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

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Abbreviations: IP_3, 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 μM) and bell-shaped. The concentrations of ATP, ATP-γS, ADP, and ADP-βS that showed maximal IL-10 release were 100, 10, 100, and 100 μM 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 Ca^{2+} 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 $P2Y_1$ antagonist) or 5'-AMPS (a $P2Y_{11}$ antagonist), indicating that both the $P2Y_1$ and $P2Y_{11}$ receptors are major receptors involved in IL-10 expression. In addition, we found that inhibition of IL-10 production by high concentrations of ATP-γS (100 μM) was restored by TNP-ATP (an antagonist of the $P2X_1$, $P2X_3$, and $P2X_4$ receptors), and that IL-10 production by 2-meSADP was restored by 2meSAMP (a $P2Y_{12}$ receptor antagonist) or pertusis toxin (PTX; a G_i protein inhibitor), indicating that the $P2X_1$, $P2X_3$, $P2X_4$ receptor group, or the $P2Y_{12}$ receptor, negatively modulate the $P2Y_{11}$ receptor or the $P2Y_1$ 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 ($P2X_1$-$P2X_7$) causes Ca^{2+} 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: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$. The $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors are preferentially activated by ADP whereas $P2Y_6$ is activated by UDP (Abbracchio *et al.,* 2006). The $P2Y_{11}$ receptor prefers ATP as an agonist (Communi *et al.,* 1997) whereas the $P2Y_2$ (Lustig *et al.,* 1993) and $P2Y_4$ receptors are equally sensitive to ATP and UTP (Communi *et al.,* 1995). The $P2Y_{14}$ receptor is activated by the nucleotide sugar UDP-glucose (Brunschweiger and Muller, 2006). The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors are coupled to G_q, promoting PLC-catalyzed generation of an inositol phosphate (IP_3) and subsequent release of intracellular calcium

(Ralevic and Burnstock, 1998). The $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors are coupled to $G_{i/o}$, inhibiting adenyl 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 $P2X_7$ receptors (Hide *et al.,* 2000). The presence of the $P2X_7$ 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-1β by activating the IL-1β-converting enzyme/caspase1 (Sanz and Di Virgilio, 2000). Further, recent studies have reported that $P2X_4$ receptors are involved in pain transmission (Tsuda *et al.,* 2003) and that during microglial activation, $P2Y_{12}$ receptors, which mediate the movement of microglial projections, are down-regulated (Haynes *et al.,* 2006), but $P2Y_6$ 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 μg/ml DNase I for 15 min at 37ºC, 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 μg/ml streptomycin, and plated on poly-D-lysine-coated T-75 culture flasks, and incubated at 37ºC in incubator with 5% CO_2/95% 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 μl of each cDNA products was amplified by PCR using specific sense and antisense primers designed from the cDNA sequences for P2 receptors: $P2X_1$, 5'-AGAGGCACTACTACAAGCAGAA-3' (sense) and 5'-GGTAAGGCTGTGGGAAAGA-3' (antisense); $P2X_2$, 5'-GAATCAGAGTGCAACCCCAA-3' (sense) and 5'-TCACAGGCCATCTACTTGAG-3' (antisense); $P2X_3$, 5'-TTAAGTTTGCTGGACAGGAT-3' (sense) and 5'-GTTCCCATATACCAGCACAT-3' (antisense); $P2X_4$, 5'-TCCCTTCTGCCCCATATTCC-3' (sense) and 5'-TTCATCTCCCCCGAAAGACC-3' (antisense); $P2X_5$, 5'-CGACCTGGTACTTATCTAC-CTC-3' (sense) and 5'-ACGTTCACAATGGCATTC-3' (antisense); $P2X_6$, 5'-GCCCAGAGCATCCTTCT-GTTC-3' (sense) and 5'-CGTGGCTGTATGTC-CCCATC-3' (antisense); $P2X_7$, 5'-AACAGTGCC-ATTCTGACC-3' (sense) and 5'-GCCACCTCTGT-AAAGTTCTC-3' (antisense); $P2Y_1$, 5'-GGCAGG-CTCAAGAAGAAGAAT-3' (sense) and 5'-ATCAC-ACATTTCTGGGGTCTG-3' (antisense); $P2Y_2$, 5'-AGCTCTTTAGCCATTTTGTG-3' (sense) and 5'-CGGAAGGAGTAATAGAGGGT-3' (antisense); $P2Y_4$, 5'-ACTGTCTTTGCTGTCTGCTT-3' (sense) and 5'-AGACAGCTATTAGCACTGGC-3' (antisense); $P2Y_6$, 5'-GTCTACCGTGAGGATTTCAA-3' (sense) and 5'-CTAGGTATCGCTGGAAGCTA-3' (antisense); $P2Y_{12}$, 5'-CTCCACCACCTACATGTTTC-3' (sense) and 5'-AAGAGGATGCTGCAGTAGAG-3' (antisense); $P2Y_{13}$, 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'-acylic dialcohol (oxidized ATP; oATP), 2'-deoxy-N^6-methyladenosine-3',5'-bisphosphate (MRS2179), 2-methylthioadenisine 5'-monophosphate (2-meSAMP), 5'-O-thiomnophosphate (5'-AMPS) (Sigma, St. Louis, MO); Ca^{2+} chelators: EGTA (Sigma, St. Louis, MO), bisaminophenoxyethane tetraacetic acid-acetoxymethyl ester (BAPTA-AM; Calbiochem, San Diego, CA); IP_3 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 ± SEM of triplicate samples. A value of $P < 0.05$ was considered statistically significant.

Results

Characterization of ATP-(or ATP-γS)-induced IL-10 release, and ADP-(or ADP-βS)-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 μM) of ATP, ADP, ATP-γS (a hydrolysis-resistant analog of ATP), or ADP-βS (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-γS that showed maximal IL-10 release were different. ATP-induced IL-10 release peaked at an ATP concentration of 100 μM (811.51 ± 29.59 pg/ml IL-10) and was sustained to 1,000 μM (750.15 ± 5.66 pg/ml). On the other hand, ATP-γS-induced IL-10 release peaked at an ATP-γS concentration of 10 μM (930.65 ± 30.94 pg/ml) but dropped to 480.88 ± 18.52 pg/ml at 100 μM ($P < 0.01$). In the case of ADP, ADP-induced or ADP-βS-induced IL-10 release peaked at a concentration of 100 μM, but treatment with 1,000 μM ADP (IL-10 release of 186.27 ± 20.70 pg/ml) or 1,000 μM ADP-βS (IL-10 release of 475.10 ± 30.96 pg/ml) appeared to induce less IL-10 release than did treatment with 100 μM ADP (485.26 ± 20.33 pg/ml) or 100 μM ADP-βS (721.43 ± 35.20 pg/ml) ($P < 0.01$). These results indicate that 100

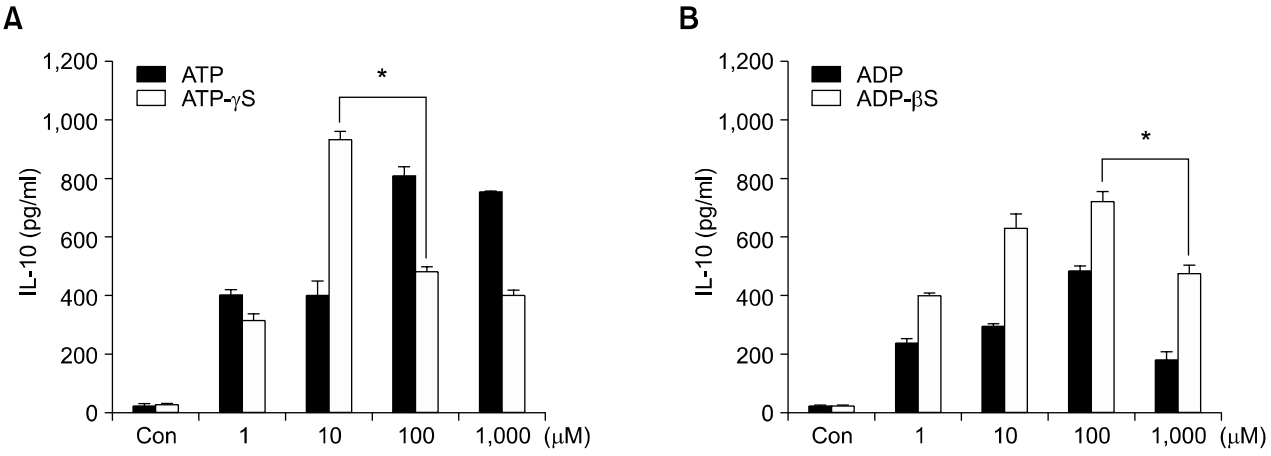

Figure 1. Characterization of ATP (or ATP-γS)- or ADP (or ADP-βS)-induced IL-10 release. Microglial cells (3×10^4 cells/well) were treated with (A) ATP or ATP-γ-S, (B) ADP or ADP-β-S at the indicated concentrations. The amount of IL-10 was measured 24 h after treatment with ATP (ATP-γ-S) or ADP (ADP-β-S).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

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 $P2X_1$ (434 bp), $P2X_3$ (272 bp), $P2X_4$ (489 bp), and $P2X_7$ (358 bp) receptor mRNAs (Figure 2A). Similarly, amplified PCR products of the expected sizes were obtained for the $P2Y_1$ (411 bp), $P2Y_2$ (244 bp), $P2Y_4$ (149 bp), $P2Y_6$ (325 bp), $P2Y_{12}$ (168 bp), and $P2Y_{13}$ (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 ($P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$) and P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$) (Light *et al.,* 2006). At this point, we could not test the expression of $P2Y_{11}$ receptor, because rat $P2Y_{11}$ receptor has not been cloned.

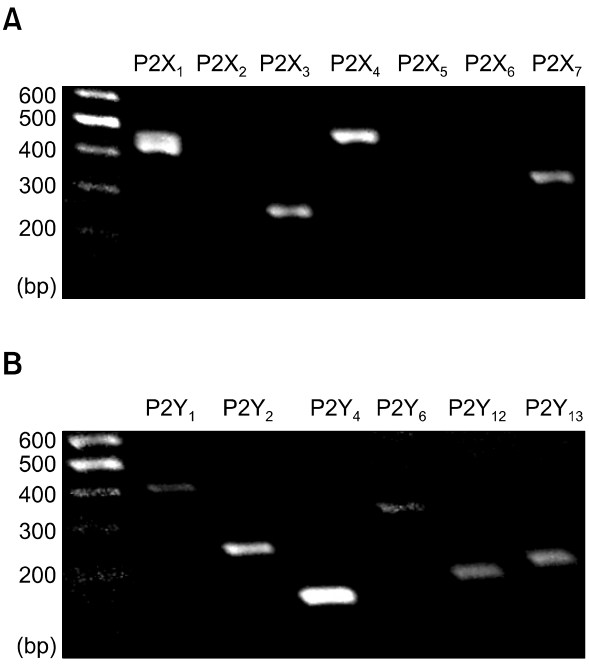

Figure 2. Expression of P2X and P2Y receptors mRNA. RT-PCR analysis of P2 receptor mRNA expression in microglial cells was done with primers specific for distinct P2 receptors subtypes. cDNA products were analyzed by 1.5% agarose gel electrophoresis. A representive gel with ethidium bromide-stained cDNA 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 $P2Y_1$ and $P2Y_{12}$ receptors, and both $P2X_7$ agonist BzATP and dATP are potent $P2Y_{11}$ receptor agonists, these results indicate that purinergic modulation of IL-10 ex-

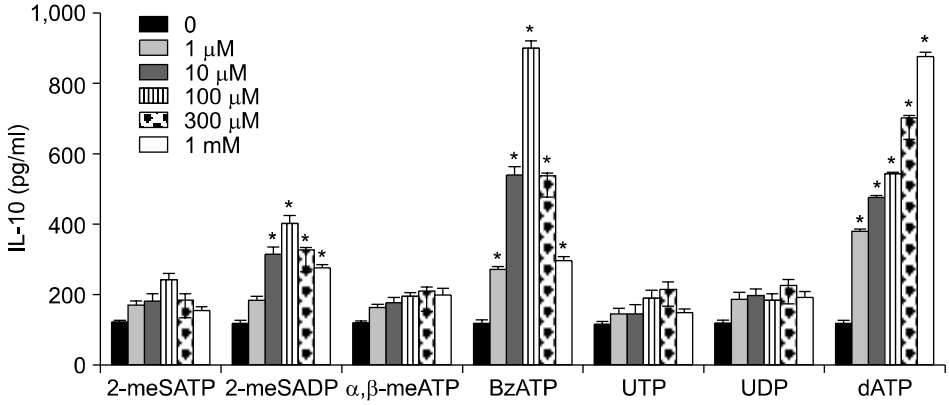

Figure 3. Effects of the P2 receptor agonist on the release of IL-10 from microglial cells. Microglial cells (3×10^4 cells/well) were treated with 2-meSATP, 2-meSADP, α,β-meATP, BzATP, UTP, UDP, and dATP at the indicated concentrations. The amount of IL-10 was measured 24 h after treatment. Data shown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

pression may be mediated by $P2X_7$, $P2Y_1$, $P2Y_{11}$, and $P2Y_{12}$ 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 $P2X_7$, $P2Y_1$, $P2Y_{11}$, or $P2Y_{12}$ receptors, and an antagonist of the $P2X_1$, $P2X_3$, and $P2X_4$ receptors. ATP-γS (100 μM)-induced IL-10 release was restored by TNP-ATP (an antagonist of the $P2X_1$, $P2X_3$, and $P2X_4$ receptors) (Figure 4A). 2-meSADP (100 μM)-induced IL-10 release was inhibited by MRS2179 (a $P2Y_1$ antagonist), but 2meSAMP (a $P2Y_{12}$ antagonist) up-regulated 2-meSADP (100 μM)-induced IL-10 release (Figure 4B). On the other hand, BzATP (300 μM)-induced IL-10 release was not restored by oATP (a $P2X_7$ antagonist) (Figure 4C). BzATP (100 μM)-induced IL-10 release was inhibited by 5'-AMPS (a $P2Y_{11}$ antagonist) (Figure 4D). These results indicate that both the $P2Y_1$ and $P2Y_{11}$ receptors are major receptors involved in IL-10 expression, and that the $P2X_1$, $P2X_3$, $P2X_4$ receptor group, or the $P2Y_{12}$ receptor, negatively modulates the $P2Y_{11}$ receptor or the $P2Y_1$ receptor, respectively.

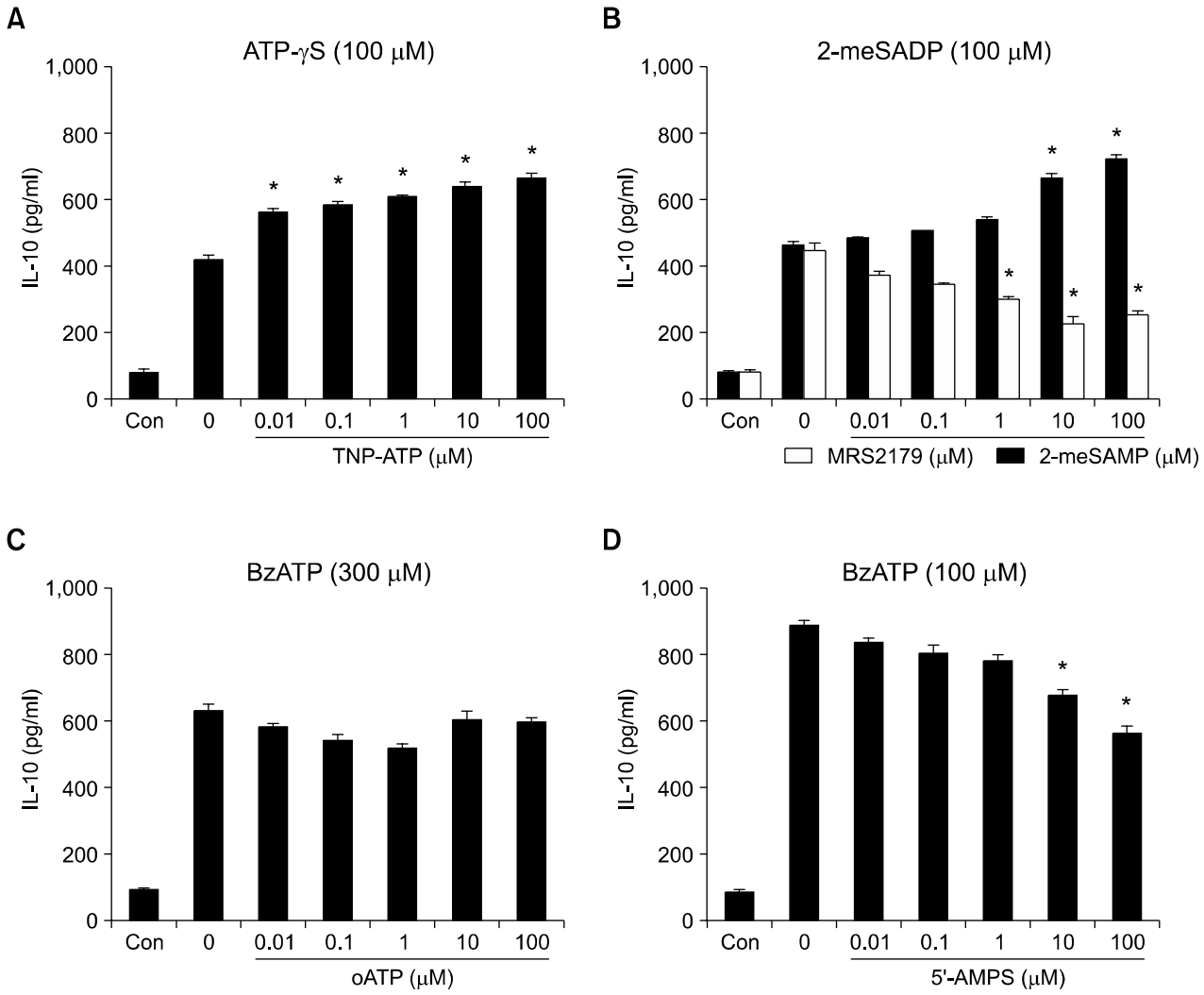

Figure 4. Effects of the P2 receptor antagonist on the release of IL-10 from microglial cells. Microglial cells (3×10^4 cells/well) were pretreated with TNP-ATP, MRS2179, 2-meSAMP, oATP, or 5'-AMPS for 30 min at the indicated concentrations, then treated with ATP-γ-S (100 μM), 2-meSADP (100 μM), and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

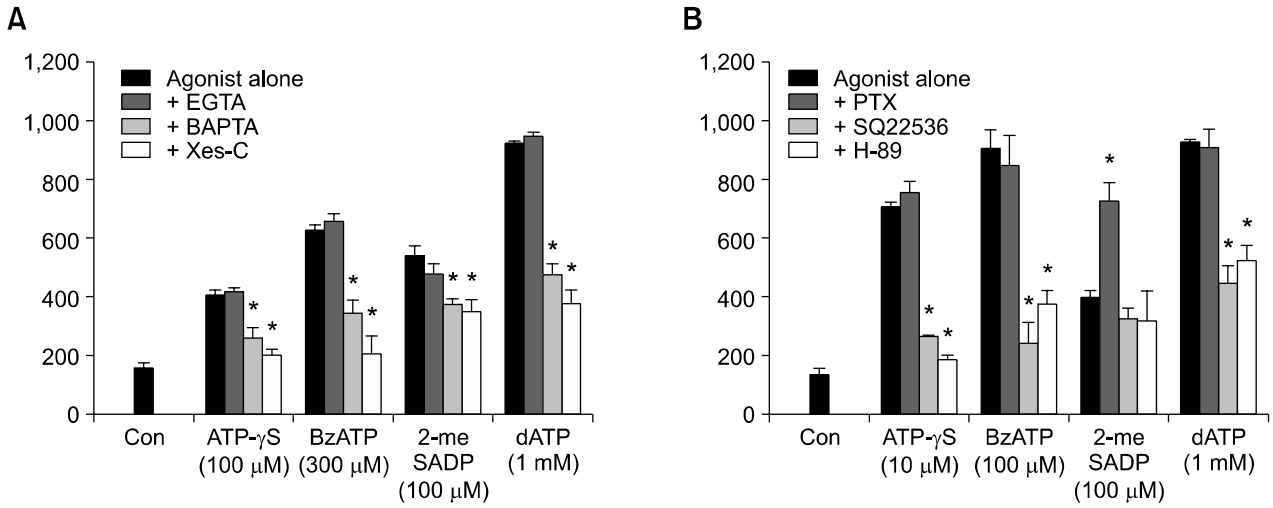

Figure 5. Effects of the calcium chelators and inhibitor of IP_3, adenylate cyclase, or PKA on IL-10 release from microglial cells. Microglial cells (3×10^4 cells/well) were pre-treated with EGTA (500 μM, 20 min), BAPTA-AM (50 μM, 1 h), Xes-C (100 nM, 20 min), PTX (500 ng/ml, 1 h), SQ22536 (10 μM, 20 min), or H-89 (10 μM, 20 min), then treated with ATP-γ-S, BzATP, 2-meSADP, and dATP at the indicated concentrations. The amount of IL-10 was measured 24 h after treatment. Data shown are mean ± SEM of triplicate samples. The figure shows one representative of three independent experiments. *$P < 0.01$ compared to agonist alone.

Effects of Ca^{2+} chelators and the inhibitors of IP_3, adenylate cyclase, and PKA on IL-10 release from microglial cells

We investigated the effects of BAPTA-AM (a membrane permeable Ca^{2+} chelator) and Xes-C (an IP_3 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 Ca^{2+} 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 Ca^{2+}-free medium, indicating that Ca^{2+} influx through $P2X_1$, $P2X_3$, $P2X_4$, $P2X_7$ 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 Ca^{2+} 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 $P2Y_1$ and $P2Y_{11}$ receptors are major receptors involved in extracellular ATP-(or ADP)-mediated IL-10 production, and $P2X_1$, $P2X_3$, $P2X_4$ receptor group, or the $P2Y_{12}$ receptor, negatively modulate the $P2Y_{11}$ receptor or the $P2Y_1$ receptor, respectively.

The $P2Y_1$ and $P2Y_{11}$ receptors are coupled to G_q, promoting PLC-catalyzed generation of an IP_3, and subsequent release of intracellular calcium (Ralevic and Burnstock, 1998). Therefore, we investigated the effects of BAPTA-AM and Xes-C (an IP_3 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 Ca^{2+} release contributes to IL-10 production (Figure 5A). In accord with these results, previous study demonstrated that Ca^{2+} and calmodulin (CaM) stimulated adenylate cyclase (Simonds, 1999), and that prostaglandin E_2 (PGE_2), 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 $P2Y_{11}$ 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 $P2Y_{11}$ receptors activate adenylate cyclase and contribute to cAMP formation (Torres *et al.,* 2002). Recent studies showed that ATP-γS enhanced LPS-induced IL-10 production in human monocyte-derived dendritic cells (Marteau *et al.,* 2004), and that ATP-γS and BzATP inhibited the production of TNF-α, IL-8, and MIP-1β 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 $P2Y_{11}$ receptor subtypes in our study, as BzATP (100 μM)-induced IL-10 release was only partially inhibited by 5'-AMPS (a $P2Y_{11}$ antagonist) (Figure 4D), and $P2Y_{11}$ receptor-transcripts have not been found in rats and mice (von Kügelgen, 2006). To date, the $P2Y_{11}$ 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 $P2X_1$, $P2X_3$, and $P2X_4$ receptors) up-regulated ATP-γS (100 μM)-induced IL-10 production (Figure 4A). These results suggest that there is reciprocal crosstalk between the $P2Y_{11}$ and P2X receptors, through which plasma membrane depolarization by Na^+ and Ca^{2+} 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 Ca^{2+} entry resulting from P2Y activation (Wang *et al.,* 2000). Therefore, down-regulation of IL-10 production by 100 μM ATP-γS may be mediated by $P2X_1$, $P2X_3$, and $P2X_4$ receptors-mediated inhibition of Ca^{2+} entry through SOC.

Unlike the G_q-coupled $P2Y_1$ receptor, the ADP-selective $P2Y_{12}$ and $P2Y_{13}$ receptors both use G_i proteins to inhibit adenylate cyclase (Communi *et al.,* 2001). In accord with these data, our results showed that 100 μM 2-meSADP-induced IL-10 production was up-regulated by 2meSAMP (a $P2Y_{12}$ antagonist) and PTX (a $P2Y_{12}$ receptor inhibitor), but down-regulated by MRS2179 (a $P2Y_1$ antagonist) (Figure 4B and 5B), indicating reciprocal crosstalk between $P2Y_1$ and $P2Y_{12}$ receptors. Crosstalk between the downstream pathways of $P2Y_1$ and $P2Y_{12}$ 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 $P2Y_1$ and $P2Y_{11}$. Further, IL-10 production is modulated by crosstalk between $P2Y_1$ and $P2Y_{12}$ receptors, and $P2Y_{11}$ and P2X receptors.

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