

Cross talk between P2 purinergic receptors modulates extracellular ATP-mediated interleukin-10 production in rat microglial cells

Dong Reoyl Seo^{1*}, Soo Yoon Kim^{1,2*},
Kyung You Kim¹, Hwan Goo Lee^{1,2},
Ju Hyun Moon^{1,2}, Jae Souk Lee^{1,2},
Se Hoon Lee^{1,2}, Seung U. Kim^{3,4}
and Yong Beom Lee^{1,2,3,5}

¹Neuroscience Graduate Program

²BK21 Division of Cell Transformation and Restoration

³Brain Disease Research Center

Ajou University School of Medicine

Suwon 443-721, Korea

⁴Division of Neurology, Department of Medicine
University of British Columbia

Vancouver, British Columbia, Canada V6T 1Z3

⁵Corresponding author: Tel, 82-31-219-4558;

Fax, 82-31-216-6381; E-mail, yblee@ajou.ac.kr

*These authors contributed equally to this work.

Accepted 19 November 2007

Abbreviations: IP₃, inositol phosphates; M-MLV, Moloney murine leukemia virus; ROS, reactive oxygen species

Abstract

Previously we demonstrated that ATP released from LPS-activated microglia induced IL-10 expression in a process involving P2 receptors, in an autocrine fashion. Therefore, in the present study we sought to determine which subtype of P2 receptor was responsible for the modulation of IL-10 expression in ATP-stimulated microglia. We found that the patterns of IL-10 production were dose-dependent (1, 10, 100, 1,000 μ M) and bell-shaped. The concentrations of ATP, ATP- γ S, ADP, and ADP- β S that showed maximal IL-10 release were 100, 10, 100, and 100 μ M respectively. The rank order of agonist potency for IL-10 production was 2'-3'-O-(4-benzoyl)-benzoyl ATP (BzATP) = dATP > 2-methylthio-ADP (2-meSADP). On the other hand, 2-methylthio-ATP (2-meSATP), α,β -methylene ATP (α,β -meATP), UTP, and UDP did not induce the release of IL-10 from microglia. Further, we obtained evidence of crosstalk between P2 receptors, in a situation where intracellular Ca²⁺ release and/or cAMP-activated PKA were the main contributors to extracellular ATP-(or

ADP)-mediated IL-10 expression, and IL-10 production was down-regulated by either MRS2179 (a P2Y₁ antagonist) or 5'-AMPS (a P2Y₁₁ antagonist), indicating that both the P2Y₁ and P2Y₁₁ receptors are major receptors involved in IL-10 expression. In addition, we found that inhibition of IL-10 production by high concentrations of ATP- γ S (100 μ M) was restored by TNP-ATP (an antagonist of the P2X₁, P2X₃, and P2X₄ receptors), and that IL-10 production by 2-meSADP was restored by 2meSAMP (a P2Y₁₂ receptor antagonist) or pertussis toxin (PTX; a G_i protein inhibitor), indicating that the P2X₁, P2X₃, P2X₄ receptor group, or the P2Y₁₂ receptor, negatively modulate the P2Y₁₁ receptor or the P2Y₁ receptor, respectively.

Keywords: adenosine diphosphate; adenosine triphosphate; calcium; cyclic AMP; cyclic AMP-dependent protein kinases; inositol 3-phosphate; interleukin-10; microglia; receptors, purinergic P2

Introduction

Extracellular purine (ATP, ADP) and pyrimidine (UTP, UDP) nucleotides mediate their effects via P2 receptors (Ralevic and Burnstock, 1998). P2 receptors consist of ligand-gated intrinsic ion channels, P2X receptors, and G-protein-coupled P2Y receptors. Stimulation of P2X receptors (P2X₁-P2X₇) causes Ca²⁺ and Na⁺ influx from the extracellular space with accompanying plasma membrane depolarization (Khakh and North, 2006). The P2Y receptors are composed of eight subtypes in mammals: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄. The P2Y₁, P2Y₁₂, and P2Y₁₃ receptors are preferentially activated by ADP whereas P2Y₆ is activated by UDP (Abbracchio *et al.*, 2006). The P2Y₁₁ receptor prefers ATP as an agonist (Communi *et al.*, 1997) whereas the P2Y₂ (Lustig *et al.*, 1993) and P2Y₄ receptors are equally sensitive to ATP and UTP (Communi *et al.*, 1995). The P2Y₁₄ receptor is activated by the nucleotide sugar UDP-glucose (Brunschweiler and Muller, 2006). The P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors are coupled to G_q, promoting PLC-catalyzed generation of an inositol phosphate (IP₃) and subsequent release of intracellular calcium

(Ralevic and Burnstock, 1998). The P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors are coupled to G_{i/o}, inhibiting adenylyl cyclase (Hollopeter *et al.*, 2001).

Recent studies have suggested that activated immune cells secrete ATP into the extracellular space, to release cytokines by activation of P2 receptors (Di Virgilio and Solini, 2002). Extracellular ATP is known to be a potential mediator of inducible iNOS expression (Ohtani *et al.*, 2000) and TNF- α release in rat microglia, via P2X₇ receptors (Hide *et al.*, 2000). The presence of the P2X₇ receptor in microglia is consistent with involvement of this receptor in the inflammatory process [e.g., in the production of proinflammatory cytokines, nitric oxide, and reactive oxygen species (ROS)] and cytotoxicity (Di Virgilio *et al.*, 1999; Parvathenani *et al.*, 2003). In addition, ATP evokes the production of IL-6, a mediator of inflammation, in the microglial cell line MG-5 (Shigemoto-Mogami *et al.*, 2001), and induces maturation and release of IL-1 β by activating the IL-1 β -converting enzyme/caspase-1 (Sanz and Di Virgilio, 2000). Further, recent studies have reported that P2X₄ receptors are involved in pain transmission (Tsuda *et al.*, 2003) and that during microglial activation, P2Y₁₂ receptors, which mediate the movement of microglial projections, are down-regulated (Haynes *et al.*, 2006), but P2Y₆ receptors are up-regulated and trigger phagocytosis (Koizumi *et al.*, 2007). Together, these results suggest that P2 receptors play a critical role in brain inflammation by regulating the production of inflammatory mediators and microglial activation.

Our previous study demonstrated that ATP released from LPS-activated microglia, and metabolites of ATP, induced IL-10 expression in a process involving P2 receptors, in an autocrine fashion, indicating that P2 receptors also have a function that counteracts the effects of proinflammatory mediators by participating in the production of the anti-inflammatory cytokine IL-10 (Seo *et al.*, 2004). Therefore, we sought to determine which subtype of P2 receptors is responsible for the modulation of IL-10 expression in ATP-stimulated microglia.

Materials and Methods

Microglial cell culture

Microglial cultures were prepared from the brains of 3 day-old Sprague-Dawley rats as described previously (Kim *et al.*, 2002). Briefly, whole brains were dissected into small cubes, incubated in D-PBS (JBI, Daegu, Korea) containing 0.1% trypsin and 40 μ g/ml DNase I for 15 min at 37°C, and dissociated into single cells by gentle pipetting.

Dissociated cells were suspended in DMEM (JBI) containing 5% horse serum, 5 mg/ml glucose, 100 U/ml penicillin and 100 μ g/ml streptomycin, and plated on poly-D-lysine-coated T-75 culture flasks, and incubated at 37°C in incubator with 5% CO₂/95% air atmosphere. After 2-4 weeks of growth in flasks, microglia floating in the medium were collected and grown in separate 6- or 96-well plates.

RT-PCR analysis

To determine which subtypes of P2X and P2Y receptors are expressed by rat microglia, microglial cells (1×10^6 cells/well) were plated into 6-well plates in DMEM (JBI) containing 5% horse serum (JBI). Total RNA was extracted using Trizol (Life Technologies, Rockville, MD). Total RNA from each sample was subjected to DNase I treatment and then processed for the first strand cDNA synthesis using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies). 5 μ l of each cDNA products was amplified by PCR using specific sense and antisense primers designed from the cDNA sequences for P2 receptors: P2X₁, 5'-AGAGGCACTACTACAAGCAGAA-3' (sense) and 5'-GGTAAGGCTGTGGGAAAGA-3' (antisense); P2X₂, 5'-GAATCAGAGTGCAACCCCAA-3' (sense) and 5'-TCACAGGCCATCTACTTGAG-3' (antisense); P2X₃, 5'-TTAAGTTTGCTGGACAGGAT-3' (sense) and 5'-GTTCCCATATACCAGCACAT-3' (antisense); P2X₄, 5'-TCCCTTCTGCCCCATATTCC-3' (sense) and 5'-TTCATCTCCCCCGAAAGACC-3' (antisense); P2X₅, 5'-CGACCTGGTACTTATCTAC-CTC-3' (sense) and 5'-ACGTTTACAATGGCATTCT-3' (antisense); P2X₆, 5'-GCCCAGAGCATCCTTCT-GTTC-3' (sense) and 5'-CGTGGCTGTATGTC-CCCATC-3' (antisense); P2X₇, 5'-AACAGTGCC-ATTCTGACC-3' (sense) and 5'-GCCACCTCTGT-AAAGTTCTC-3' (antisense); P2Y₁, 5'-GGCAGG-CTCAAGAAGAAGAAT-3' (sense) and 5'-ATCAC-ACATTTCTGGGGTCTG-3' (antisense); P2Y₂, 5'-AGCTCTTTAGCCATTTTGTG-3' (sense) and 5'-CGGAAGGAGTAATAGAGGGT-3' (antisense); P2Y₄, 5'-ACTGTCTTTGCTGTCTGCTT-3' (sense) and 5'-AGACAGCTATTAGCACTGGC-3' (antisense); P2Y₆, 5'-GTCTACCGTGAGGATTTCAA-3' (sense) and 5'-CTAGGTATCGCTGGAAGCTA-3' (antisense); P2Y₁₂, 5'-CTCCACCACCTACATGTTTC-3' (sense) and 5'-AAGAGGATGCTGCAGTAGAG-3' (antisense); P2Y₁₃, 5'-TGCACTTTCTCATCCGTGGT-3' (sense) and 5'-GGCAGGGAGATGAGGAACAT-3' (antisense).

Measurement of IL-10

To evaluate the effects of ATP on IL-10 production,

microglial cells (3×10^4 cells/well) were plated into 96-well plates in DMEM containing 5% horse serum. The amount of IL-10 in the supernatant was measured by ELISA. To assess which purinergic receptor was involved in the microglial IL-10 production, microglia cells were treated with P2 receptor agonists: ATP, ADP, adenosine 5'-O-(3-thiotriphosphate) (ATP- γ -S), adenosine 5'-O-(2-thiodiphosphate) (ADP- β -S), 2-methylthio-ATP (2-meSATP), 2-methylthio-ADP (2-meSADP), α,β -methylene ATP (α,β -meATP), 2',3'-(benzoyl-4-benzoyl)-ATP (BzATP), UTP, UDP, or dATP (Sigma, St. Louis, MO); P2 receptor antagonists: trinitrophenyl-substituted ATP (TNP-ATP), adenosine 5'-triphosphate 2',3'-acyclic dialcohol (oxidized ATP; oATP), 2'-deoxy-N⁶-methyladenosine-3',5'-bisphosphate (MRS2179), 2-methylthioadenosine 5'-monophosphate (2-meSAMP), 5'-O-thiomnophosphate (5'-AMPS) (Sigma, St. Louis, MO); Ca²⁺ chelators: EGTA (Sigma, St. Louis, MO), bisaminophenoxyethane tetraacetic acid-acetoxymethyl ester (BAPTA-AM; Calbiochem, San Diego, CA); IP₃ inhibitor, Xes-C (Sigma, St. Louis, MO); adenylate cyclase inhibitor, SQ22536 (Sigma, St. Louis, MO); PKA inhibitor, H-89 (Sigma, St. Louis, MO); G_i protein inhibitor, pertussis toxin (PTX; Sigma, St. Louis, MO). For IL-10 assay, we used Cytosets kit for rat IL-10 (Biosource, Camarillo, CA) according to the manufacturer's protocol.

Statistical analysis

All statistical comparisons in this study were performed using one-way ANOVA with the Tukey-Kramer multiple comparisons test, and data are expressed

as mean \pm SEM of triplicate samples. A value of $P < 0.05$ was considered statistically significant.

Results

Characterization of ATP-(or ATP- γ -S)-induced IL-10 release, and ADP-(or ADP- β -S)-induced IL-10 release

To characterize IL-10 expression by ATP-stimulated microglia, microglial cells were treated with various concentrations (1, 10, 100, 1,000 μ M) of ATP, ADP, ATP- γ -S (a hydrolysis-resistant analog of ATP), or ADP- β -S (a hydrolysis-resistant analog of ADP). We found that the patterns of IL-10 production were dose-dependent and bell-shaped (Figure 1). Interestingly, the concentrations of ATP and ATP- γ -S that showed maximal IL-10 release were different. ATP-induced IL-10 release peaked at an ATP concentration of 100 μ M (811.51 ± 29.59 pg/ml IL-10) and was sustained to 1,000 μ M (750.15 ± 5.66 pg/ml). On the other hand, ATP- γ -S-induced IL-10 release peaked at an ATP- γ -S concentration of 10 μ M (930.65 ± 30.94 pg/ml) but dropped to 480.88 ± 18.52 pg/ml at 100 μ M ($P < 0.01$). In the case of ADP, ADP-induced or ADP- β -S-induced IL-10 release peaked at a concentration of 100 μ M, but treatment with 1,000 μ M ADP (IL-10 release of 186.27 ± 20.70 pg/ml) or 1,000 μ M ADP- β -S (IL-10 release of 475.10 ± 30.96 pg/ml) appeared to induce less IL-10 release than did treatment with 100 μ M ADP (485.26 ± 20.33 pg/ml) or 100 μ M ADP- β -S (721.43 ± 35.20 pg/ml) ($P < 0.01$). These results indicate that 100

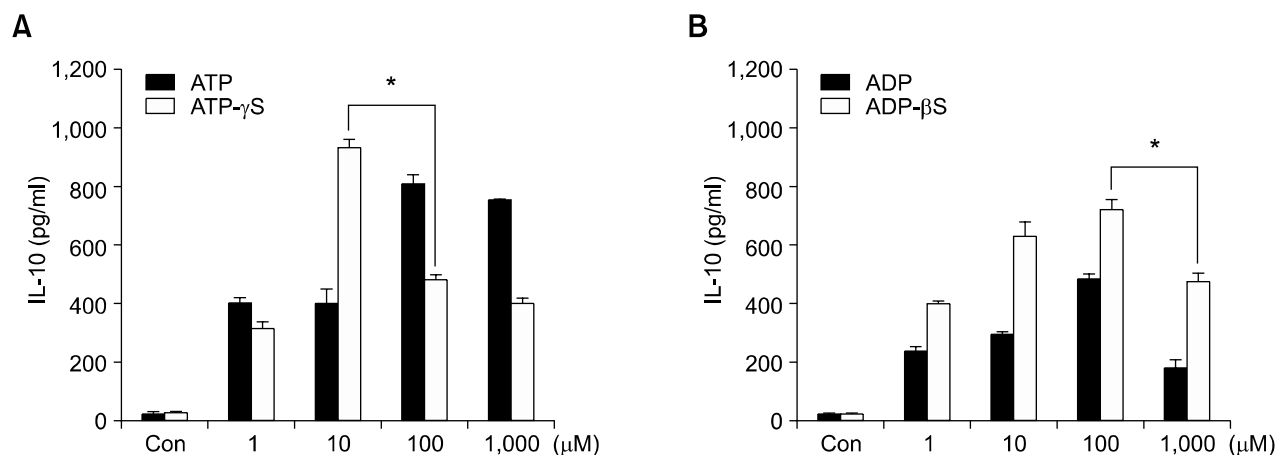


Figure 1. Characterization of ATP (or ATP- γ -S)- or ADP (or ADP- β -S)-induced IL-10 release. Microglial cells (3×10^4 cells/well) were treated with (A) ATP or ATP- γ -S, (B) ADP or ADP- β -S at the indicated concentrations. The amount of IL-10 was measured 24 h after treatment with ATP (ATP- γ -S) or ADP (ADP- β -S). Data shown are mean \pm SEM of triplicate samples. The figure shows one representative of five independent experiments. * $P < 0.01$ compared to 10 μ M ATP- γ -S or 100 μ M ADP- β -S.

μM ATP- γS or 1,000 μM ADP- βS inhibit IL-10 production by affecting distinct subtypes of the P2 receptor involved in IL-10 expression. We found no loss of cell viability in the presence of either 100 μM ATP- γS or 1,000 μM ADP- βS (data not shown).

Expression of mRNAs encoding the P2X and P2Y receptors

To determine which subtypes of P2X and P2Y

receptors are expressed by rat microglia, mRNA was isolated from microglial cells and analyzed by RT-PCR. Amplified PCR products of the expected sizes were obtained for P2X₁ (434 bp), P2X₃ (272 bp), P2X₄ (489 bp), and P2X₇ (358 bp) receptor mRNAs (Figure 2A). Similarly, amplified PCR products of the expected sizes were obtained for the P2Y₁ (411 bp), P2Y₂ (244 bp), P2Y₄ (149 bp), P2Y₆ (325 bp), P2Y₁₂ (168 bp), and P2Y₁₃ (185 bp) receptor mRNAs from microglial cell total mRNA (Figure 2B). A recent study reported that microglia express various receptors for ATP including both P2X receptors (P2X₃, P2X₄, P2X₅, P2X₇) and P2Y receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₂, P2Y₁₃) (Light *et al.*, 2006). At this point, we could not test the expression of P2Y₁₁ receptor, because rat P2Y₁₁ receptor has not been cloned.

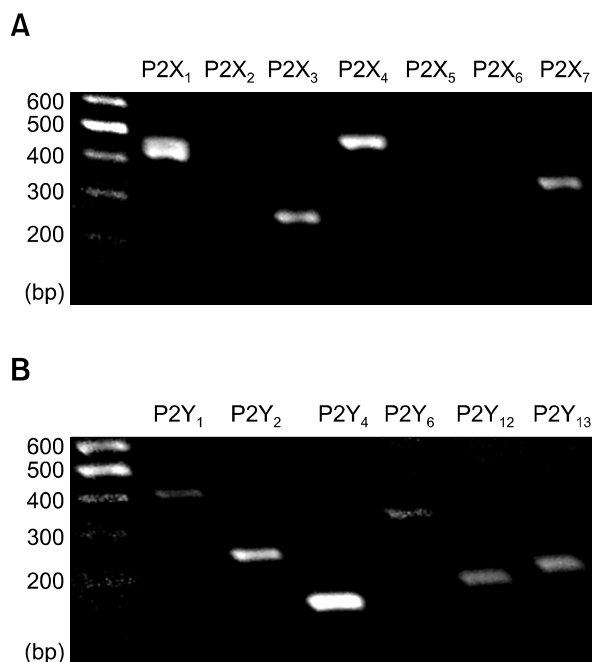


Figure 2. Expression of P2X and P2Y receptors mRNA. RT-PCR analysis of P2 receptor mRNA expression in microglial cells was done with primers specific for distinct P2 receptors subtypes. cDNA products were analyzed by 1.5% agarose gel electrophoresis. A representative gel with ethidium bromide-stained cDNA fragments of the P2 receptors.

Effects of P2 receptor agonists on the release of IL-10 from microglial cells

We next examined the effects of various concentrations (1, 10, 100, 300, 1,000 μM) of agonists (2-meSATP, 2-meSADP, α,β -meATP, BzATP, UTP, UDP, dATP) of the microglia-expressed P2 receptors (identified by RT-PCR) on the release of IL-10 from microglia. The agonists 2-meSADP, BzATP, and dATP increased the release of IL-10 in a dose-dependent manner, but 2-meSATP, α,β -meATP, UTP, and UDP did not (Figure 3). 2-meSADP and BzATP showed the bell-shaped paradigm which has maximal IL-10 release at 100 μM . On the other hand, dATP-induced IL-10 release was sustained as agonist concentrations rose to 1 mM. The rank order of agonist potency was BzATP = dATP > 2-meSADP. Because 2-meSADP is an agonist for both P2Y₁ and P2Y₁₂ receptors, and both P2X₇ agonist BzATP and dATP are potent P2Y₁₁ receptor agonists, these results indicate that purinergic modulation of IL-10 ex-

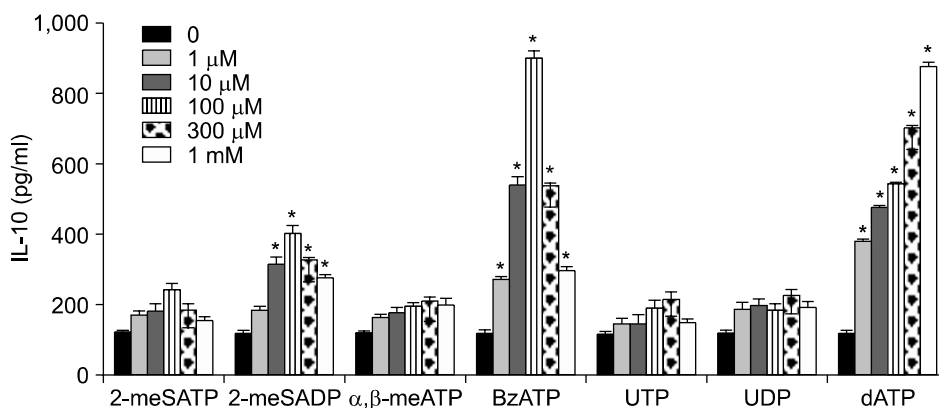


Figure 3. Effects of the P2 receptor agonist on the release of IL-10 from microglial cells. Microglial cells (3×10^4 cells/well) were treated with 2-meSATP, 2-meSADP, α,β -meATP, BzATP, UTP, UDP, and dATP at the indicated concentrations. The amount of IL-10 was measured 24 h after treatment. Data shown are mean \pm SEM of triplicate samples. The figure shows one representative of five independent experiments. * P < 0.01 compared to control.

pression may be mediated by P2X₇, P2Y₁, P2Y₁₁, and P2Y₁₂ receptors.

Effects of P2 receptor antagonists on the release of IL-10 from microglial cells

To determine which subtype of P2 receptor was responsible for the modulation of IL-10 expression, we then examined the effects of the specific antagonist of the P2X₇, P2Y₁, P2Y₁₁, or P2Y₁₂ receptors, and an antagonist of the P2X₁, P2X₃, and P2X₄ receptors. ATP- γ S (100 μ M)-induced IL-10 release was restored by TNP-ATP (an antagonist of the P2X₁, P2X₃, and P2X₄ receptors) (Figure 4A).

2-meSADP (100 μ M)-induced IL-10 release was inhibited by MRS2179 (a P2Y₁ antagonist), but 2meSAMP (a P2Y₁₂ antagonist) up-regulated 2-meSADP (100 μ M)-induced IL-10 release (Figure 4B). On the other hand, BzATP (300 μ M)-induced IL-10 release was not restored by oATP (a P2X₇ antagonist) (Figure 4C). BzATP (100 μ M)-induced IL-10 release was inhibited by 5'-AMPS (a P2Y₁₁ antagonist) (Figure 4D). These results indicate that both the P2Y₁ and P2Y₁₁ receptors are major receptors involved in IL-10 expression, and that the P2X₁, P2X₃, P2X₄ receptor group, or the P2Y₁₂ receptor, negatively modulates the P2Y₁₁ receptor or the P2Y₁ receptor, respectively.

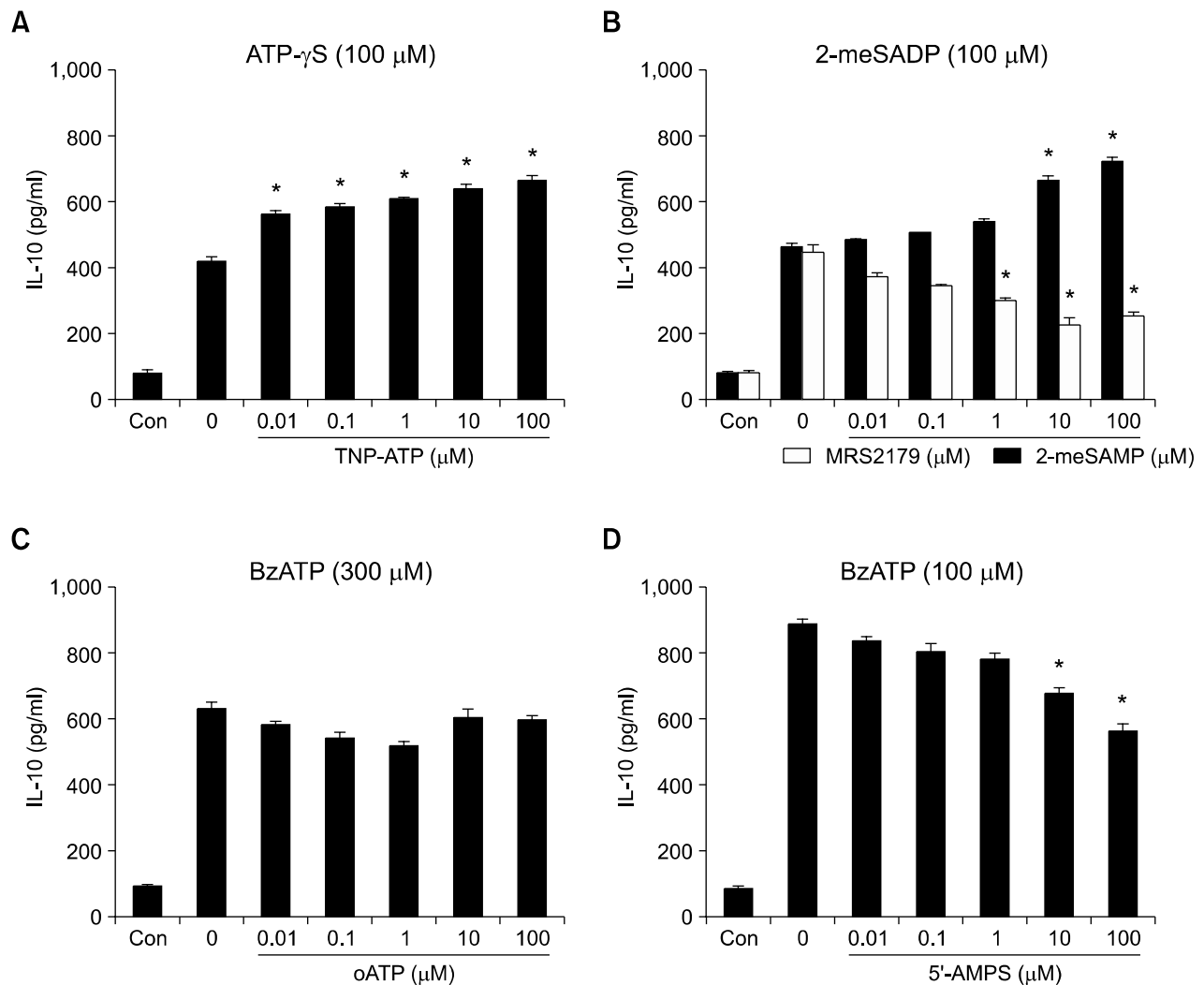


Figure 4. Effects of the P2 receptor antagonist on the release of IL-10 from microglial cells. Microglial cells (3×10^4 cells/well) were pretreated with TNP-ATP, MRS2179, 2-meSAMP, oATP, or 5'-AMPS for 30 min at the indicated concentrations, then treated with ATP- γ -S (100 μ M), 2-meSADP (100 μ M), and BzATP (100 or 300 μ M). The amount of IL-10 was measured 24 h after treatment. Data shown are mean \pm SEM of triplicate samples. The figure shows one representative of five independent experiments. * $P < 0.01$ compared to the agonist alone.

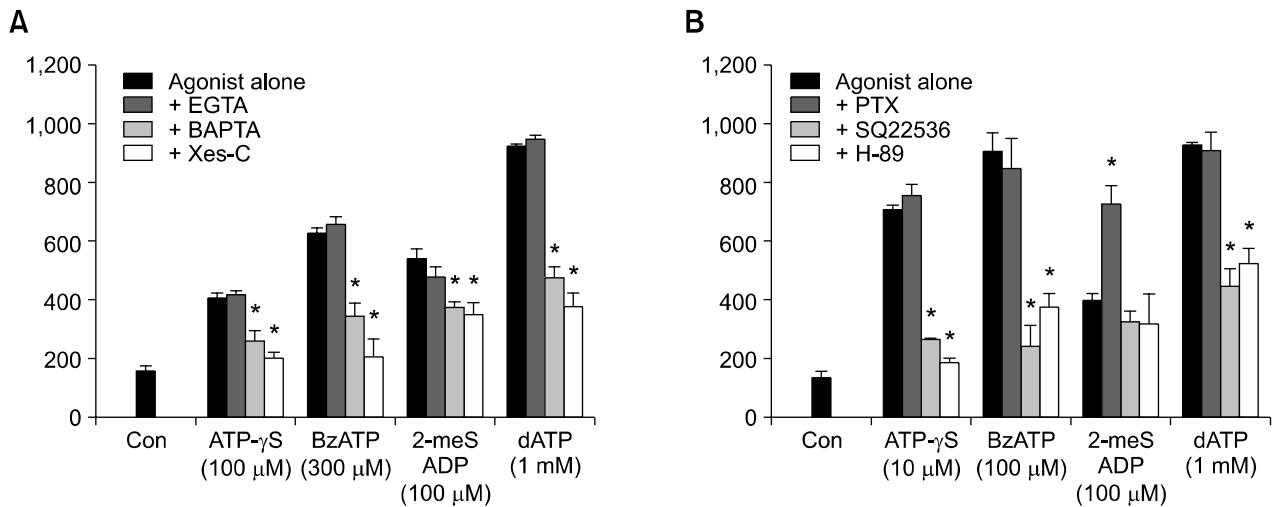


Figure 5. Effects of the calcium chelators and inhibitor of IP₃, adenylate cyclase, or PKA on IL-10 release from microglial cells. Microglial cells (3×10^4 cells/well) were pre-treated with EGTA (500 μM, 20 min), BAPTA-AM (50 μM, 1 h), Xes-C (100 nM, 20 min), PTX (500 ng/ml, 1 h), SQ22536 (10 μM, 20 min), or H-89 (10 μM, 20 min), then treated with ATP-γS, BzATP, 2-meSADP, and dATP at the indicated concentrations. The amount of IL-10 was measured 24 h after treatment. Data shown are mean \pm SEM of triplicate samples. The figure shows one representative of three independent experiments. * $P < 0.01$ compared to agonist alone.

Effects of Ca²⁺ chelators and the inhibitors of IP₃, adenylate cyclase, and PKA on IL-10 release from microglial cells

We investigated the effects of BAPTA-AM (a membrane permeable Ca²⁺ chelator) and Xes-C (an IP₃ inhibitor) on the IL-10 production. As expected, ATP-γS (100 μM), BzATP (300 μM), dATP (1 mM), and 2-meSADP (100 μM)-induced IL-10 expression was down-regulated with BAPTA-AM or Xes-C treatment, indicating that intracellular Ca²⁺ release contributes the IL-10 production. However, IL-10 production by ATP-γS (100 μM) or BzATP (300 μM) was not affected when the cells were pre-incubated in EGTA-containing Ca²⁺-free medium, indicating that Ca²⁺ influx through P2X₁, P2X₃, P2X₄, P2X₇ receptor group did not inhibit the IL-10 production (Figure 5A). As can be seen in Figure 5B, ATP-γS (10 μM), BzATP (100 μM), and dATP (1 mM)-induced IL-10 production was down-regulated by SQ22536 (an adenylate cyclase inhibitor) or H-89 (a PKA inhibitor), and 2-meSADP (100 μM)-induced IL-10 production was more up-regulated by pertussis toxin (PTX; a G_i protein inhibitor). These results suggest that intracellular Ca²⁺ release and/or cAMP-activated PKA are the main contributors to extracellular ATP-(or ADP)-mediated IL-10 expression.

Discussion

In this study, we presented evidences for crosstalk

between P2 receptors in a situation where P2Y₁ and P2Y₁₁ receptors are major receptors involved in extracellular ATP-(or ADP)-mediated IL-10 production, and P2X₁, P2X₃, P2X₄ receptor group, or the P2Y₁₂ receptor, negatively modulate the P2Y₁₁ receptor or the P2Y₁ receptor, respectively.

The P2Y₁ and P2Y₁₁ receptors are coupled to G_q, promoting PLC-catalyzed generation of an IP₃, and subsequent release of intracellular calcium (Ralevic and Burnstock, 1998). Therefore, we investigated the effects of BAPTA-AM and Xes-C (an IP₃ inhibitor) on IL-10 production. As expected, the IL-10 expression induced by ATP-γS (100 μM), BzATP (300 μM), dATP (1 mM), or 2-meSADP (100 μM) was down-regulated by BAPTA-AM or Xes-C treatment, indicating that intracellular Ca²⁺ release contributes to IL-10 production (Figure 5A). In accord with these results, previous study demonstrated that Ca²⁺ and calmodulin (CaM) stimulated adenylate cyclase (Simonds, 1999), and that prostaglandin E₂ (PGE₂), which was shown to accumulate cAMP in microglia, enhanced the LPS-induced IL-10 expression by microglia (Patrizio *et al.*, 1996; Aloisi *et al.*, 1999).

Previous work has shown that agonists for the P2Y₁₁ receptor are ATP-γS, BzATP, ATP, and ADP-βS (Communi *et al.*, 1999). Indeed, exposure to these nucleotides increased the release of IL-10 (Figures 1 and 3), and IL-10 release mediated by ATP-γS (10 μM), BzATP (100 μM), and dATP (1 mM) was down-regulated by an adenylate cyclase inhibitor, SQ22536, and a PKA inhibitor, H-89 (Figure 5B). These results are consistent with

previous findings that P2Y₁₁ receptors activate adenylate cyclase and contribute to cAMP formation (Torres *et al.*, 2002). Recent studies showed that ATP- γ S enhanced LPS-induced IL-10 production in human monocyte-derived dendritic cells (Marteau *et al.*, 2004), and that ATP- γ S and BzATP inhibited the production of TNF- α , IL-8, and MIP-1 β in human mast cells through a G_s-coupled receptor (Feng *et al.*, 2004). At this point, we can not rule out the involvement of other P2Y₁₁ receptor subtypes in our study, as BzATP (100 μ M)-induced IL-10 release was only partially inhibited by 5'-AMPS (a P2Y₁₁ antagonist) (Figure 4D), and P2Y₁₁ receptor-transcripts have not been found in rats and mice (von K  gelgen, 2006). To date, the P2Y₁₁ receptor is the only cloned ATP-binding P2Y family member known to stimulate G_s proteins (Communi *et al.*, 1999).

Data from work with agonists indicated that the P2X receptors did not contribute to IL-10 production (Figure 3), but TNP-ATP (an antagonist of the P2X₁, P2X₃, and P2X₄ receptors) up-regulated ATP- γ S (100 μ M)-induced IL-10 production (Figure 4A). These results suggest that there is reciprocal crosstalk between the P2Y₁₁ and P2X receptors, through which plasma membrane depolarization by Na⁺ and Ca²⁺ entry from the extracellular space negatively modulates IL-10 expression. Previous study showed that depolarization induced by P2X receptor-mediated Na⁺ influx inhibited store-operated channels (SOC)-mediated Ca²⁺ entry resulting from P2Y activation (Wang *et al.*, 2000). Therefore, down-regulation of IL-10 production by 100 μ M ATP- γ S may be mediated by P2X₁, P2X₃, and P2X₄ receptors-mediated inhibition of Ca²⁺ entry through SOC.

Unlike the G_q-coupled P2Y₁ receptor, the ADP-selective P2Y₁₂ and P2Y₁₃ receptors both use G_i proteins to inhibit adenylate cyclase (Communi *et al.*, 2001). In accord with these data, our results showed that 100 μ M 2-meSADP-induced IL-10 production was up-regulated by 2meSAMP (a P2Y₁₂ antagonist) and PTX (a P2Y₁₂ receptor inhibitor), but down-regulated by MRS2179 (a P2Y₁ antagonist) (Figure 4B and 5B), indicating reciprocal crosstalk between P2Y₁ and P2Y₁₂ receptors. Cross-talk between the downstream pathways of P2Y₁ and P2Y₁₂ receptors was demonstrated in the regulation of platelet aggregation and astrocytes cell death (Hardy *et al.*, 2004; Mamedova *et al.*, 2006).

In conclusion, our study provides evidence that extracellular ATP-(or ADP)-mediated IL-10 production in microglia is mediated via P2Y₁ and P2Y₁₁. Further, IL-10 production is modulated by crosstalk between P2Y₁ and P2Y₁₂ receptors, and P2Y₁₁ and P2X receptors.

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the Brain Disease Research Center at Ajou University.

References

- Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 2006;58:281-341
- Aloisi F, De Simone R, Columba-Cabezas S, Levi G. Opposite effects of interferon- γ and prostaglandin E₂ on tumor necrosis factor and interleukin-10 production in microglia: a regulatory loop controlling microglia pro- and anti-inflammatory activities. *J Neurosci Res* 1999;56:571-80
- Brunschweiler A, Muller CE. P2 receptors activated by uracil nucleotides—an update. *Curr Med Chem* 2006;13:289-312
- Communi D, Piroton S, Parmentier M, Boeynaems JM. Cloning and functional expression of a human uridine nucleotide receptor. *J Biol Chem* 1995;270:30849-52
- Communi D, Govaerts C, Parmentier M, Boeynaems JM. Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J Biol Chem* 1997; 272:31969-73
- Communi D, Robaye B, Boeynaems JM. Pharmacological characterization of the human P2Y₁₁ receptor. *Br J Pharmacol* 1999;128:1199-206
- Communi D, Gonzalez NS, Detheux M, Brezillon S, Lannoy V, Parmentier M, Boeynaems JM. Identification of a novel human ADP receptor coupled to G_i. *J Biol Chem* 2001;276: 41479-85
- Di Virgilio F, Sanz JM, Chiozzi P, Falzoni S. The P2Z/P2X7 receptor of microglial cells: a novel immunomodulatory receptor. *Prog Brain Res* 1999;120:355-68
- Di Virgilio F, Solini A. P2 receptors: new potential players in atherosclerosis. *Br J Pharmacol* 2002;135:831-42
- Feng C, Mery AG, Beller EM, favot C, Boyce JA. Adenine nucleotides inhibit cytokine generation by human mast cells through a G_s-coupled receptor. *J Immunol* 2004;173:7539-47
- Hardy AR, Jones ML, Mundell JS, Poole AW. Reciprocal cross-talk between P2Y₁ and P2Y₁₂ receptors at the level of calcium signaling in human platelets. *Blood* 2004;104: 1745-52
- Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D. The P2Y₁₂ receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci* 2006; 9:1512-9
- Hide I, Tanaka M, Inoue A, Nakajima K, Kohsaka S, Inoue K, Nakata Y. Extracellular ATP triggers tumor necrosis factor- α release from rat microglia. *J Neurochem* 2000;75:965-72

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001;409:202-7

Khakh BS, North RA. P2X receptors as cell-surface ATP sensors in health and disease. *Nature* 2006;442:527-32

Kim KY, Kim MY, Choi HS, Jin BK, Kim SU, Lee YB. Thrombin induces IL-10 production in microglia as a negative feedback regulator of TNF- α release. *Neuroreport* 2002;13:849-52

Koizumi S, Shigemoto-Mogami Y, Nasu-Tada K, Shinozaki Y, Ohsawa K, Tsuda M, Joshi BV, Jacobson KA, Kohsaka S, Inoue K. UDP acting at P2Y₆ receptors is a mediator of microglial phagocytosis. *Nature* 2007;446:1091-5

Light AR, Wu Y, Huguen RW, Guthrie PB. Purinergic receptors activating rapid intracellular Ca²⁺ increases in microglia. *Neuron Glia Biol* 2006;2:125-38

Lustig KD, Shiao AK, Brake AJ, Julius D. Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc Natl Acad Sci USA* 1993;90:5113-7

Mamedova LK, Gao ZG, Jacobson KA. Regulation of death and survival in astrocytes by ADP activating P2Y₁ and P2Y₁₂ receptors. *Biochem Pharmacol* 2006;72:1031-41

Marteau F, Communi D, Boeynaems JM, Gonzalez NS. Involvement of multiple P2Y receptors and signaling pathways in the action of adenine nucleotides diphosphates on human monocyte-derived dendritic cells. *J Leukoc Biol* 2004;76:796-803

Ohtani Y, Minami M, Satoh M. Expression of inducible nitric oxide synthase mRNA and production of nitric oxide are induced by adenosine triphosphate in cultured rat microglia. *Neurosci Lett* 2000;293:72-4

Parvathenani LK, Tertyshnikova S, Greco CR, Roberts SB, Robertson B, Posmantur R. P2X₇ mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. *J Biol*

Chem 2003;278:13309-17

Patrizio M, Costa T, Levi G. Interferon- γ and lipopolysaccharide reduce cAMP responses in cultured glial cells: reversal by a type IV phosphodiesterase inhibitor. *Glia* 1996;14:94-100

Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev* 1998;50:413-92

Sanz JM, Di Virgilio F. Kinetics and mechanism of ATP-dependent IL-1 β release from microglial cells. *J Immunol* 2000;164:4893-8

Seo DR, Kim KY, Lee YB. Interleukin-10 expression in lipopolysaccharide-activated microglia is mediated by extracellular ATP in an autocrine fashion. *Neuroreport* 2004;15:1157-61

Shigemoto-Mogami Y, Koizumi S, Tsuda M, Ohsawa K, Kohsaka S, Inoue K. Mechanisms underlying extracellular ATP-evoked interleukin-6 release in mouse microglial cell line, MG-5. *J Neurochem* 2001;78:1339-49

Simonds WF. G protein regulation of adenylate cyclase. *Trends Pharmacol Sci* 1999;20:66-73

Torres B, Zambon AC, Insel PA. P2Y₁₁ receptors activate adenylyl cyclase and contribute to nucleotide-promoted cAMP formation in MDCK-D₁ cells. A mechanism for nucleotide-mediated autocrine-paracrine regulation. *J Biol Chem* 2002;277:7761-5

Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K. P2X₄ receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 2003;424:778-83

von Kügelgen I. Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacol Ther* 2006;110:415-32

Wang X, Kim SU, van Breemen C, McLarnon JG. Activation of purinergic P2X receptors inhibits P2Y-mediated Ca²⁺ influx in human microglia. *Cell Calcium* 2000;27:205-12