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

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Abstract

The heterologous expression and activation of the human P2Y₂ nucleotide receptor (P2Y₂R) in human 1321N1 astrocytoma cells stimulates α -secretase-dependent cleavage of the amyloid precursor protein (APP), causing extracellular release of the non-amyloidogenic protein sAPP α . To determine whether a similar response occurs in a neuronal cell, we analyzed whether P2Y₂R-mediated production of sAPP α occurs in rat primary cortical neurons (rPCNs). In rPCNs, P2Y₂R mRNA and receptor activity were virtually absent in quiescent cells, whereas overnight treatment with the pro-inflammatory cytokine interleukin-1 β (IL-1 β) upregulated both P2Y₂R mRNA expression and receptor activity by four-fold. The upregulation of the P2Y₂R was abrogated by pre-incubation with Bay 11-7085, an I κ B- α phosphorylation inhibitor, which suggests that P2Y₂R mRNA transcript levels are regulated through NF- κ B signaling. Furthermore, the P2Y₂R agonist UTP enhanced the release of sAPP α in rPCNs treated with IL-1 β or transfected with P2Y₂R cDNA. UTP-induced release of sAPP α from rPCNs was completely inhibited by pretreatment of the cells with the metalloproteinase inhibitor TAPI-2 or the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, and was partially inhibited by the MAPK/ERK kinase (MEK) inhibitor U0126 and the protein kinase C (PKC) inhibitor GF109203. These data suggest that P2Y₂R-mediated release of sAPP α from cortical neurons is directly dependent on ADAM10/17 and PI3K activity, whereas ERK1/2 and PI3K activity may indirectly regulate APP processing. These results demonstrate that elevated levels of pro-inflammatory cytokines associated with neurodegenerative diseases, such as IL-1 β , can enhance non-amyloidogenic APP processing through upregulation of the P2Y₂R in neurons.

INTRODUCTION

Inflammation is a central component of several chronic neurological diseases including Alzheimer's disease (Skaper, 2007). Various inflammatory mediators, such as cytokines, chemokines, proteases and protease inhibitors, have been detected in the brains of patients with Alzheimer's disease (Parihar and Hemnani, 2004). Interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) are rapidly upregulated in a variety of tissues in response to pro-

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inflammatory stimuli and amplify downstream signaling cascades that mediate inflammation (Stoll *et al.*, 2002). In Alzheimer's disease, IL-1 β regulates the increased expression and processing of amyloid precursor protein (APP) (Griffin *et al.* 1994), the phosphorylation of tau (Sheng *et al.*, 2000), the induction of secretory phospholipase in astrocytes (Moses *et al.*, 2006) and the increased production of acetylcholinesterase (AChE) in neurons (Li *et al.*, 2000; Sue and Griffin, 2006). These inflammatory processes result in neuronal stress and injury, which in turn promotes microglial cell activation and IL-1 β overexpression in a self-propagating cycle (reviewed by Standridge, 2006). Recent studies, however, indicate that certain aspects of the inflammatory response, specifically constitutive IL-1 β production, may have therapeutic potential for alleviating amyloid pathologies (Wyss-Coray *et al.*, 2001; Shafteel *et al.*, 2007).

Nucleotides co-released with neurotransmitters can activate a widely distributed family of nucleotide receptors in the central nervous system (CNS). P2 nucleotide receptors (*i.e.*, ionotropic P2X receptors 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, and neuronal cell apoptosis and survival (Burnstock, 2000; Ciccarelli *et al.*, 2001; Weisman *et al.*, 2005). The P2Y₂ nucleotide receptor (P2Y₂R) is distinguished among other human P2 receptors by its ability to be activated equipotently by ATP and UTP. The P2Y₂R is upregulated in response to stress or injury in various cell types, including activated thymocytes, salivary gland epithelial cells, and vascular smooth muscle and endothelial cells (Koshiba *et al.*, 1997; Turner *et al.*, 1997; Seye *et al.*, 1997 & 2002).

Previously, we demonstrated that the heterologously expressed G protein-coupled P2Y₂R mediates the α -secretase-dependent release of sAPP α from human 1321N1 astrocytoma cells that are devoid of endogenous P2Y receptors (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 protein called sAPP α , and essentially precludes the generation of neurotoxic A β peptide from the same APP molecule (Kojro and Fahrenholz, 2005). Several zinc metalloproteinases, including a disintegrin and metalloproteinase (ADAM) 9 and 10, tumor necrosis factor- α convertase (TACE/ADAM17), and the aspartyl protease β -secretase beta-site APP cleaving enzyme (BACE2) can cleave APP at the α -secretase site (Allinson *et al.*, 2003). While the proteolytic cleavage of APP is constitutive, it can also be increased through activation of protein kinase C (PKC) or other signaling pathways by substances such as G protein-coupled receptor (GPCR) agonists (Nitsch *et al.*, 1997; Kirazov *et al.*, 1997). It has been postulated that the proteolytic products of APP can modulate 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 whether P2Y₂R activation can modulate 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 Neurobasal medium (B27 medium without cortex antioxidants) were obtained from Gibco-BRL (Carlsbad, CA). Rabbit anti-rat extracellular signal-regulated kinase 1 and 2 (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 sAPP α 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 Plus Mini Kit was obtained from Qiagen (Chatsworth, CA). The First Strand cDNA Synthesis Kit was obtained from Roche (Indianapolis, IN). Real-time polymerase chain reaction (PCR) was performed on an Applied Biosystems 7500 Real-Time PCR machine with TaqMan Gene Expression assay probes (*P2Y₂R* NM_017255.1 and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*, NM_017008.3) and TaqMan Universal PCR Master Mix (2X) (Foster City, CA). The GenCarrier^{TM}2 Transfection Reagent Kit was obtained from Epoch Biolabs (Sugar Land, TX). Nucleotides and all other biochemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Primary cell culture of cortical neurons

Experimental procedures for cell cultures of 95% pure 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 HGD MEM 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 HGD MEM. The resulting cell suspension was filtered through a sterilized 75 mm cell strainer (Becton Dickinson, Franklin Lakes, NJ) and the cells were seeded on plastic culture plates precoated with poly-D-lysine (0.1 μ g/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 Neurobasal medium, and Neurobasal medium to 500 ml) and 15 μ g/ml 5-fluoro-2'-deoxyuridine was added to retard glial cell proliferation. The neurons were used for experiments after 7 days in culture (DIV7). Unless otherwise stated, DIV7 cells were treated for 24 h at 37 °C in serum-free DMEM with or without IL-1 β at the indicated concentration. The next day, cells were either harvested or the medium was replaced with fresh serum-free DMEM containing UTP, PMA, or other compounds, as indicated in the figure legends.

Real-Time and Reverse Transcription-PCR analysis of *P2Y₂R* mRNA expression

Total RNA was isolated from rPCNs using the RNeasy Plus Mini Kit (Qiagen). cDNA was synthesized from 500 ng purified RNA using the First Strand cDNA Synthesis Kit for RT-PCR (AMV; Roche). Specific oligonucleotide primers were designed to selectively amplify cDNA for *P2Y₂R* and *GAPDH*, as previously described (Wang *et al.*, 2005). The amplification was performed using 35 cycles of denaturation for *P2Y₂R* and 30 cycles for *GAPDH* at 95 °C for 30 sec, with annealing at 60 °C for 30 sec 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. Ten percent of the synthesized cDNA was used as a template in a 50 μ l real-time PCR. For TaqMan quantitative real-time PCR analysis, the probes were labeled on the 5' end with 6-carboxy-fluorescein phosphoramidite (FAM) (for *P2Y₂R*) or VIC (for *GAPDH*), and at the 3' end with minor groove binder (MGB) dye as the quencher. The *GAPDH* gene was used as a stable endogenous control. The samples were run in quadruplicate for the *P2Y₂R* target and the endogenous *GAPDH* control. After computing the relative amounts of target gene and

endogenous control for each sample, the final amount of target gene in the sample was calculated as a ratio of the amounts of *P2Y₂R* to *GAPDH*, using Applied Biosystems software. Relative mRNA levels of the control were normalized to 1.

Single cell calcium assay

The intracellular free calcium concentration, $[Ca^{2+}]_i$, was quantified in single cells using the Ca^{2+} -sensitive fluorescent dye fura-2, and the 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, $CaCl_2$ 2, $MgCl_2$ 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 undetectable levels of endogenous P2Y₂R were transiently transfected with 1 µg of human P2Y₂ receptor cDNA using the GenCarrier™2 Transfection Reagent Kit (Epoch Biolabs), according to the manufacturer's instructions. The transiently-transfected cells were cultured for an additional 24–48 h in Neurobasal medium plus B27-AO Neurobasal medium and used to detect sAPPα release.

Western blot analysis

rPCNs were plated on 6-well plates and grown until DIV7. Cells were serum-starved and incubated in 1 ml of serum-free DMEM with or without various compounds for specified time periods, as indicated in the figure legends. At the end of the incubation period, the medium was collected and centrifuged at $12,000 \times g$ for 5 min to remove cellular debris. The supernatant, representing conditioned medium, was stored at –80 °C until further use. To measure the level of secreted APPα, 200 µl of supernatant were 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). The cells were washed twice with ice-cold PBS, and lysed in 2X Laemmli sample buffer (200 µl/well).

Cell lysate and conditioned medium were heated for 5 min at 65 °C, 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-rat actin antibodies for 2 h at room temperature, or 1:1000 dilution of anti-sAPPα antibodies 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 sAPPα release were expressed as a percentage of controls (*i.e.*, total kinase or actin, respectively).

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 5.0. Statistical significance was determined by one-way ANOVA followed by Newman-Keuls multiple comparison tests or 2-way ANOVA followed by Bonferroni post-tests. Differences were considered to be statistically significant when $p < 0.05$ (*) and not significant when $p > 0.05$ (n.s.).

RESULTS

Upregulation of functional P2Y₂Rs in rat primary cortical neurons (rPCNs) 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). 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 real-time PCR analysis to examine whether this cytokine could also increase P2Y₂R mRNA expression in rPCNs. After overnight incubation with IL-1 β (0–100 ng/ml), P2Y₂R mRNA expression was upregulated in a dose-dependent manner (Figures 1A and B). IL-1 β did not significantly increase the expression of P2Y₄R or P2Y₆R mRNA (data not shown). Furthermore, P2Y₂R mRNA upregulation was abrogated in a concentration-dependent manner by a 30 min pre-incubation with Bay 11–7082 (Figure 1B), an I κ B- α phosphorylation inhibitor (Pierce *et al.*, 1997), which suggests that I κ B/NF- κ B signaling pathway regulates transcriptional activation of the P2Y₂R. Stimulation of IL-1 β -treated rPCNs with the P2Y₂R agonist UTP caused an increase in [Ca²⁺]_i (Figure 1C) and activation of the mitogen-activated protein kinases (MAPKs) ERK1/2 (Figures 2A and B), indicating that functional expression of the P2Y₂R is also upregulated by IL-1 β in rPCNs. Co-incubation of rPCNs with TNF- α and IL-1 β did not further enhance P2Y₂R-mediated calcium mobilization (data not shown) or ERK1/2 phosphorylation as compared to IL-1 β alone (Figures 2C and D).

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

Overnight treatment of rPCNs with IL-1 β (1–100 ng/ml) caused a dose-dependent increase in sAPP α release when IL-1 β treated neurons were stimulated with UTP (100 μ M) (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 TACE/ADAM17 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 inhibited by the selective metalloproteinase inhibitor TAPI-2 (Figure 5), suggesting that P2Y₂R-mediated APP processing in rPCNs is likely mediated by ADAM10/17.

P2Y₂R-mediated sAPP α release is partially dependent on 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 in rPCNs is dependent on activation of PKC. The general PKC inhibitor, GF109203, partially inhibited UTP-induced sAPP α release in IL-1 β -treated neurons (Figure 6). Similarly, GF109203 significantly inhibited PMA-induced sAPP α release (Figure 6). These results indicate that P2Y₂R-mediated sAPP α release is partially dependent on PKC.

P2Y₂R-mediated sAPP α release is dependent on PI3K

The release of sAPP α induced by insulin requires activation of the PI3K/Akt pathway (Solano *et al.*, 2000). Since P2Y₂Rs activate PI3K in HeLa cells, endothelial cells and rat primary astrocytes (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. As shown in Figure 7, incubation of IL-1 β -treated rPCNs with the selective PI3K inhibitor LY294002 completely inhibited UTP-induced sAPP α release, but had no effect on constitutive release of sAPP α in IL-1 β -treated rPCNs. These results suggest that UTP-induced sAPP α release in rPCNs is mediated through the PI3K/Akt signaling pathway.

P2Y₂R-mediated release of sAPP α is partially dependent on PKC

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 expressing human P2Y₂R cDNA (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 (1 μ M), an inhibitor of the MAPK/ERK kinase (MEK), partially inhibited UTP-induced sAPP α release, but not constitutive release of sAPP α in IL-1 β -treated rPCNs (Figure 8). These results suggest that P2Y₂R-mediated sAPP α release in neurons is partially dependent on ERK1/2 activation.

DISCUSSION

In the present study, we demonstrated that the pro-inflammatory cytokine IL-1 β upregulated expression of the P2Y₂R via the I κ B- α /NF- κ B signaling pathway in rat primary cortical neurons. Furthermore, we showed that activation of the P2Y₂R in IL-1 β -treated neurons enhanced the release of sAPP α , a non-amyloidogenic 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.

Previous studies have demonstrated that P2Y₂R mRNA expression and function are upregulated in stressed or injured tissue in salivary gland epithelium (Turner *et al.*, 1998, Ahn *et al.*, 2000, Schrader *et al.*, 2005), in intimal lesions of rat aorta (Seye *et al.*, 1997) and in endothelial and smooth muscle cells of collared rabbit carotid arteries (Seye *et al.*, 2002). P2Y₂R mRNA expression is also upregulated in smooth muscle cells by IL-1 β (Hou *et al.*, 2000), a cytokine that mediates CNS inflammation in a mouse model of Alzheimer's disease (Brugg *et al.*, 1995; Hauss-Wegrzyniak *et al.*, 1998). Furthermore, activation of the P2Y₂R expressed in human 132N1 astrocytoma cells induces sAPP α release (Camden *et al.*, 2005). The present study demonstrates for the first time that IL-1 β -mediated P2Y₂R upregulation and subsequent P2Y₂R-mediated sAPP α release occurs in primary neurons. These results are consistent with reports that chronic inflammatory responses can enhance microglial cell-mediated amyloid-beta clearance and reduce plaque burden in murine models of AD (Wyss-Coray *et al.*, 2001; Shaftel *et al.*, 2007).

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 at least three proteases 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 ($A\beta$), the main component of senile plaques found in the brains of patients with Alzheimer's disease (Martins *et al.*, 1991). Cleavage of APP by α - and γ -secretases causes the release of sAPP α , thereby preventing secretion of amyloidogenic $A\beta$ (Furukawa *et al.* 1996; Mattson, 1997; Kojro and Fahrenholz, 2005). Although sAPP α is constitutively secreted, release of this protein is modulated by neural activity and can be increased by agonists of various receptors (Robert *et al.*, 2001; Canet-Aviles *et al.*, 2002; Camden *et al.*, 2005). The studies presented here show that both IL-1 β -induced upregulation of P2Y₂Rs in primary neurons (Figure 3) and transfection of neurons with P2Y₂R cDNA (Figure 4) significantly increase UTP-stimulated sAPP α release, suggesting an important physiological role for the P2Y₂R in Alzheimer's disease pathology. IL-1 β alone also significantly enhances sAPP α release and reduces sAPP β and $A\beta_{40/42}$ release in human neuroblastoma SK-N-SH cells (Tachida *et al.*, 2008), although IL-1 β alone did not enhance sAPP α release in rPCNs (Figure 3).

Responses to extracellular UTP in mammalian tissues can potentially be mediated by 3 P2Y receptor subtypes, P2Y₂, P2Y₄, and/or P2Y₆ (Brunschweiler and Müller, 2006). P2Y₄ receptors are endogenously expressed in rat neurons and have an agonist potency order of ITP = ATP = UTP = ATP γ S = 2-MeSATP = Ap₄A (Bogdanov *et al.*, 1998). Therefore, it is reasonable to suggest that P2Y₄Rs could contribute to the enhanced release of sAPP α evoked by UTP. In primary neurons without IL-1 β treatment, P2Y₄R mRNA is expressed at higher levels than P2Y₂R mRNA (data not shown). However, there have been no studies to suggest that the P2Y₄R can activate metalloproteinases, whereas P2Y₂R activation has been shown to stimulate ADAM10/17 activity (Camden *et al.*, 2005). The small percentage of rPCNs responding to UTP with an increase in $[Ca^{2+}]_i$ in the absence of IL-1 β stimulation (Figure 1C) may be due to P2Y₄R activation. Nonetheless, in the absence of IL-1 β pre-treatment, UTP did not significantly increase sAPP α release from rPCNs (Figure 3), suggesting that the P2Y₄R does not contribute to APP processing. Moreover, since IL-1 β does not increase P2Y₄ or P2Y₆ receptor mRNA expression in rPCNs (data not shown), it is most likely that the P2Y₂R mediates UTP-induced sAPP α release in rPCNs treated with IL-1 β .

There are three major isoforms of amyloid precursor protein (APP₇₇₀, APP₇₅₁ and APP₆₉₅; Kitaguchi *et al.*, 1988) resulting from alternative splicing of APP mRNA. APP₆₉₅ is the predominant isoform in the brain (Tanaka *et al.*, 1989), and 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 that express high levels of APP₆₉₅, astrocytes synthesize relatively higher levels of APP₇₇₀ and APP₇₅₁, as expected for these Kunitz protease inhibitor (KPI) containing isoforms. In our study, soluble APP α released into the UTP-containing medium of P2Y₂R-expressing rPCNs was detected as a doublet of 100–120 kDa (data not shown), as has been described for proteolytic degradation of APP 695 (Johnson *et al.*, 1988). These results support the notion that soluble APP α released from rPCNs is not due to glial cell contamination in the neuronal preparation (LeBlanc *et al.*, 1991 & 1996 & 1997; Lahiri *et al.*, 1994; Parvathy *et al.*, 1998; Solano *et al.*, 2000; Ma *et al.*, 2005).

The data presented here also suggest that UTP induces APP processing in IL-1 β -treated rPCNs through activation of the metalloproteinases ADAM10/17 (Figure 5). Another enzyme that is inhibited by TAPI-2, TNF-converting enzyme, is not involved in the regulated release of sAPP α from neuronal cells (Parkin *et al.*, 2002).

Stimulation of GPCRs has been shown to regulate APP processing by protein kinase C (PKC)-dependent signaling pathways (Nitsch *et al.*, 1992). However, APP processing can

also be regulated by increases in $[Ca^{2+}]_i$, and activation of phospholipase A2 (PLA2), protein kinase A and tyrosine kinases (reviewed in Mills and Reiner, 1999). In human 1321N1 astrocytoma cells expressing human P2Y₂R cDNA, UTP-induced sAPP α release was independent of P2Y₂R-mediated activation of PKC (Camden *et al.*, 2005). Our results with pharmacological inhibitors of PI3K, PKC and ERK1/2 indicated that P2Y₂R-mediated sAPP α release from IL-1 β -treated neurons requires PI3K activity (Figure 7) and is partially dependent on PKC and ERK1/2 activation (Figure 6 and Figure 8). The MAPK/ERK1/2 signaling pathway has been implicated in the regulation of both PKC-dependent and PKC-independent APP catabolism in PC12 cells, human embryonic kidney cells, and cortical neurons (Mills *et al.*, 1997; Desdouits-Magnen *et al.*, 1998). PI3K has also been reported to regulate α -secretase-dependent APP processing (Solano *et al.*, 2000; Fu *et al.*, 2002), and IL-1 β increased the release of sAPP α via ERK1/2-dependent activation of α -secretase cleavage in neuroglioma U251 cells independent of PI3K (Ma *et al.*, 2005). In SH-SY5Y cells, sAPP α release is induced by muscarinic receptor activation and regulated by arachidonic acid generation via PLA2 activation (Cho *et al.*, 2006). In HEK239 cells, a direct role for G $\alpha_{q/11}$ protein in the regulation of muscarinic M3 receptor-mediated sAPP α release was shown (Kim and Kim, 2005). Thus, there is significant divergence in agonist-induced signaling pathways that regulate sAPP α release in different cell types.

The results of this study strongly suggest that IL-1 β via activation of the I κ B/NF- κ B pathway induces functional upregulation of the P2Y₂R in primary cortical neurons and subsequent activation of the P2Y₂R signaling cascade by UTP results in a significant increase in ADAM10/17-mediated sAPP α release which is dependent on PI3K and partially dependent on PKC and ERK1/2 activities. Together, these lines of evidence suggest that under inflammatory conditions in the brain where IL-1 β levels are increased (Rothwell and Luheshi, 2000; Allan and Rothwell, 2001; Caquevel *et al.*, 2004; Colangelo *et al.*, 2003), functional P2Y₂R expression in neuronal cells may serve a neuroprotective role by promoting non-amyloidogenic APP processing. Since P2Y₂R expression in glial cells has been postulated to regulate chronic inflammatory responses seen in Alzheimer's disease (Weisman *et al.*, 2005), future studies are required to evaluate how glial cell-mediated chronic inflammation can be used to promote neuroprotective sAPP α release from neurons in response to P2Y₂R activation during the progression of Alzheimer's disease.

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Fig. 1a

Fig. 1b

Fig. 1c

Figure 1. IL-1 β upregulates P2Y₂ receptor mRNA and function in rPCNs

(A) Rat primary cortical neurons (rPCNs) at DIV7 were treated with the indicated concentration of IL-1 β for 24 h at 37 °C in serum-free DMEM and P2Y₂R mRNA levels were detected by real-time PCR using GAPDH as a control. RT + or – indicates usage of reverse transcriptase in the PCR. (B) The effect on IL-1 β -induced P2Y₂R mRNA expression of a 30 min pretreatment of rPCNs with the indicated concentration of the I κ B- α phosphorylation inhibitor Bay11 is shown. Real-time PCR data was expressed as a ratio of P2Y₂R to GAPDH mRNA levels (n=6). Bay11 preincubation alone did not significantly change P2Y₂R mRNA expression levels as compared to the untreated control (data not shown). * represents significant differences ($p < 0.05$), as compared to control conditions, and # represents significant differences ($p < 0.05$), as compared to rPCNs treated with 100 ng/ml IL-1 β . (C) IL-1 β -treated cells on coverslips were loaded with fura-2 and coverslips were positioned on the stage of an inverted epifluorescence microscope for measurement of $[Ca^{2+}]_i$, as described in the “Materials and Methods”. Then, rPCNs were incubated in PSS with UTP (100 μ M), and the time-dependent increase in $[Ca^{2+}]_i$ was determined. Results are expressed as the percentage of rPCNs responding to UTP. Data represent the means $\pm$ S.E.M. of results from at least three experiments where “n.s.” represents no significant differences ($p > 0.05$) and * represents significant differences ($p < 0.05$), as compared to rPCNs treated without IL-1 β .

Fig. 2a

Fig. 2b

Fig. 2c

Fig. 2d

Figure 2. UTP stimulates phosphorylation of ERK1/2 in IL-1β-treated rPCNs

rPCNs were pretreated with the indicated concentration of IL-1β and/or TNF-α in serum-free DMEM for 24 h at 37 °C. Then, the medium was removed and cells were incubated for 10 min in serum-free DMEM with or without 100 μM UTP at 37 °C. (A, C) Cell lysates were subjected to Western blot analysis for phosphorylated ERK1/2 and total ERK1/2. (B, D) UTP-induced ERK1/2 phosphorylation was expressed as the ratio of phosphorylated ERK1/2 to total ERK1/2 after subtracting the basal level of ERK1/2 phosphorylation in the absence of UTP. The increase of ERK1/2 phosphorylation induced by IL-1β and/or TNF-α in rPCNs was expressed as a ratio of the UTP-stimulated rPCNs in the absence of cytokine pretreatment. Data represent the means ± S.E.M. of results from three experiments where “n.s.” represents no significant differences and * represents significant differences ($p < 0.05$), as compared to rPCNs treated without cytokines.

Fig. 3a

Fig. 3b

Figure 3. UTP induces sAPP α release in rPCNs pre-treated with IL-1 β

rPCNs were pretreated with the indicated concentration of IL-1 β in serum-free DMEM for 24 h at 37 °C. Then, the medium was removed and cells were incubated for 2 h at 37 °C in serum-free DMEM with or without 100 μ M UTP or 1 μ M PMA, a positive control. (A) The medium was collected and assayed by Western blot analysis for sAPP α release, whereas the actin level in cell lysate was determined as a control. (B) UTP- and PMA-induced sAPP α release in rPCNs with or without IL-1 β pretreatment is expressed relative to the actin level in cell lysate, after subtracting basal sAPP α release in the absence of IL-1 β and UTP 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.

Fig. 4a

Fig. 4b

Figure 4. P2Y₂ receptor expression in rPCNs enhances α -secretase-mediated APP processing induced by UTP

On DIV5, rPCNs were transfected with human P2Y₂R cDNA (– indicates untransfected controls). On DIV7, the neurons were incubated for 2 h in serum-free DMEM with or without 100 μ M UTP or 1 μ M PMA at 37 °C. (A) sAPP α release into the medium was determined by Western blot analysis. (B) sAPP α release is expressed relative to the actin level in cell lysate. 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.

Fig. 5a

Fig. 5b

Figure 5. A metalloprotease inhibitor prevents UTP-induced sAPP α release from rPCNs
rPCNs were pretreated with 25 ng/ml IL-1 β in serum-free DMEM for 24 h at 37 °C. The medium was replaced with fresh serum-free DMEM and the cells were incubated at 37 °C for 30 min with or without the metalloprotease inhibitor TAPI-2 (10 μ M) followed by a 2 h incubation with or without UTP (100 μ M). (A) The release of sAPP α into the medium was analyzed by Western blot analysis. (B) sAPP α release is expressed relative to the actin level in cell lysate. 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 analyzed by 2-way ANOVA followed by Bonferroni post-tests.

Fig. 6a

Fig. 6b

Figure 6. UTP-induced sAPPα release is partially dependent on PKC activation in IL-1β-treated rPCNs

rPCNs were pretreated with 25 ng/ml IL-1β in serum-free DMEM for 24 h at 37 °C. The medium was replaced with fresh serum-free DMEM and the cells were incubated at 37 °C for 30 min with or without GF109203 (10 μM) followed by a 2 h incubation with or without UTP (100 μM) or PMA (1 μM). (A) The release of sAPPα into the medium was analyzed by Western blot analysis. (B) sAPPα release is expressed relative to the actin level in cell lysate. Data represent the means ± S.E.M. of results from three experiments, where * represents significant differences ($p < 0.05$) between the indicated sample groups analyzed by 2-way ANOVA followed by Bonferroni post-tests.

Fig. 7a

Fig. 7b

Figure 7. Inhibition of PI3K prevents UTP-induced sAPP α release in IL-1 β -treated rPCNs
rPCNs were pretreated with 25 ng/ml IL-1 β in serum-free DMEM for 24 h at 37 °C. The medium was replaced with fresh serum-free DMEM and the cells were incubated at 37 °C for 30 min with or without LY294002 (1 μ M) followed by a 2 h incubation with or without UTP (100 μ M). (A) The release of sAPP α into the medium was analyzed by Western blot analysis. (B) sAPP α release is expressed relative to the actin level in cell lysate. 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 analyzed by 2-way ANOVA followed by Bonferroni post-tests.

Fig. 8a

Fig. 8b

Figure 8. UTP-stimulated sAPP α release in IL-1 β -treated rPCNs is partially dependent on ERK1/2 activation

rPCNs were pretreated with 25 ng/ml IL-1 β in serum-free DMEM for 24 h at 37 °C. The medium was replaced with fresh serum-free DMEM and the cells were incubated at 37 °C for 30 min with or without U0126 (1 μ M) followed by a 2 h incubation with or without UTP (100 μ M). (A) The release of sAPP α into the medium was analyzed by Western blot analysis. (B) sAPP α release is expressed relative to the actin level in cell lysate. 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 analyzed by 2-way ANOVA followed by Bonferroni post-tests.