Extracellular ATP or ADP Induce Chemotaxis of Cultured Microglia through $G_{i/o}$ -Coupled P2Y Receptors

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The initial microglial responses that occur after brain injury and in various neurological diseases are characterized by microglial accumulation in the affected sites of brain that results from the migration and proliferation of these cells. The early-phase signal responsible for this accumulation is likely to be transduced by rapidly diffusible factors. In this study, the possibility of ATP released from injured neurons and nerve terminals affecting cell motility was determined in rat primary cultured microglia. Extracellular ATP and ADP induced membrane ruffling and markedly enhanced chemokinesis in Boyden chamber assay. Further analyses using the Dunn chemotaxis chamber assay, which allows direct observation of cell movement, revealed that both ATP and ADP induced chemotaxis of microglia. The elim-

ination of extracellular calcium or treatment with pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid, suramin, or adenosine-3'-phosphate-5'-phosphosulfate did not inhibit ATP- or ADP-induced membrane ruffling, whereas AR-C69931MX or pertussis toxin treatments clearly did so. As an intracellular signaling molecule underlying these phenomena, the small G-protein Rac was activated by ATP and ADP stimulation, and its activation was also inhibited by pretreatment with pertussis toxin. These results strongly suggest that membrane ruffling and chemotaxis of microglia induced by ATP or ADP are mediated by $G_{i/o}$ -coupled P2Y receptors.

Key words: microglia; ATP; ADP; membrane ruffling; chemotaxis; $G_{i/o}$ -coupled P2Y receptors

Accumulated evidence suggests that extracellular ATP functions in various tissues and cells (Dubyak and El-Moatassim, 1993). The roles of extracellular ATP as a neurotransmitter and neuromodulator in the CNS have been well documented. For example, ATP induces excitation and increases in calcium in various neurons in the brain (Edwards et al., 1992; Shen and North, 1993; Chen et al., 1994; Inoue et al., 1995; Nabekura et al., 1995). In addition to the role played by ATP in neurons, effects of ATP on glial cells have also been demonstrated. In astrocytes, for example, DNA synthesis, process formation, and the increase in the expression of glial fibrillary acidic protein (Neary et al., 1994), arachidonic acid release (Chen and Chen, 1998), Erk activation (Neary et al., 1999), and calcium wave propagation (Scemes et al., 2000) were reported to be stimulated by ATP. Ca^{2+} release from internal stores by ATP stimulation was also reported in oligodendrocytes (Kirischuk et al., 1995). This evidence suggests diverse roles of extracellular ATP in the CNS.

Reports have shown that ATP stimulates microglia, another kind of glial cell in the CNS, to release various biologically active substances, such as interleukin-1 β (Ferrari et al., 1996, 1997), plasminogen (Inoue et al., 1998), and tumor necrosis factor- α (Hide et al., 2000). Microglial cell death was also demonstrated after stimulation with high-dose ATP (Ferrari et al., 1999). After

neuronal damage, microglia migrate to the affected sites, where they function to secrete a variety of cytokines and neurotrophic factors, to express major histocompatibility complex antigen class II, and in certain cases to perform phagocytic activities (Kreutzberg, 1996; Thomas, 1999). For microglia to function in the proper region of the brain, there must be a mechanism underlying the regulation of microglial motility. In fact, in regions of the facial nucleus affected with neuronal damage after facial nerve axotomy, for instance, the accumulation of microglia resulting from their proliferation and migration was clearly observed (Streit et al., 1988; Graeber et al., 1998; Ito et al., 1998). The search for factors influencing microglial motility is an important issue in understanding the function of microglia in brain pathology. To date, transforming growth factor- β , complement 5a (C5a), epidermal growth factor, and various kinds of chemokines have been reported to enhance microglial motility, and these factors have been postulated as possible chemoattractants for microglia in brain (Yao et al., 1990; Hayashi et al., 1995; Nolte et al., 1996, 1997; Cross and Woodroffe, 1999). In the present study, we demonstrated that extracellular ATP and ADP induced membrane ruffling and cell migration in the manner of chemoattraction, possibly mediated by $G_{i/o}$ -coupled P2Y receptors.

MATERIALS AND METHODS

Microglial culture. Rat primary cultured microglia were prepared according to the method described previously (Nakajima et al., 1992). In brief, mixed glial culture was prepared from the cerebral cortex of neonatal Wistar rats and maintained for 12–23 d in DMEM (Life Technologies, Grand Island, NY) with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA). Microglia were obtained as floating cells over the mixed glial culture. The floating cells were collected by a gentle shake and transferred to appropriate dishes or glasses, then the microglia attached to them were used for various assays.

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Membrane ruffling. The thus-prepared microglia were attached to glass coverslips (Matsunami, Osaka, Japan) coated with 100 $\mu\text{g}/\text{ml}$ of poly-L-lysine (Sigma, St. Louis, MO). After attachment for 2 hr, microglia were washed with serum-free DMEM and starved for 4 hr in the same medium. They were then stimulated with ATP (Yamasashyoyu, Chiba, Japan), ADP (Sigma), UTP (Wako Pure Chemical Industries, Osaka, Japan), and adenosine (Sigma) at 50 μM , or recombinant murine macrophage colony stimulating factor (M-CSF) (R & D Systems, Minneapolis, MN) at 100 ng/ml for 5 min at 37°C. In the control, cells were treated with PBS instead of nucleotides. The reaction was stopped by the addition of PBS containing 3.7% formaldehyde. After fixation for 5 min and washing with PBS, the cells were permeabilized with PBS containing 0.1% Triton X-100 for 5 min and washed three times with PBS. To visualize membrane ruffling, cells were stained with Texas Red-conjugated phalloidin (Texas Red-X Phalloidin) (Molecular Probes, Eugene, OR) and observed under the fluorescence microscope PROVIS AX (Olympus, Tokyo, Japan). In the experiments to determine the effects of inhibitors, cells were preincubated with suramin (Wako) (300 μM), pyridoxal-phosphate-6-azophenyl-2',4-disulphonic acid tetrasodium salt (PPADS) (Research Biochemicals International, Natick, MA) (300 μM), adenosine-3'-phosphate-5'-phosphosulfate (A3P5PS) (Sigma) (300 μM), or AR-C69931MX (AstraZeneca UK Limited, London, UK) (1 μM) for 10 min after starvation and then stimulated with nucleotides. The cells were also preincubated with pertussis toxin (PTx) (Sigma) (50 ng/ml) for 4 hr.

To evaluate extracellular calcium dependency, attached microglia were washed with Ca^{2+} -containing balanced salt solution (BSS) [(in mM): 150 NaCl, 5.0 KCl, 1.2 MgCl_2 , 25 HEPES, 10 D-glucose, and 1.2 CaCl_2] or Ca^{2+} -free BSS [in mM: 150 NaCl, 5.0 KCl, 1.2 MgCl_2 , 25 HEPES, 10 D-glucose, and 1 EGTA] (Inoue et al., 1998) and stimulated with nucleotides in the same BSS.

Chemokinesis assay using the Boyden chamber. Chemokinesis of microglia was assessed using the Boyden chamber (Neuroprobe, Bethesda, MD) according to the method described previously (Yokomizo et al., 1997). In brief, polycarbonate filters (5 μm pore) were coated with 10 $\mu\text{g}/\text{ml}$ fibronectin (Sigma) in PBS for 60 min. A dry coated filter was installed in the Boyden chamber, whose bottom wells were filled with serum-free DMEM containing nucleotides at the various concentrations indicated. Freshly prepared microglia were suspended in serum-free DMEM containing nucleotides at the same concentration as that in each bottom well, and the cell suspension was placed into the top wells (2.5×10^4 cells/well). The chamber was kept in a CO_2 incubator at 37°C for 90 min. The filter was removed and stained with 0.05% crystal violet, 12% formaldehyde, and 10% ethanol in PBS. The cells on the top side of the filter were wiped off, and the number of cells that had migrated to the bottom side was measured at 595 nm with a BioLumin 960 fluorescence/absorbance microassay reader (Pharmacia-LKB, Uppsala, Sweden).

Chemotaxis assay using the Dunn chemotaxis chamber. Chemotaxis was assessed by the Dunn chemotaxis chamber (Weber Scientific International Ltd., Teddington, UK), which allows direct observation of cell movement. The assay was performed according to the method described in the previous report (Webb et al., 1996). In brief, microglia were attached to square coverslips for 2 hr, washed three times with serum-free DMEM, and kept for 4–16 hr until the chemotaxis assay was performed. After starvation, the coverslip was placed over the chamber, whose outer and inner wells were filled with DMEM. The coverslip was sealed with a 1:1 mixture of molten paraffin wax and Vaseline around three edges to leave a slit for exchange of the medium in the outer well. To observe the chemically directed cell migration, the medium in the outer well was exchanged through the slit for DMEM containing 50 μM ATP or ADP. Then the last edge of the coverslip was sealed immediately, and the chamber was set on the stage of a microscope (ECLIPSE TE300; Nikon, Tokyo, Japan), which was maintained at 37°C. Control experiments were performed under the condition in which both outer and inner wells were filled with DMEM.

A region of the bridge was viewed via a CCD video camera (Hamamatsu Photonics, Hamamatsu, Japan), and the phase-contrast images were recorded every 5 min during 1 hr of observation using imaging software (fishPPC; Hamamatsu Photonics). The straight distance between the starting point and the point of a cell reached after 1 hr was measured by plotting the point of the microglial nucleus on a computer display using drawing software. The distance and direction were shown as x,y coordinates on scatter diagrams whose x -axis was positioned parallel to the outer edge of the bridge.

Rac translocation. To determine the translocation of Rac to the region

of membrane ruffling, double staining was performed after stimulating the cells with ATP or ADP. After fixation, the cells were incubated with mouse anti-human Rac antibody (Upstate Biotechnology, Lake Placid, NY) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (BioSource, Sunnyvale, CA). F-actin was stained with Texas Red-conjugated phalloidin. Images were obtained with a confocal laser-scanning microscope CLSM2010 (Pharmacia-LKB).

Pull-down assay of Rac. Activated Rac was measured by the method described previously (Manser et al., 1998; Ohsawa et al., 2000). In brief, the microglia attached to dishes for 2 hr were washed with serum-free DMEM and starved for 4 hr with or without pertussis toxin (50 ng/ml). The starved microglia were stimulated with 50 μM ATP or ADP for 1 min, and lysed in lysis buffer A (25 mM HEPES, pH 7.3, 0.15 M NaCl, 5 mM MgCl_2 , 0.5 mM EGTA, 20 mM β -glycerophosphate, 0.5% Triton X-100, 4% glycerol, 10 mM NaF, 2 mM sodium orthovanadate, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin). The lysates were centrifuged at $12,000 \times g$ to remove debris. Part of the supernatant was mixed with SDS sample buffer, and the remainder was incubated for 30 min at 4°C with glutathione *S*-transferase (GST)-fused p21-activated kinase (PAK) that was coupled to Glutathione Sepharose 4B beads (Pharmacia Biotech). After incubation, the samples were centrifuged at $500 \times g$ and washed twice with lysis buffer A. The precipitated beads were boiled with SDS sample buffer. The total cell lysates and pull-down samples were subjected to SDS-PAGE using 10–20% gradient gel (Daichi Pure Chemicals, Tokyo, Japan), immunoblotted with anti-Rac antibody and horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Pharmacia), and visualized with ECL Western blotting detection reagents (Pharmacia).

RESULTS

ATP- and ADP-induced membrane ruffling in microglia

We first examined extracellular ATP in terms of morphology and ruffle formation in microglia after 5 min of stimulation with either 50 μM ATP, ADP, UTP or adenosine by staining with Texas Red-conjugated phalloidin. Freshly prepared microglia showed the ramified morphology after 4 hr of incubation in serum-free medium. After the addition of PBS (control), there was no change in morphology (Fig. 1*A*). However, as shown in Figure 1*B*, the cells were spread and showed amoeboid-like morphology at 5 min after ATP stimulation. The structure of membrane ruffling was clearly observed by staining with phalloidin. The prominent morphological change that occurred with ruffle formation was also detected with extracellular ADP (Fig. 1*C*). Although UTP induced obvious morphological change, ruffle formation was not significant (Fig. 1*D*). The morphological changes of microglia induced by ATP, ADP, and UTP were observed in almost all of the stimulated cells. By contrast, adenosine induced neither morphological change nor ruffle formation (data not shown).

ATP- and ADP-enhanced chemokinesis of microglia in the Boyden chamber

Membrane ruffles are structures that are found primarily at the front edges of migrating cells (Lauffenburger and Horwitz, 1996). To investigate whether the nucleotides that induce membrane ruffling act as a chemoattractant for microglia, a chemotaxis assay using the Boyden chemotaxis chamber was initially performed. In assessing chemotaxis in the Boyden chamber, we usually observe cell migration under two assay conditions, one in which the ligand is in only the bottom compartment and the other in which it is in both the bottom and top compartments. We preliminarily compared the two kinds of microglial migration stimulated by ATP and found that cell migration was enhanced in both cases (data not shown). This preliminary result indicated that ATP-induced cell migration detected in the Boyden chamber is chemokinesis (enhanced migration without chemical gradient). Although we could not evaluate ATP-induced chemotaxis in this assay system, we collected data on the chemokinesis induced by nucleotides. As

Figure 1. Nucleotide-induced membrane ruffling in microglia. The cells were stimulated with PBS (*A*) or 50 μ M ATP (*B*), ADP (*C*), or UTP (*D*) for 5 min. After fixation, the cells were stained with Texas Red-conjugated phalloidin. ATP and ADP clearly induced membrane ruffling (indicated by arrows). Scale bar, 20 μ m.

Figure 2. Nucleotide-induced chemokinesis of microglia in the Boyden chamber. The cells were exposed to ATP (circle), ADP (square), or UTP (triangle) for 90 min. Nucleotides were added to both top and bottom wells at the concentrations indicated. The absorbance of the stained cells on the bottom side of the filter was measured with a plate reader. Each point and vertical line represent the mean and SD for three wells. We confirmed that the three independent experiments showed the same tendency.

shown in Figure 2, ATP clearly promoted the chemokinesis of microglia in a dose-dependent manner. ADP exerted a more marked effect than ATP on the chemokinesis of microglia, whereas UTP had no effect (Fig. 2). Unlike nucleotide-induced cell migration, we were able to assess the chemotaxis of microglia in the Boyden chamber by stimulation with C5a. C5a-induced cell migration could be detected only when the ligand was in the bottom compartment. Under this condition, almost the same extent of cell migration was observed between ATP (50 μ M)- and

Figure 3. Nucleotide-induced chemotaxis of microglia in the Dunn chemotaxis chamber. *A*, Vector diagrams of cell displacement at 60 min after setting up the chamber. The cells were incubated in the absence of nucleotides (*a*) or in the presence of 50 μ M ATP (*b*), ADP (*c*), or UTP (*d*) in the outer well. The position of the outer well of the chamber is vertically upward. All diagrams were obtained from a representative experiment using the same lot of microglial culture. We confirmed that the three independent experiments showed the same tendency. *B*, Displacement and morphological change at 5 and 30 min after setting up the chamber. The cells were incubated in the presence of 50 μ M ATP in the outer well. The images of the same area are presented such that the position of the outer well of the chamber is vertically upward. Arrowheads indicate membrane ruffles.

C5a (1 μ M)-induced chemotaxis at 90 min of incubation (data not shown).

ATP- and ADP-induced chemotaxis of microglia in the Dunn chemotaxis chamber

To evaluate whether the nucleotides induced chemotaxis of microglia, we performed another cell migration assay using the Dunn chemotaxis chamber, which allows direct observation of cell movement. The displacements of cells after 1 hr of incubation were plotted as x, y coordinates on scatter diagrams (Fig. 3*A*). As shown in Figure 3*Aa*, microglia have weak motility in the absence of ligands. Compared with that, the great majority of cells that were incubated in the gradient of ATP or ADP migrated toward the source of ligands (Fig. 3*Ab, Ac*), whereas UTP did not induce migration (Fig. 3*Ad*). Two-tailed Student's t tests showed that none of the mean values of x components that were measured for

Figure 4. Effect of extracellular calcium deprivation on membrane ruffling induced by ATP or ADP. Cells were stimulated with PBS (*A, D*), 50 μ M ATP (*B, E*), or 50 μ M ADP (*C, F*) in BSS with (*A–C*) or without (*D–F*) calcium. After stimulation, the cells were fixed and stained with Texas Red-conjugated phalloidin. Arrowheads indicate membrane ruffles. Scale bar, 20 μ m.

the cells stimulated with ATP (4.07 μ m), ADP (7.68 μ m), or UTP (–1.76 μ m) was significantly different from that of control cells without stimulation (1.58 μ m) ($p > 0.05$). By contrast, the mean value of the y component that was measured for cells stimulated with UTP (1.29 μ m) was not significantly different from that without nucleotides (–0.91 μ m), whereas each of the mean y components that was measured for the cells stimulated with ATP (53.04 μ m) or ADP (51.30 μ m) was significantly different from the control ($p < 0.0001$). Representative images obtained 5 min and 30 min after setting the coverslip in the chamber are presented in Figure 3*B*. The cells produced membrane ruffles at 5 min, as indicated by arrowheads, and migrated to the ATP gradient (vertically upward) at 30 min. These results indicated that extracellular ATP and ADP promote microglial motility in the manner of chemoattraction, which may have been mediated by P2 receptors.

Determination of subtype of P2 receptors

P2 receptors have been divided into two types, ionotropic P2X and G-protein-coupled P2Y receptors (Barnard et al., 1997). P2X receptors act as ligand-gated, nonselective cation channels. We have previously demonstrated that ATP-induced plasminogen release from microglia depends on extracellular- Ca^{2+} influx via P2X₇ receptors (Inoue et al., 1998). To identify the types of P2 receptors involved in ATP- and ADP-induced membrane ruffling, we examined the effect of extracellular calcium deprivation. Unlike the plasminogen release (Inoue et al., 1998), ATP and ADP induced membrane ruffling in Ca^{2+} -free BSS as well as in Ca^{2+} -containing BSS (Fig. 4). These results suggest that the membrane ruffling induced by ATP and ADP is not dependent on extracellular Ca^{2+} influx via ionotropic P2X receptors, but is mediated by P2Y receptors.

In our experiments, ATP and ADP acted as potent agonists, whereas UTP exerted only a slight effect on membrane ruffling. Some reports have shown that human P2Y₁, among various P2Y receptors, is activated by ADP and that mouse P2Y₂ is equally activated by ATP and UTP (Lustig et al., 1993; Léon et al., 1997). Although some discrepancy exists regarding the potency of UTP, we speculated that P2Y₁ or P2Y₂ might be a possible receptor for ADP- or ATP-induced membrane ruffling and cell migration. We examined this possibility using three kinds of selective antagonists, suramin, PPADS, and A3P5PS. Charlton et al. (1996) reported that suramin caused antagonistic effects on turkey P2Y₁

and human P2Y₂ expressed in human astrocytoma cell line 1321N1, whereas PPADS antagonized the effects via P2Y₁ and not P2Y₂. A3P5PS was reported as a selective antagonist of the P2Y₁ receptor (Boyer et al., 1996). By contrast, pretreatment with a rather high dose (300 μ M) of PPADS, suramin, or A3P5PS did not inhibit the ATP- and ADP-induced membrane ruffling in microglia, as shown in Figure 5. These data suggest that neither P2Y₁ nor P2Y₂ plays a main role in the ADP- and ATP-evoked responses in microglia.

Inhibition of membrane ruffling by AR-C69931MX and pertussis toxin

P2T_{AC} receptors, which are highly sensitive to ADP but distinct from P2Y₁, have recently been predicted to be present in platelets (Daniel et al., 1998), although they have not yet been cloned. Therefore, we examined the effect of AR-C69931MX, a potent and selective antagonist against P2T_{AC} receptors (Ingall et al., 1999; Ishii-Watabe et al., 2000), on ATP- and ADP-induced membrane ruffling. Pretreatment with 1 μ M AR-C69931MX completely inhibited ATP- and ADP-induced membrane ruffling in microglia, whereas the same treatment did not inhibit M-CSF-induced membrane ruffling (Fig. 6). The antagonistic effect of AR-C69931MX was further confirmed against the poorly hydrolyzable analog ATP γ S (50 μ M) (data not shown). These receptors were also reported to be PTx-sensitive (Daniel et al., 1998). To verify the possibility of this type of P2 receptor being involved, we further examined the effects of PTx on ATP- and ADP-induced membrane ruffling and chemokinesis. The 4 hr pretreatment with PTx at 50 ng/ml completely inhibited membrane ruffling induced by ATP and ADP (Fig. 7), whereas M-CSF-induced membrane ruffling, which is well known to be mediated by a tyrosine kinase receptor, Fms, was not affected by PTx. Furthermore, the pretreatment with PTx also inhibited ATP-induced chemokinesis in the Boyden chamber assay (Fig. 8). These results strongly suggest that G_{i/o}-coupled P2Y receptors, most likely P2T_{AC} receptors, are involved in the membrane ruffling and chemotaxis of microglia induced by ATP or ADP, although precise identification of the specific receptor remains unclear.

ATP- and ADP-induced Rac activation in microglia

Finally, to determine whether ATP- and ADP-induced membrane ruffling and chemotaxis of microglia are mediated by G_{i/o}-coupled P2Y receptors, intracellular signaling was investigated. It

Figure 5. Effects of PPADS, suramin, and A3P5PS on nucleotide-induced membrane ruffling. Microglia were stimulated with 50 μ M ATP (*B, E, H, K*) or ADP (*C, F, I, L*) for 5 min after 10 min of pretreatment with PBS (*A–C*), 300 μ M suramin (*D–F*), 300 μ M PPADS (*G–I*), or 300 μ M A3P5PS (*J–L*). After fixation, the cells were stained with Texas Red-conjugated phalloidin. Arrowheads indicate membrane ruffles. Scale bar, 20 μ m.

has been well established that the Rho family of small G-proteins are key molecules in the reorganization of actin cytoskeleton (Hall, 1998). Among the Rho family, Rac is known to be activated when cells form membrane ruffles and lamellipodia (Ridley et al., 1992; Ohsawa et al., 2000). Activated Rac was found to be translocated to the membrane after stimulation (Ridley et al., 1992; Bokoch et al., 1994). Thus, we performed double staining to examine the translocation of Rac after ATP or ADP stimulation by using Texas Red-conjugated phalloidin (red) and anti-Rac antibody visualized with FITC-conjugated secondary antibody (green) (Fig. 9*A*). We could detect the translocation of Rac to the

ruffling region colocalized with phalloidin staining 5 min after stimulation.

To confirm Rac activation, activated Rac was biochemically measured. Because activated Rac is known to bind to PAK kinase (Manser et al., 1998), a pull-down assay was performed using GST-fused PAK to detect the GTP-bound form of Rac. In samples from cells that were stimulated with ATP or ADP for 1 min, more of the active form of Rac was pulled down as compared with that in control cells (Fig. 9*B*). At the same time, we confirmed that the total amount of Rac in the cell lysates in controls was the same as that in the nucleotide-treated cells (Fig. 9*B*). Moreover, pre-

Figure 6. Effect of AR-C69931MX on nucleotide-induced membrane ruffling. Microglia were stimulated with 50 μ M ATP (*B, F*) or ADP (*C, G*) or 100 ng/ml M-CSF (*D, H*) for 5 min after 10 min of pretreatment with PBS (*A–D*) or 1 μ M AR-C69931MX (*E–H*). After fixation, the cells were stained with Texas Red-conjugated phalloidin. Arrowheads indicate membrane ruffling. Scale bar, 20 μ m.

Figure 7. Effects of PTx on nucleotide-induced membrane ruffling. Microglia were stimulated with PBS (*A, E*), 50 μ M ATP (*B, F*), 50 μ M ADP (*C, G*), or 100 ng/ml M-CSF (*D, H*) for 5 min after 4 hr of pretreatment with PBS (*A–D*) or 50 ng/ml pertussis toxin (*E–H*). After fixation, the cells were stained with Texas Red-conjugated phalloidin. Arrowheads indicate membrane ruffles. Scale bar, 20 μ m.

Figure 8. Effect of PTx on ATP-induced chemokinesis in the Boyden chamber. The chemokinesis assay was performed in the presence of 50 μ M ATP with (white column) or without (black column) pertussis toxin treatment. Cells were pretreated with 50 ng/ml PTx for 4 hr.

treatment of the cells with PTx completely blocked the activation of Rac induced by ATP or ADP (Fig. 10). These results further support the idea that ATP- and ADP-induced membrane ruffling and chemotaxis are mediated by $G_{i/o}$ -coupled P2Y receptors.

DISCUSSION

In the present study, we demonstrated that extracellular ATP and ADP strongly enhanced the formation of membrane ruffles and chemotaxis of microglia. Nucleotide-induced chemotaxis has been reported in rat mast cells (McCloskey et al., 1999) and human neutrophils (Verghese et al., 1996) by using the Boyden chamber assay. We found that ATP and ADP enhanced chemokinesis in the Boyden chamber assay. Although we could not determine whether nucleotides played a role as chemoattractants on microglia by the Boyden chamber assay, the Dunn chemotaxis chamber, which allows the direct observation of cell migration, revealed that the nucleotides had the potency to induce chemotaxis of microglia. The reason we were unable to detect the chemotactic activity of nucleotides in the Boyden chamber assay seems to be partly that the low molecular weight nucleotides easily diffuse from the bottom to the top compartment of the Boyden chamber. Considering the strong effect of ADP, it is highly probable that the effects of ATP actually depend on the metabolite ADP, which may be easily produced during incubation. However, the possibility is very slight because ATP γ S (10 μ M) had the same effect as ATP (data not shown).

Considering the physiological effect of nucleotides on microglia, it is important to identify P2 receptor subtypes. P2 receptors have been divided into two types, ionotropic P2X, and G-protein-

Figure 9. Rac activation induced by ATP and ADP. *A*, Translocation of Rac. The cells were stimulated with PBS (*a–c*), 50 μ M ATP (*d–f*), or 50 μ M ADP (*g–i*) for 5 min and stained with Rac antibody and FITC-conjugated anti-mouse IgG (*a, d, g*) and Texas Red-conjugated phalloidin (*b, e, h*). Merged photographs show the colocalization of Rac and F-actin in the ruffling region (*f, i*), as indicated by arrows. Scale bar, 10 μ m. *B*, Pull-down assay of activated Rac. Cells were stimulated with PBS (control), 50 μ M ATP, or 50 μ M ADP for 1 min, and pull-down assay was performed as described in Materials and Methods. Although the total amount of Rac in the cell lysate was the same for each stimulation, activated Rac was increased in the pull-down samples from ATP- and ADP-stimulated cells.

coupled P2Y receptors (Barnard et al., 1997). Although no study has clarified the profile of P2-receptor subtype expression in microglia, the existence of several kinds of P2Y and P2X receptors has been suggested from electrophysiological studies (Nören-

Figure 10. Effect of PTx on the nucleotide-induced activation of Rac. Pull-down assay was performed, as described in Materials and Methods. Microglia were stimulated with PBS (*control*), 50 μ M ADP, 50 μ M ATP, or 100 ng/ml M-CSF for 1 min after 4 hr of treatment with (+) or without (–) PTx at 50 ng/ml.

berg et al., 1997; Visentin et al., 1999). The biological effects of ATP on microglia have been suggested to be mediated mainly by P2X₇ receptors (Ferrari et al., 1996, 1997, 1999; Inoue et al., 1998; Hide et al., 2000). The present study, however, suggests the involvement of P2Y receptors in nucleotide-induced ruffle formation and cell migration, based on the experiments of extracellular-calcium elimination and AR-C69931MX or PTx treatment.

To date, seven G-protein-coupled P2Y receptors have been cloned in mammalian species, some of which have not been fully characterized in nature (Alexander et al., 1999). Suramin, PPADS, and A3P5PS are commonly used for the pharmacological classification of the subtypes of P2Y receptors. Suramin is an antagonist for both P2Y₁ and P2Y₂, whereas PPADS antagonizes P2Y₁ but not P2Y₂ (Charlton et al., 1996). A3P5PS is a potent and selective antagonist of P2Y₁ (Boyer et al., 1996). Although we examined the effects of these antagonists on ATP- and ADP-induced membrane ruffling in microglia, we did not observe any inhibitory effect. Based on the previous reports described above, neither P2Y₁ nor P2Y₂ would be involved in nucleotide-induced membrane ruffling of microglia.

Recently, G_{i/o}-coupled P2Y receptors, designated P2TAC receptors, which are highly sensitive to ADP, were predicted in platelets (Daniel et al., 1998), although they have not yet been cloned. Based on the results showing that the effects of nucleotides on microglial motility were completely blocked by either AR-C69931MX or PTx pretreatment, this type of G_{i/o}-coupled receptor is postulated as a candidate for the P2Y receptors involved in membrane ruffling and chemotaxis. However, there are a few reports suggesting that P2Y₂ is partially sensitive to PTx in the stable expression system of 1321N1 astrocytoma cells (Parr et al., 1994) and in human erythroleukemia cells (Baltensperger and Porzig, 1997). P2Y₁ was also suggested to be PTx-sensitive in astrocytes (Chen et al., 1998), whereas Schachter et al. (1997) clarified the uncoupling of P2Y₁ to G_{i/o} by using the system of expression in 1321N1 cells. Under these circumstances, in which contradictory reports have been issued, the specific G_{i/o}-coupled subtype of P2Y receptors involved in the present effects cannot be defined. Further characterization of P2Y receptors, including cloning of the P2TAC receptor is needed.

With regard to the intracellular signaling downstream of the G_{i/o}-coupled P2Y receptors, we were able to detect Rac activation in microglia when the cells formed membrane ruffles after ATP or ADP stimulation. Furthermore, PTx-induced inhibition of Rac activation indicates a signal cascade from G_{i/o}-coupled-P2Y re-

ceptors to Rac activation. Rac is a member of the Rho family of small G-proteins, and Rac activation is known to induce lamellipodia and membrane ruffles in various kinds of cells (Ridley et al., 1992; Hall, 1998). There has been cumulative evidence suggesting of the activation of Rac via heterotrimeric G-proteins. Ma et al. (1998) suggested that cytoskeletal reorganization by fMLP (N-formyl-Met-Leu-Phe) is dependent on Rac, Vav, a guanine exchange factor of Rac, and phosphoinositide 3-kinase γ (PI3K γ), in Cos-7SH cells. Although Rac activation still occurred in neutrophils obtained from PI3K γ and PLC β 2/3 knock-out mice, chemokine-mediated chemotaxis was impaired in PI3K γ knock-out mice (Li et al., 2000). Such signaling molecules may be involved in microglia. The possibility of the present findings having been physiologically involved with the function of microglia in the pathological states of the brain may be considered.

In brain with damaged neurons and astrocytes, large amounts of nucleotides are released from these cells (Dubyak and El-Moatassim, 1993; Neary et al., 1996). The extracellular nucleotides, which are easily diffused and rapidly catalyzed by ATPases, may play a role in modulating the microglial function of the brain in the early phase of pathology. We presented a novel effect of nucleotides on microglia, that is, the induction of chemotaxis via G_{i/o}-coupled P2Y receptors. Considering former studies that have revealed the biological effects of nucleotides on microglia such as the release of IL-1 β , plasminogen, and TNF- α via P2X₇ (Ferrari et al., 1996, 1997, 1999; Inoue et al., 1998; Hide et al., 2000), our results suggest that two distinct P2X and P2Y receptor subtypes are involved in the diverse functions of microglia, such as scavenging and neuroprotective actions, in pathological states.

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