia. In the present studies, we used a silicone collar placed around rabbit carotid arteries to induce vascular injury and intimal thickening. Collar placement caused rapid upregulation of P2Y₂ receptor mRNA and receptor activity (*i.e.*, UTP-induced increases in the intracellular calcium concentration) in medial SMCs before appearance of neointima. Neointimal development was significantly enhanced by perivascular infusion of UTP (100 μmol/L) and neointima were infiltrated by macrophages. UTP also stimulated osteopontin expression *in situ* in collared arteries and in cultured SMCs in a dose-dependent manner. Furthermore, P2Y₂ antisense oligonucleotide inhibited osteopontin expression induced by UTP suggesting that this response to UTP was specifically mediated by P2Y₂ receptor subtype. In conclusion, these findings indicate for the first time a role for the UTP/ATP receptor, P2Y₂, in development of intimal hyperplasia associated with atherosclerosis and restenosis.

INTRODUCTION

Atherosclerosis is an immunoinflammatory process that involves complex interactions between the vessel wall and blood components primarily affecting the large conduit arteries. Endothelial dysfunction is thought to be a key factor in the development of vascular disease (Ross 1993; Ferns *et al.*, 1991; Wang *et al.*, 1992). Previous studies suggest that an intact endothelium is important in maintaining a low proliferative state of smooth muscle cells (SMCs) under normal conditions (Gordon, 1986). In arterial injury, nucleotides are released from endothelial cells, SMCs, and various blood cells and can bind to P2 receptors to modulate proliferation and migration of SMCs, which is known to be involved in intimal hyperplasia that accompanies atherosclerosis and postangioplasty restenosis (Erlinge *et al.*, 1993). The aim of this study was to determine whether activation of the P2Y₂R promotes intimal hyperplasia under conditions that maintain the vascular endothelium.

MATERIALS AND METHODS

Cell culture

Rabbit SMCs obtained from normal carotid arteries by enzymatic dissociation were maintained in culture and used from passages 4 to 6. Phosphorothioate-modified oligonucleotides to rabbit P2Y₂R were synthesized and purified by Integrated DNA Technologies (ADT). Sequences including translation initiation site were as follows: sense 5'-GGGCAATGGCACTCTTCCCTCACCTG

ACA-3', antisense 5'-TGTCAGGTGAGGGAAGAGTGCCATTGCCC-3'.

Sequences were checked for uniqueness with the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST). SMCs were incubated with 0.1 or 1 $\mu\text{mol/L}$ *P2Y₂R* sense or antisense S-oligonucleotides for 6 h in serum-free medium containing 1.4% DOTAP liposomal reagent (Roche Diagnostics). Serum-free medium was added, and cells were cultured for an additional 20 h. FITC-conjugated *P2Y₂R* antisense and sense oligonucleotides were used to quantify cellular oligonucleotide uptake by SMCs. Cells were rendered quiescent by a 24 h incubation in serum-free medium before stimulation with nucleotides.

Collar implantation

The collared rabbit carotid artery model was employed to induce arterial stress (Figure 1). Protocols conformed to Animal Care and Use guidelines of the University of Missouri-Columbia. New Zealand White rabbits (42 animals) were anesthetized subcutaneously with ketamine (33 mg/kg body weight) and xylazine (7 mg/kg body weight). Rabbits were maintained under respiration with oxygen and 0.2% isoflurane. A nonocclusive, biologically inert, flexible silicone collar (20 mm long; inlet/outlet diameter 1.8 mm; Silicone MED-4211, Nusil Technology) was placed around a carotid artery (Figure 1), and the contralateral artery was sham operated, *i.e.*, isolated from surrounding connective tissue and exposed to similar stretch. Arteries were collected at day 3 (n = 12), 7 (n = 14), or 14 (n = 14). Two collars per animal were implanted in another series of experiments, and each was

connected to an osmotic minipump (Alzet 2 ML2; Alza Corporation) implanted subdermally in the thoracic region. The pump continuously delivered (5 μ l/h) UTP (100 μ mol/l) or PBS (mmol/l: NaCl 154, Na₂HPO₄ 8, NaH₂PO₄ 2, EDTA 0.2) for 3 (n = 14), 7 (n = 14), and 14 (n = 14) days. After surgery, analgesic (Buprenex; 0.1 to 0.5 mg/kg body weight) was administered.

Immunoblotting

Cultured cells were solublized in 2X Laemmli sample buffer (120 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% sucrose, 1 mmol/L EDTA, 50 mmol/L dithiothreitol, 0.003% bromophenol blue). Rabbit aortas were homogenized with a polytron homogenizer in buffer containing 0.9% NaCl, 20 mmol/L Tris-HCl, pH 7.6, 1 mmol/L PMSF in 0.2% Triton X-100. Equivalent amounts of protein (100 μ g) were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Detection of OPN (Chemicon International) was performed with rat anti-human OPN (1:1000 dilution) and horseradish-peroxidase-conjugated goat anti-rat IgG (1:1500 dilution) as primary and secondary antibodies, respectively. For signal normalization, membranes were reprobbed with anti-mouse α -tubulin antibody (1:1000 dilution; Santa Cruz Biotechnology).

RESULTS

Effect of local UTP application on OPN expression

Immunoblots of sham-operated arteries indicated lack of OPN expression (Figure 2A, Lane 5), whereas collared arteries at day 14 expressed OPN (Figure 2A, Lane 2).

Local UTP infusion greatly increased OPN expression (Figure 4A, Lane 4) compared with PBS (Figure 2A, Lane 3).

UTP-induced OPN expression is mediated by P2Y₂Rs in SMCs

UTP exposure for 8 h stimulated OPN expression in vascular SMCs (Figure 2B) in a dose-dependent manner (Figure 2C). ATP and 10% FBS (positive control) induced OPN expression, whereas ADP, 2-MeSATP, and UDP did not (Figure 2B), which suggests that P2Y₂Rs mediate OPN expression. To conclusively demonstrate the role of P2Y₂Rs, SMCs incubated with 0.1 or 1 µg of P2Y₂R antisense or sense oligonucleotides were stimulated with 50 µmol/L UTP for 8 h. Antisense (Figure 2D, Lanes 5 and 6) but not sense (Figure 2D, Lanes 2 and 3) P2Y₂R oligonucleotides inhibited UTP-induced OPN expression in SMCs.

DISCUSSION

Proliferation of SMCs is a hallmark of vascular diseases such as atherosclerosis and restenosis following angioplasty (Hanke *et al.*, 1990). Nucleotides released from various arterial and blood cells (Di Virgilio and Solini, 2002) can bind to P2 nucleotide receptors. In our present studies, *in vivo* experiments showed that the P2Y₂ receptor was functionally upregulated in both SMCs and endothelial cells after collar implantation (Seye *et al.*, 2002). Moreover, intimal thickening of collared rabbit carotid arteries was greatly enhanced by *in situ* UTP application (Seye *et al.*, 2002) and was closely associated with osteopontin (OPN) expression in medial SMCs (Figure 2A). *In vitro* studies showed that both UTP and ATP increased OPN

expression in cultured SMCs whereas ADP, UDP, and 2-MeSATP were ineffective (Figures 2B-C), which suggests a role for the P2Y₂R in which ATP and UTP are equipotent, and is confirmed by the inhibition of UTP-induced OPN expression in cultured SMCs after P2Y₂R antisense oligonucleotide treatment (Figure 2D).

Extracellular nucleotides may also cause cell recruitment by inducing lymphocyte and macrophage adhesion to human pulmonary artery endothelial cells, as demonstrated *in vitro* (Parker *et al.*, 1996). In collared rabbit carotid arteries, we found that local UTP delivery via an osmotic pump induced intimal accumulation of macrophages mediated by P2Y₂ receptors (data not shown; Seye *et al.*, 2002).

In conclusion, these studies demonstrate for the first time that extracellular UTP and the P2Y₂R contribute to the development of arterial lesions associated with atherosclerosis and restenosis.

Figure 1. Collared rabbit carotid artery model. New Zealand White rabbits (42 animals) were anesthetized and (A) a nonocclusive, biologically inert, flexible silicone collar (20 mm long; inlet/outlet diameter 1.8 mm; Silicone MED-4211, Nusil Technology) was (B) placed around a carotid artery, and the contralateral artery was sham operated, *i.e.*, isolated from surrounding connective tissue and exposed to similar stretch.

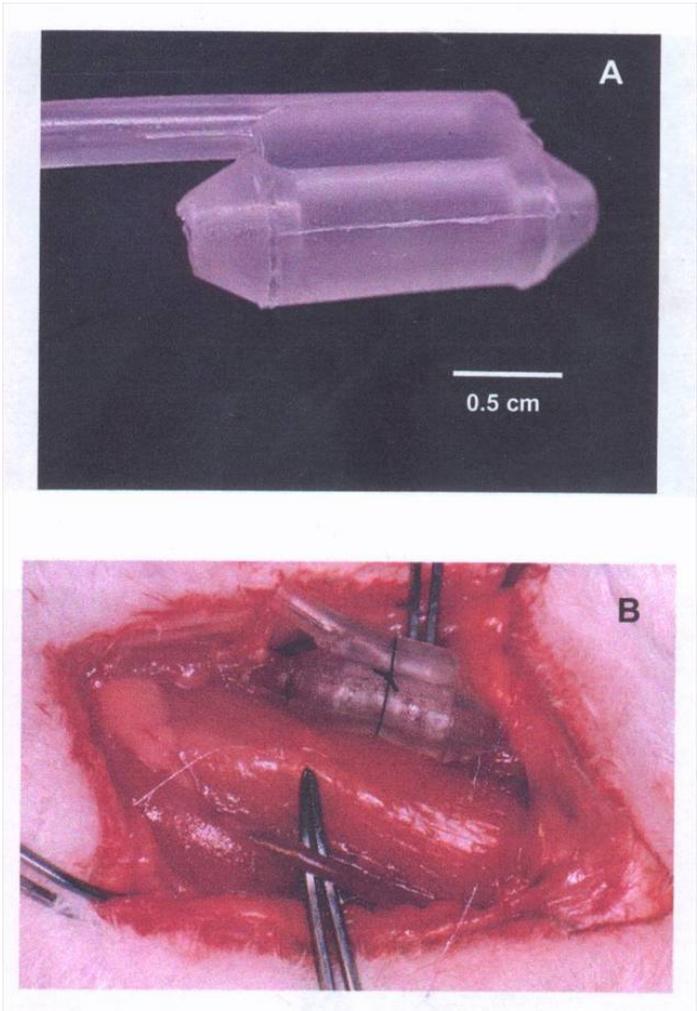
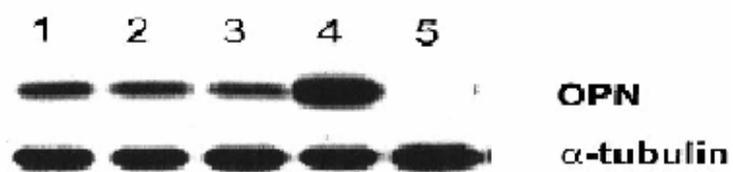
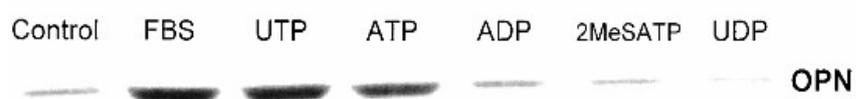


Figure 2. Nucleotide-induced OPN expression in collared carotid arteries and SMC cultures. A. Collared arteries connected to an osmotic minipump were continuously infused with UTP or saline for 14 days. Lane 1, control (kidney); 2, collar without infusion; 3, collar with PBS, 4, collar with UTP; 5, sham-operated artery. Blots were reprobbed with anti- α -tubulin antibody. B-C. SMCs were stimulated with 10% FBS or (B) 50 μ mol/L nucleotide or (C) the indicated UTP concentration. Immunoblot analysis was performed with anti-OPN antibodies. Data represent 3 (A) or 5 (B) experiments. D. SMCs incubated with P2Y₂R sense or antisense S-oligonucleotides for 6 h in DMEM containing 1.4% DOTAP liposomal reagent were cultured for an additional 16 h in serum-free medium before 50 μ M UTP treatment. Cells were incubated with serum-free medium (Lane 1); 0.1 (Lane 2) or 1 (Lane 3) μ g of sense oligonucleotide; serum (Lane 4); or 0.1 (Lane 5) or 1 (Lane 6) μ g of antisense oligonucleotide.

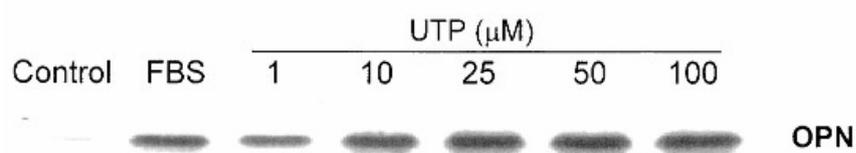
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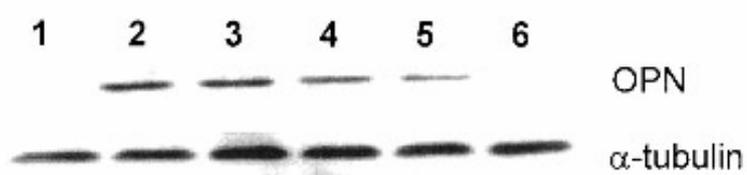
B



C



D



A-2. P2Y₂ RECEPTOR ACTIVITY IS MODULATED BY THE ECTO-NUCLEOTIDASE NTPASE (CD39)

Unpublished data; Gendron *et al.*, (2002) *Curr Drug Targets*. (3):229-245.

ABSTRACT

Extracellular nucleotides initiate various cellular and physiological responses through P2 nucleotide receptors. Another important factor affecting extracellular nucleotide signaling is ecto-nucleoside triphosphate diphosphohydrolase (NTPDase; CD39) now considered as the main ecto-nucleotidase responsible for degrading tri- and diphosphonucleosides to monophosphate derivatives through sequential hydrolysis. Since the concentration and duration of extracellular nucleotides are key regulators of P2 nucleotide receptor function, the influence of ecto-nucleotidases is always considered as one of the major ways to modulate P2 receptor function. In the present studies, the NTPDase inhibitor BG0136 and several cell model systems including COS-7 cells, HEK cells and human 1321N1 astrocytoma cells stably transfected with *P2Y₂R* cDNA were employed to understand the complex interrelationship between P2Y₂ receptors and ecto-nucleotidases. Treatment with 350 μM BG0136 resulted in a 30-50% decrease in UTP-stimulated ERK1/2 activity in COS-7, HEK cells and P2Y₂R-transfected 1321N1 cells. In contrast, the COS-7/CD39 cell transfectants demonstrated a decrease in UTP-stimulated inositol phosphate release compared to native COS-7 cells. The variable effects of BG0136 on P2Y₂ receptor signal propagation in cells expressing CD39 cDNA observed in our studies suggests that BG0136 may represent a new P2Y₂R antagonist.

INTRODUCTION

It was suggested that inhibition of the ecto-nucleotidases responsible for the hydrolysis of extracellular nucleotides could be an important factor in the modulation of purinergic signaling. This study concerns the inhibition of NTPDase and its possible impact on different physiological systems. Several enzymes are involved in the metabolism and control of extracellular nucleotide concentrations (Beaudoin *et al.*, 1996 & 2000; Gordon 1986; Zimmermann 1992 & 2000; Ziganshin *et al.*, 1994; Plesner *et al.*, 1995; Vlajkovic *et al.*, 1998). The enzymes described above may also play an important role in the dephosphorylation of extracellular nucleotides (Zimmermann 2000; Beaudoin *et al.*, 1996; Culic *et al.*, 1990). In the past, modulation of nucleotide-induced cell signaling was focused on nucleotide receptors. Ecto-nucleotidases, more particularly the E-NTPDases family of ecto-enzymes, are now considered as potential new targets for P2 receptor modulation. Inhibition or modulation of E-NTPDases may prolong the effect of nucleotides at their respective receptors and/or modulate cell-to-cell interaction (Imai *et al.*, 1999; Clifford *et al.*, 1997; Dzhandzhugazyan *et al.*, 1998). New molecules have been synthesized to inhibit ecto-nucleotidases and particularly the E-NTPDases, such as P2 receptor agonists/antagonists and molecules related to the suramin-Evans blue families. Based on the structure-activity requirements, as described by Wittenburg *et al.* (Wittenburg *et al.*, 1996), we report here the possible antagonistic property of BG0136 (1-naphthol-3, 6-disulfonic acid, disodium salt) towards the P2Y₂ receptor.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Dulbecco's Modified Eagle's medium (DMEM), penicillin (100 units/ml), and streptomycin (100 units/ml) were obtained from Gibco-BRL (Carlsbad, CA, USA). Rabbit anti-rat ERK1/2, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and HRP-conjugated goat anti-mouse IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PERK antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). The Precision Plus Protein Standards and nitrocellulose membranes (0.45 mm) were obtained from Bio-Rad (Hercules, CA, USA). LumiGLO chemiluminescent substrates were obtained from New England Biolabs (Beverly, MA, USA). Nucleotides and all other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell Culture and Transfection

Human 1321N1 astrocytoma cells (Parr *et al.*, 1994), African Green Monkey Kidney Fibroblasts (COS-7 cells) and Human Embryonic Kidney (HEK) cells were cultured in DMEM (GIBCO BRL) containing 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The retroviral vector, pLXSN, was used for stable expression of the *P2Y₂R* construct in 1321N1 cells as described previously (Erb *et al.*, 1995). In brief, the recombinant *P2Y₂R-pLXSN* construct was used to transfect PA317 amphotrophic packaging cells for production of viral vectors. Then, 1321N1 cells

were infected with the viral vectors and selected for neomycin resistance with 1 mg/ml G418 (GIBCO BRL).

IP Assay

To measure accumulation of IPs, COS-7 cells with or without expression of CD39 were plated in 12-well dishes and incubated in the presence or absence of the specified antibodies for 24 h in 0.7 ml serum-free and inositol-free DMEM containing 2 μ Ci of [3 H]-inositol (20 Ci/mmol). The cells were washed three times and incubated for 30 min at 37 °C in 1 ml of LiCl solution (15 mM glucose, 25 mM Hepes, 110 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.9 mM MgCl₂, and 30 mM LiCl) followed by stimulation with 0.1 μ M UTP for 10 min. The reactions were stopped by aspiration and cell extracts were obtained by addition of 0.3 ml of ice-cold 10% perchloric acid for 15 min. The extracts were neutralized with 0.6 ml of 2 M NaOH and 225 mM Hepes. The IPs in the neutralized extracts were separated from inositol and glycerophosphoinositol on Dowex columns, as described previously (Berridge *et al.*, 1983). Radioactivity in the IP fraction was determined by liquid scintillation spectrometry using Ecolume scintillation fluid.

Western blot analysis

Cells cultured in 6-well plates were treated with nucleotides in the presence or absence of inhibitors as indicated. The cells were washed twice with ice-cold PBS, and lysed for 5 min in 200 μ l of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 2% (w/v) sodium dodecyl sulfate (SDS), and 1 mM sodium orthovanadate). Lysate (30

mg of protein) was added to Laemmli sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% (w/v) SDS, 1.8% (v/v) β -mercaptoethanol and 0.003% (w/v) bromophenol blue). Samples were heated for 5 min at 96 °C, and subjected to 7.5% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for protein immunoblotting. After overnight blocking at 4 °C with 5% (w/v) fat-free milk in TBS-T (10 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 0.1% (v/v) Tween-20), membranes were incubated with either 1:1000 dilution of anti-ERK1/2 or anti-phospho-ERK1/2 antibodies for 2 h at room temperature, followed by incubation with HRP-conjugated anti-rabbit or anti-mouse IgG antibodies (1:1000 dilution in TBS-T containing 5% (w/v) fat-free milk) for 1 h at room temperature. Protein immunoreactivity was visualized on autoradiographic film using the LumiGlo Chemiluminescence System (New England BioLabs), according to the manufacturer's instructions. The protein bands detected on X-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad, Hercules, CA). The activation levels of ERK1/2 were calculated as a percentage of control (*i.e.*, total ERK1/2).

RESULTS

To determine the effect of CD39 on P2 nucleotide receptor-mediated signaling pathways, COS-7 and HEK cells were transfected with CD39. UTP-stimulated inositol phosphate release (Figure 1A) and ERK phosphorylation (Figure 1B and 2) were both inhibited in CD39-transfected COS-7 and HEK cells compared to untransfected cells, indicating that the expression and activation of E-NTPDase by

UTP inhibited UTP-stimulated P2Y₂ nucleotide receptor signaling pathways. A recently developed compound, 1-hydroxynaphthalene-3, 6-disulfonic acid (BG0136) has been shown to inhibit bovine spleen NTPDase with a K_i of 350 μM. However, BG0136, the inhibitor of E-NTPDase also inhibited UTP-stimulated ERK phosphorylation in both CD39-transfected COS-7 and HEK cells (Figure 1B and 2), indicating the possible direct interaction of BG0136 with the P2Y₂ nucleotide receptor.

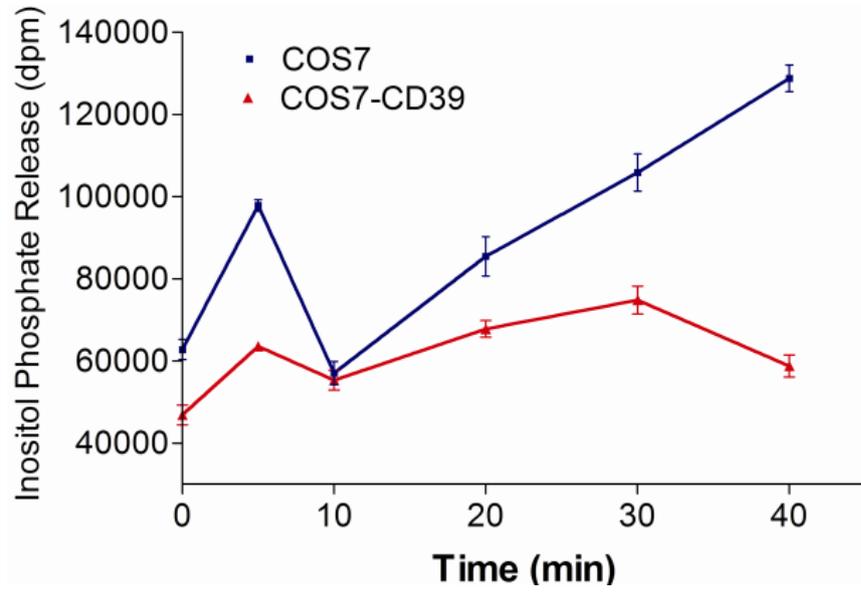
To investigate whether BG0136 could interact with P2 purinoceptors, human 1321N1 astrocytoma cells which do not express endogenous P2Y₂Rs were stably transfected with human *P2Y₂R*. Pretreatment of cell transfectants with 350 μM BG0136 resulted in greater than 50% inhibition of ATP- and UTP- stimulated ERK1/2 phosphorylation in hP2Y₂-1321N1 cells (Figure 3). Since this cell line has been found to have very little ectonucleotidase activity (Gendron *et al.*, 2002), it appears that BG0136 could be either acting directly on the P2Y₂ receptor or on some other component(s) in the ERK signaling pathway.

Altogether, our results obtained with BG0136 and CD39-transfected COS-7, HEK and hP2Y₂-1321N1 cells showed that transfection with ectonucleotidase inhibits UTP-stimulated signaling mediated by P2Y₂ nucleotide receptors, including inositol phosphate release and ERK1/2 activation, whereas BG0136 inhibits UTP/ATP-stimulated ERK phosphorylation mediated by P2Y₂Rs, possibly by acting as a P2Y₂ receptor antagonist. Hence, even if BG0136 displays inhibitory activity towards E-NTPDase, it should be considered as a potential new P2Y₂ nucleotide

receptor antagonist. However, further studies need to be done with this compound to determine its molecular specificity for P2 nucleotide receptor subtypes and ectonucleotidases.

Figure 1. Effect of BG0136 on UTP-stimulated inositol phosphate (IP) release and ERK1/2 activation in COS-7 cells with or without CD39 transfection. African Green Monkey Kidney Fibroblast (COS-7) Cells with (CD39/COS7) or without CD39 transfection were incubated with 350 μ M BG0136 for 5 min at 37 °C, followed by stimulation with 10 μ M UTP at 37 °C for the indicated time. IP release was measured by inositol assay and the phosphorylation of ERK1/2 was determined by Western blot analysis in which total ERK1/2 levels served as a loading control.

A



B

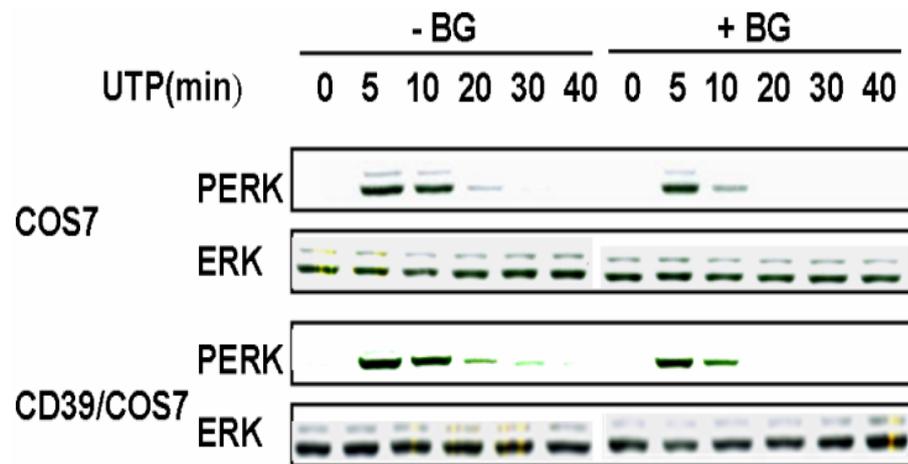


Figure 2. Effect of BG0136 on UTP-stimulated ERK1/2 activation in HEK cells with or without CD39 transfection. Human Embryonic Kidney (HEK) cells with or without CD39 transfection were incubated with 350 μ M BG0136 for 5 min at 37 °C, followed by stimulation with 10 μ M UTP at 37 °C for the indicated time, and the phosphorylation of ERK1/2 was determined by Western blot analysis in which total ERK1/2 levels served as a loading control.

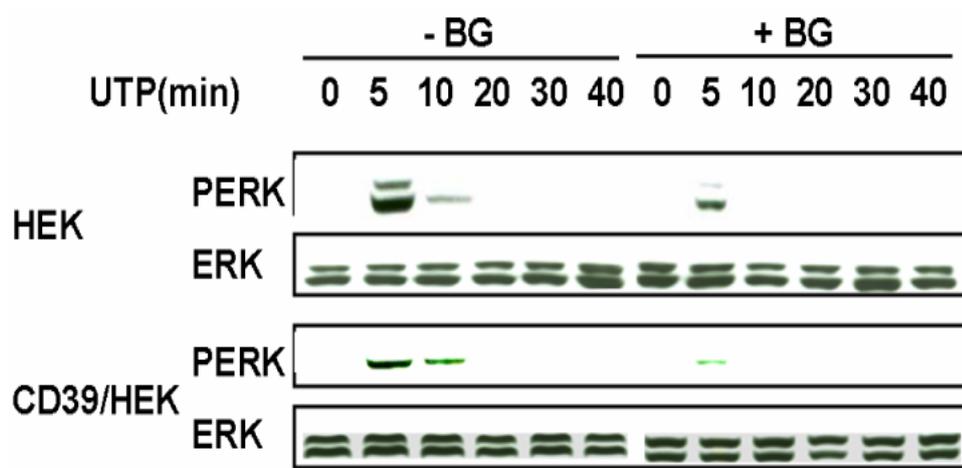
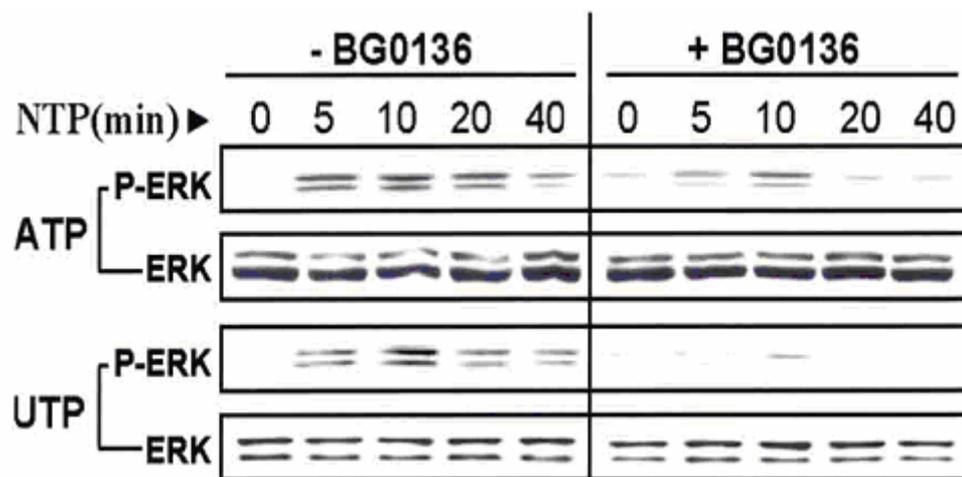


Figure 3. Effect of BG0136 on nucleotide-stimulated ERK activation in human 1321N1 astrocytoma cells expressing the human P2Y₂ nucleotide receptor.

Human 1321N1 astrocytoma cells, stably transfected with the human P2Y₂R cDNA, were incubated with 350 μM BG0136 for 5 min at 37 °C, followed by stimulation with 10 μM ATP or UTP at 37 °C for the indicated time. Phosphorylation of ERK1/2 was determined by Western blot analysis in which total ERK levels served as a loading control.



**A-3. AN RGD SEQUENCE IN THE P2Y₂ RECEPTOR INTERACTS WITH
α_vβ₃ INTEGRINS AND IS REQUIRED FOR G₀-MEDIATED SIGNAL
TRANSDUCTION**

Erb *et al.*, (2001) *J Cell Biol.* 153(3):491-501.

ABSTRACT

The P2Y₂ nucleotide receptor (P2Y₂R) contains the integrin-binding domain arginine-glycine-aspartic acid (RGD) in its first extracellular loop, raising the possibility that this P2Y₂R interacts directly with an integrin through its RGD domain. In this project, we tested whether an antibody raised against the α_v integrin subunit could interfere with UTP-stimulated P2Y₂R signal transduction such as inositol phosphates accumulation. Human 1321N1 cells expressing the P2Y₂R were incubated in the presence or absence of specific anti-α_v integrin antibody for 24 h in serum-free and inositol-free DMEM containing [³H]-inositol. Cells were then used to measure UTP-stimulated inositol phosphate (IP) release. The results indicate that overnight treatment with anti-α_v antibody partially inhibits IP formation, consistent with the partial inhibition of UTP-induced FAK and ERK activation by anti-α_v antibody and the decreased calcium response mediated by RGE mutant P2Y₂Rs as compared to wild-type P2Y₂Rs (Erb *et al.*, 2001). In conclusion, these studies suggest that interaction of α_v integrins with the P2Y₂R is important for optimum P2Y₂R activation.

INTRODUCTION

As a G protein-coupled nucleotide receptor, activation of the P2Y₂R stimulates PLC-dependent production of IP₃ and DAG followed by calcium release and PKC activation. P2Y₂Rs can also bind to growth factor receptors (GFRs) through SH3-binding domains in the C-terminal tail of the P2Y₂R and cause transactivation of GFR signaling pathways. The present studies explore protein complex formation between integrins and the P2Y₂ nucleotide receptor (P2Y₂R), a G_o- and G_q-coupled P2 nucleotide receptor subtype that is equipotently stimulated by ATP or UTP and mediates various cellular responses (Erb *et al.* 1993; Boarder *et al.* 1995). RGD has been found in various integrin ligands, (Clark and Brugge 1995; Frenette and Wagner 1996) as the core recognition sequence for many integrins, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_{11}$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$ (Hynes 1992). Studies by Erb *et al.* (Erb *et al.*, 2001) with P2Y₂R-transfected cells indicated that a mutation of the RGD motif in the wild-type P2Y₂R to RGE decreases receptor colocalization with α_v integrins by 10-fold and the RGE-mutant P2Y₂R requires 1,000-fold higher agonist concentrations than the wild-type P2Y₂R to phosphorylate FAK, activate ERK, and initiate the PLC-dependent mobilization of intracellular Ca²⁺. Furthermore, pertussis toxin, an inhibitor of G_{i/o} proteins, partially inhibited Ca²⁺ mobilization mediated by the wild-type P2Y₂R, but not by the RGE mutant, suggesting that the RGD sequence is required for P2Y₂R-mediated G_o but not G_q activation. To address the possibility that the P2Y₂R interacts with a specific integrin, here it was investigated whether an

antibody raised against the α_v integrin subunit could interfere with UTP-stimulated P2Y₂R signal transduction.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Dulbecco's Modified Eagle's medium (DMEM), penicillin (100 units/ml), and streptomycin (100 units/ml) were obtained from Gibco-BRL (Carlsbad, CA, USA). Anti-human α_v monoclonal antibody (mAb) was purchased from Zymed Laboratories. Anti-human α_3 mAb was purchased from Santa Cruz Biotechnology, Inc. Nucleotides and all other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture and transfection

Human 1321N1 astrocytoma cells (Parr *et al.*, 1994) were cultured in DMEM (GIBCO BRL) containing 5% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The retroviral vector, pLXSN, was used for stable expression of the P2Y₂R constructs in 1321N1 cells, as described previously (Erb *et al.*, 1995). In brief, the recombinant P2Y₂R-pLXSN construct was used to transfect PA317 amphotrophic packaging cells for production of the viral vectors. Then, 1321N1 cells were infected with the viral vectors and selected for neomycin resistance with 1 mg/ml G418 (GIBCO BRL).

IP assay

To measure accumulation of IPs, 1321N1 cells expressing the P2Y₂R were plated in 12-well dishes and incubated in the presence or absence of the specified antibodies for 24 h in 0.7 ml serum-free and inositol-free DMEM containing 2 μ Ci of [³H]-inositol (20 Ci/mmol). The cells were washed three times and incubated for 30 min at 37 °C in 1 ml of LiCl solution (15 mM glucose, 25 mM Hepes, 110 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.9 mM MgCl₂, and 30 mM LiCl) followed by stimulation with 0.1 mM UTP for 10 min. The reactions were stopped by aspiration and cell extracts were obtained by addition of 0.3 ml of ice-cold 10% perchloric acid for 15 min. The extracts were neutralized with 0.6 ml of 2 M NaOH and 225 mM Hepes. The IPs in the neutralized extracts were separated from inositol and glycerophosphoinositol on Dowex columns, as described previously (Berridge *et al.*, 1983). Radioactivity in the IP fraction was determined by liquid scintillation spectrometry using Ecolume scintillation fluid.

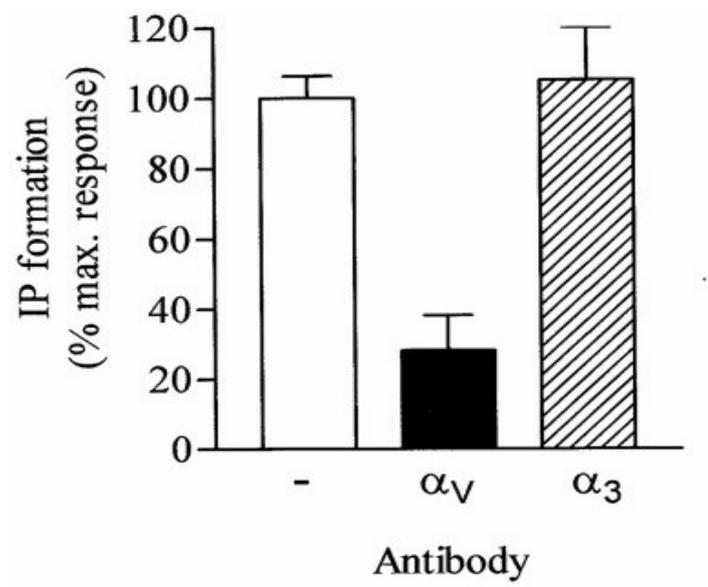
RESULTS

P2Y₂R-mediated signaling is inhibited by anti- α_V antibodies

We investigated whether an antibody raised against the α_V integrin subunit could interfere with UTP-stimulated P2Y₂R signal transduction. To analyze the effect of this antibody on the P2Y₂R calcium signaling pathway, we measured IP formation since inositol 1, 4, 5-trisphosphate (IP₃) is the second messenger for intracellular calcium mobilization (Weisman *et al.*, 1998). The results indicated that overnight treatment with anti- α_V antibody partially inhibited IP formation induced

by UTP (Figure 1), consistent with the decreased calcium response seen for the RGE mutant P2Y₂ receptor as compared with the wild-type P2Y₂R (data not shown; Erb *et al.*, 2001). Anti- α_3 integrin antibody had no effect on the ability of the P2Y₂R to stimulate calcium signaling (Figure 1). These findings suggest that interaction of α_v integrin with the P2Y₂R is important for optimum P2Y₂R activation.

Figure 1. Effect of anti-integrin antibodies on P2Y₂R-activated IP formation in 1321N1 cells. Human 1321N1 cells expressing wild-type P2Y₂Rs were incubated for 24 h with or without 100 µg/ml of the indicated antibody. The cells were then stimulated with 1 µM UTP for 10 min. Generation of IP is expressed as the percentage of maximum stimulation with UTP after background subtraction (backgrounds for control, α₃-treated, and α_v-treated samples were 18,462 ± 1,090, 15,670 ± 1,000, and 30,450 ± 3,650 dpm, respectively).



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