

Next, we investigated the effect of PI3K inhibitors on ATP-induced microglial membrane ruffling. As shown in Fig. 2A, phalloidin staining clearly demonstrated that ATP stimulation caused membrane ruffling within 5 min. Pretreatment of microglia with AR-C69931MX inhibited the ATP-induced membrane ruffling, as reported previously (Honda et al., 2001). However, exposure to 100 nM wortmannin or 50 μ M LY294002 appeared to have no effect on the membrane ruffling. To confirm the effect of PI3K inhibitors on membrane ruffling quantitatively, the increase in F-actin in cells with membrane ruffles was analyzed with a laser scanning cytometer (LSC), which is a microscope-based cytometer. ATP-stimulated and unstimulated cells were stained with an anti-Iba1 antibody and Alexa Fluor 647-conjugated phalloidin, and F-actin content was quantified by calculating the mean Alexa Fluor 647 fluorescent intensity of individual cells positive for a microglial marker protein Iba1 (Ito et al., 1998). As shown in Fig. 2B, the mean fluorescent intensity of control microglia was increased approximately 2-fold by ATP stimulation. The fluorescent intensity of ATP-stimulated cells pretreated with AR-C69931MX did not change significantly; however, the fluorescent intensity of cells pretreated with wortmannin or LY294002 was increased by ATP stimulation the same as in the control and the DMSO-treated cells. These results indicate that PI3K activation is not required for the membrane ruffling, but is necessary for induction of microglial chemotaxis.

ATP-Induced Akt Phosphorylation and Effect of Extracellular Calcium Deprivation

To determine whether the PI3K pathway in microglia is activated by ATP stimulation, we investigated Akt phosphorylation, a downstream signaling for PI3K (Bellacosa et al., 1991; Scheid and Woodgett, 2003), by Western blot analysis with a phospho-specific Akt antibody. ATP stimulation rapidly increased the level of Akt phosphorylation in a time-dependent manner (Fig. 3A), and pretreatment with 1 μ M AR-C69931MX or 100 nM wortmannin inhibited the increase in Akt phosphorylation (Figs. 3B,C). These results indicated that ATP induces activation of the PI3K/Akt cascade in microglia and that the activation is mediated by P2Y₁₂R.

Previous studies (Inoue et al., 1998; Tsuda et al., 2003) have shown that stimulation of microglia with 50 μ M ATP induces a transient increase in $[Ca^{2+}]_i$ that depends on the presence of extracellular Ca^{2+} , suggesting that ionotropic P2X receptors are responsible for the Ca^{2+} response. Use of the Ca^{2+} -sensitive fluorescent dye fura-2 revealed that the chelation of extracellular Ca^{2+} suppressed the ATP (50 μ M)-evoked increase in $[Ca^{2+}]_i$ in our cultured microglia (data not shown). To determine whether the increase in $[Ca^{2+}]_i$ had any effect on the PI3K/Akt activation, we investigated Akt phosphorylation in microglia stimulated with 50 μ M ATP in the absence of extracellular Ca^{2+} , and as shown in Fig. 4, chelation of extracellular Ca^{2+} by EGTA significantly

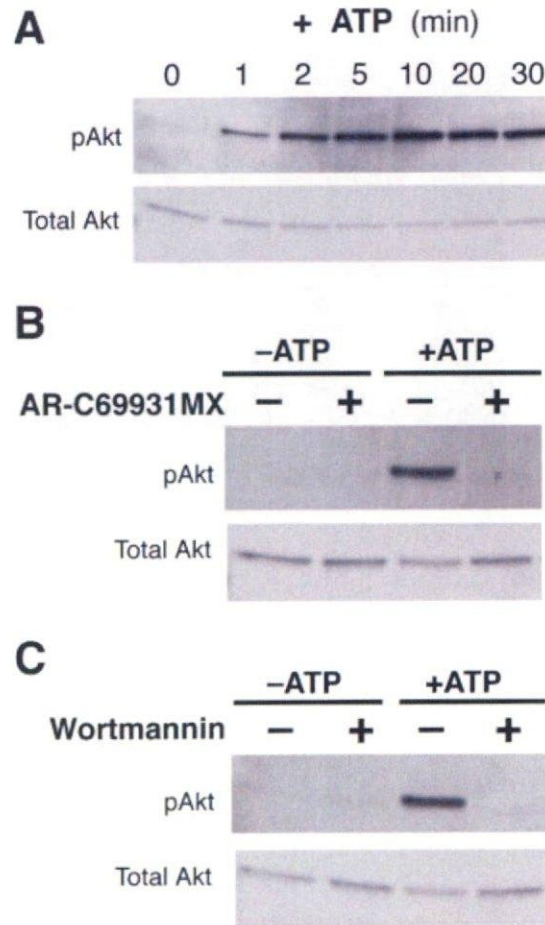


Fig. 3. ATP-induced Akt phosphorylation in microglia is dependent on PI3K activation through P2Y₁₂R. (A) Microglia were stimulated with 50 μ M ATP for the period of time indicated and then lysed in SDS sample buffer. Phosphorylated (pAkt) and total Akt in the lysates were detected by Western blot analysis. (B, C) Microglia were pretreated with 1 μ M AR-C69931MX for 10 min (B) or with 100 nM wortmannin for 20 min (C) and then stimulated with 50 μ M ATP for 5 min. Akt phosphorylation was detected by Western blot analysis. Similar results were obtained from three independent experiments.

decreased ATP-induced Akt phosphorylation. Previous studies have shown that M-CSF stimulates the Fms tyrosine kinase receptor to activate Akt in macrophages in a PI3K-dependent manner (Comalada et al., 2004; Weiss-Haljiti et al., 2004). M-CSF also stimulated Akt phosphorylation in microglia, but chelation of extracellular Ca^{2+} had no effect on it (Fig. 4). These results led us to speculate that the ATP-induced PI3K/Akt activation, which is an essential component for induction of microglial chemotaxis, was linked to an increase in $[Ca^{2+}]_i$ through the extracellular Ca^{2+} -influx via ionotropic P2X receptors.

Effect of P2XR Antagonists on ATP-Induced Microglial Chemotaxis and Akt Phosphorylation

Since primary-cultured microglia have been shown to express P2X₄R (Tsuda et al., 2003; Xiang and Burnstock,

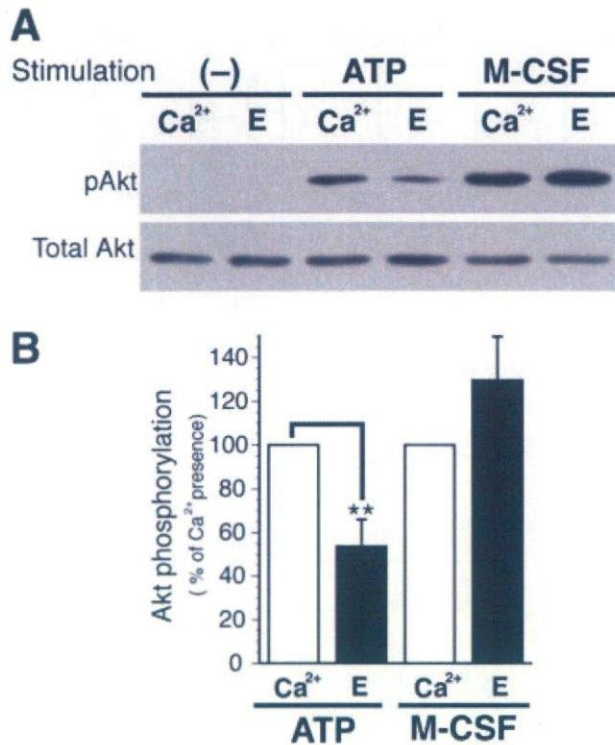


Fig. 4. Inhibitory effect of chelation of extracellular calcium on ATP-stimulated Akt phosphorylation. (A) Microglia were incubated for 30 min in BSS containing 1.2 mM Ca²⁺ (Ca²⁺) or 1 mM EGTA (E) and then stimulated with 50 μ M ATP or 100 ng/mL M-CSF for 5 min. Akt phosphorylation was detected by Western blot analysis. (B) The Akt phosphorylation level was quantified by densitometry. The results are expressed as percentage of agonist-induced phosphorylation in the presence of Ca²⁺ and are means \pm SD of three independent experiments. ** $P < 0.01$; Student's *t*-test.

2005) and P2X₇R (Ferrari et al., 1996; Nörenberg et al., 1994; Verkhatsky and Kettenmann, 1996; Walz et al., 1993), we first investigated the involvement of P2XRs in microglial chemotaxis with a P2X₁₋₄R, antagonist TNP-ATP, with a P2X_{1, 2, 3, 5, 7}R antagonist PPADS, and with a selective P2X₇R antagonist BBG. After pretreatment with an antagonist for 5 min the microglia were observed for chemotactic movement toward ATP in a Dunn chemotaxis chamber containing the antagonist. Treatment with 100 μ M TNP-ATP appeared to suppress the ATP-induced microglial chemotaxis, but 300 μ M PPADS or 1 μ M BBG had no effect (Fig. 5A). The chemotactic movement of the microglia was quantified by calculating the mean value of the total (*x, y*) distances of individual cells migrated toward ATP. As shown in Fig. 5B, the mean distance migrated by the cells pretreated with TNP-ATP was significantly shorter than the distance migrated by the control cells, but the values of the cells pretreated with PPADS or BBG were not significantly different from those of the controls. Treatment with 1 μ M AR-C69931MX also completely inhibited the chemotaxis. These results suggested that P2X₄R as well as P2Y₁₂R is involved in ATP-induced microglial chemotaxis.

We next examined the effect of the three P2XR antagonists, TNP-ATP, PPADS, and BBG, on ATP-stimu-

lated Akt phosphorylation. TNP-ATP significantly suppressed the ATP-stimulated Akt phosphorylation, but PPADS or BBG appeared to have no effect (Fig. 6). AR-C69931MX also completely inhibited Akt phosphorylation. These results suggested that ATP-induced PI3K/Akt activation is mediated by P2X₄R as well as P2Y₁₂R.

Downregulation of P2X₄R in Microglia by Short Hairpin P2X₄R RNAi

To determine whether P2X₄R is in fact involved in ATP-induced microglial chemotaxis, we suppressed P2X₄R expression in microglia with RNAi. We constructed a lentivirus vector that expresses both short hairpin (sh)-RNAi and EGFP under the control of the U6 RNA polymerase III promoter and the CMV promoter, respectively (Fig. 7A), and thus cells expressing the shRNAi should also express the EGFP reporter. Microglia were transduced with a lentivirus vector expressing a short hairpin P2X₄R RNAi (shP2X₄R) or a control vector that expressed short hairpin luciferase RNAi (shControl). Lentiviral particles were added to mixed glial cell cultures, and floating cells were collected as microglia. To confirm the suppression of P2X₄R expression by shP2X₄R, EGFP-positive cells were sorted with a flow cytometer, and expression of P2X₄R protein in the cell lysate was investigated by Western blot analysis with an anti-P2X₄R antibody. P2X₄R protein expression was markedly suppressed in the EGFP-positive cells transduced with shP2X₄R (Fig. 7B), whereas there was no difference in P2X₄R protein level between the EGFP-positive and EGFP-negative cells after transduction with shControl. P2X₇R protein expression was unaffected by transduction with shP2X₄R (Fig. 7B). Microglial RNA was isolated from the sorted cells, and P2Y₁₂R mRNA levels were analyzed by RT-PCR and normalized against actin mRNA levels. P2Y₁₂R mRNA levels increased linearly with PCR reactions for 25–35 cycles. When PCR reactions were performed for 27 cycles, there was no difference in relative level of P2Y₁₂R mRNA in EGFP-positive cells between transduction with shP2X₄R and transduction with shControl (ratio of P2Y₁₂R mRNA level in the shP2X₄R-transduced cells to the shControl-transduced cells = 1.1, Fig. 7C). We checked the P2Y₁₂R mRNA level amplified by PCR for 25 and 30 cycles and confirmed that the relative level of P2Y₁₂R mRNA in the shP2X₄R-transduced cells was the same as in the shControl-transduced cells. These results indicated that P2X₄R expression was specifically suppressed in the EGFP-positive microglia after transduction with shP2X₄R.

The increase in [Ca²⁺]_i induced by ATP (50 μ M) in microglia has been shown to be mediated by P2X₄R (Tsuda et al., 2003). To determine whether shP2X₄R interfered with P2X₄R function in the EGFP-positive microglia, the level of [Ca²⁺]_i in individual cells was monitored by imaging analysis with fura-2 after transduction with the lentivirus vectors. A 30-s application of 50 μ M ATP produced an increase in the 340/380 emission ratio of fura-2 in the EGFP-positive cells transduced with the con-

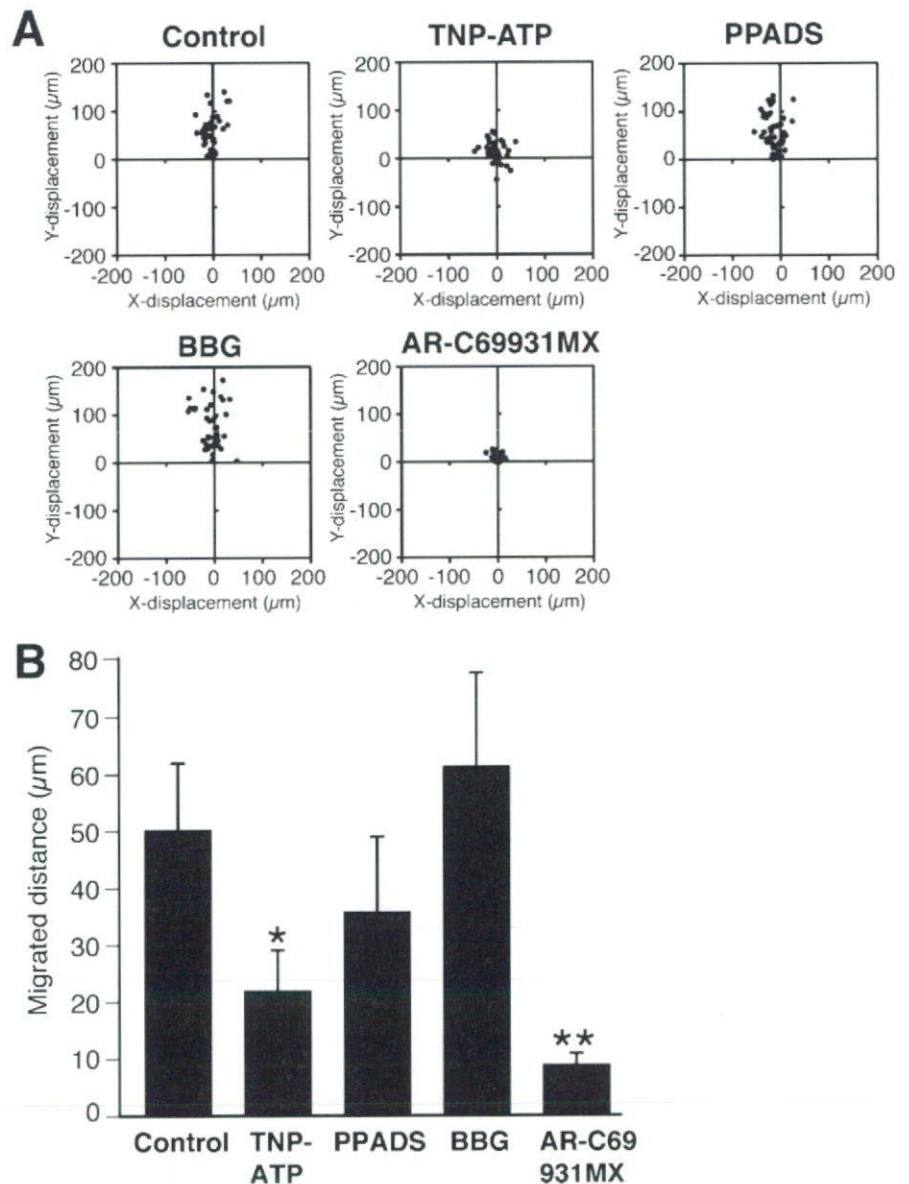


Fig. 5. Effect of P2X antagonists on ATP-induced microglial chemotaxis. (A) Microglia were pretreated with 100 μ M TNP-ATP, 300 μ M PPADS, or 1 μ M BBG for 5 min or with 1 μ M AR-C69931MX for 10 min. Microglial migration towards 50 μ M ATP was observed in the Dunn chemotaxis chamber. The distance and direction of migration by individual cells are shown as x and y coordinates on scatter diagrams. (B) Chemotaxis was quantified by measuring the (x , y) distance migrated from the starting position of cells. Data are means \pm SD of three independent experiments. * P < 0.05, ** P < 0.01, Student's t -test.

control vector (Fig. 8A left), whereas the increase in 340/380 emission ratio was significantly attenuated in the EGFP-positive cells transduced with shP2X₄R (Figs. 8A,B). These results confirmed that transduction with shP2X₄R downregulates expression of P2X₄R protein.

Effect of shP2X₄R on ATP-Induced Membrane Ruffling and Chemotaxis by Microglia

The effect of P2X₄R downregulation on ATP-induced membrane ruffling was examined in microglia transduced with the lentivirus vectors. EGFP-positive cells transduced with shP2X₄R or shControl developed membrane ruffles in response to ATP stimulation, the same as EGFP-negative cells (Fig. 9). These results indicated that shP2X₄R did not inhibit the activation of P2Y₁₂R

and suggested that P2X₄R downregulation had no effect on ATP-induced membrane ruffling.

The cells transduced with the vectors were also examined for chemotactic movement in a Dunn chemotaxis chamber. As shown in the scatter diagrams, the migration of EGFP-positive cells transduced with shP2X₄R (Fig. 10A, bottom left) was clearly inhibited in comparison with the EGFP-negative cells (bottom right). EGFP-positive cells transduced with shControl (top left) migrated toward ATP as same as the EGFP-negative cells (top right). To quantify the effect of the shRNAi on the chemotactic movement of microglia, we calculated the mean value of the (x , y) distances EGFP-positive and -negative cells migrated toward ATP (Fig. 10B). The mean distance migrated by the EGFP-positive cells transduced with shP2X₄R was significantly shorter than both the mean distance migrated by the EGFP-negative cells and the mean distance migrated by the EGFP-positive cells

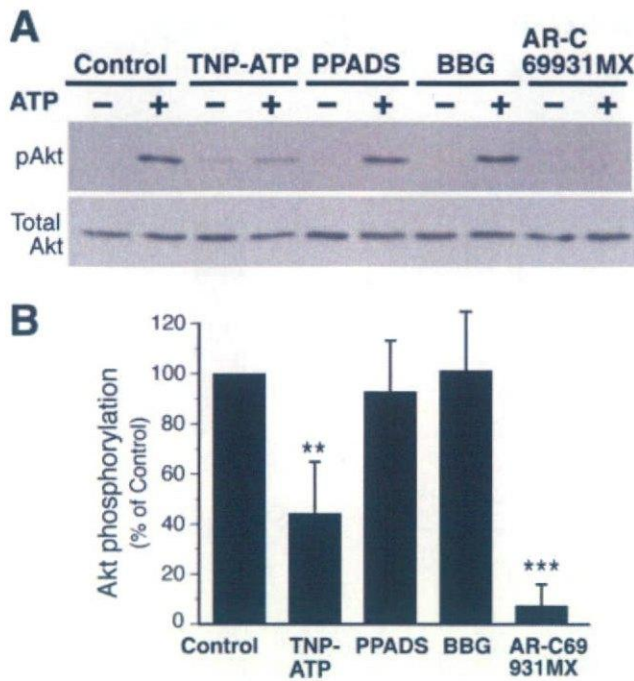


Fig. 6. Effect of P2X antagonists on ATP-stimulated Akt phosphorylation. (A) Microglia were pretreated with 100 μ M TNP-ATP, 30 μ M PPADS, or 100 nM BBG for 5 min or with 1 μ M AR-C69931MX for 10 min, and then stimulated with 50 μ M ATP for 5 min. Akt phosphorylation was detected by Western blot analysis. (B) The Akt phosphorylation level was quantified by densitometry and expressed as percentage of ATP-induced phosphorylation in control cells. The data shown are means \pm SD of three independent experiments. ** $P < 0.01$, *** $P < 0.001$; Student's *t*-test.

transduced with shControl. There was no difference in distance migrated by the EGFP-positive cells and the EGFP-negative cells after transduction with shControl. These results clearly indicated that P2X₄R is involved in ATP-induced microglial chemotaxis.

DISCUSSION

As expected from our previous findings (Honda et al., 2001), both the ATP-induced microglial membrane ruffling and chemotaxis were completely inhibited by a specific P2Y₁₂R antagonist, AR-C69931MX, (Figs. 1 and 2). In this study we further investigated the signaling pathway downstream for P2Y₁₂R and the effect of P2XR antagonists and shRNAi against P2X₄R on microglial migration, and we found that P2X₄R is also involved in ATP-induced microglial chemotaxis.

P2Y₁₂R was known to be coupled to activation of PI3K and inhibition of adenylate cyclase (Czajkowski et al., 2004; Soulet et al., 2004; Van Kolen and Slegers, 2004), and Nasu-Tada et al. (2005) recently reported that a P2Y₁₂R-mediated decrease in cyclic AMP is an important step in membrane ruffling and chemotaxis by microglia on fibronectin-coated dishes. PI3K is well known to be a key player in remodeling of the actin cytoskeleton and in regulating cell migration, including chemotaxis (Procko and McColl, 2005; Van Haastert and Devreotes, 2004). In this study we showed that PI3K inhibitors

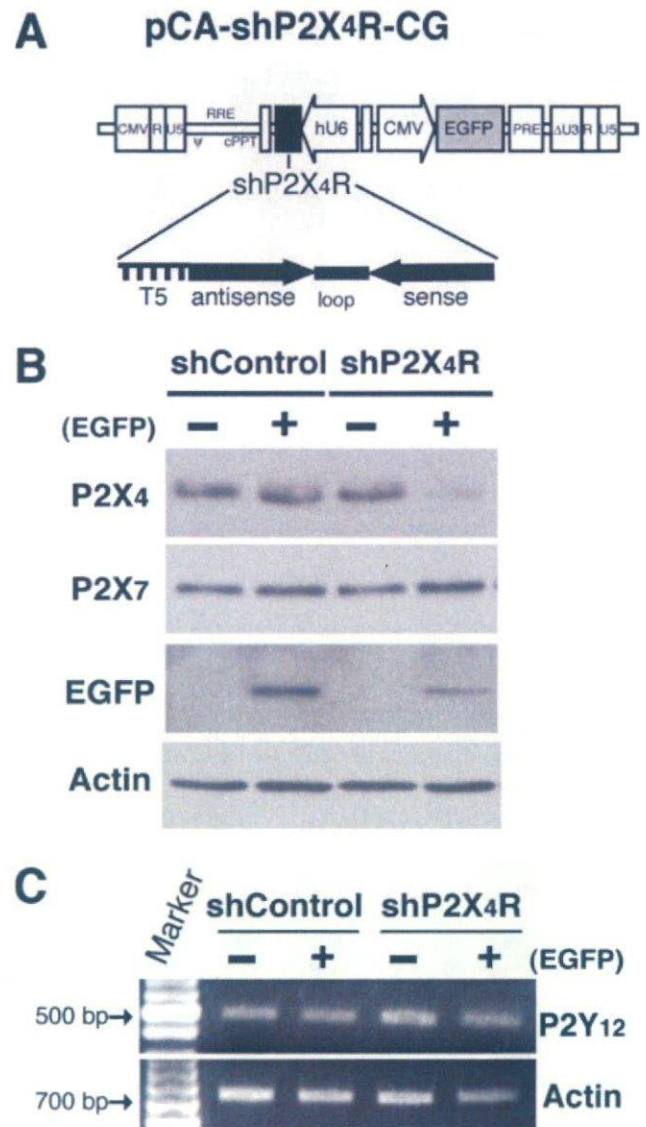


Fig. 7. shRNAi-targeted downregulation of P2X₄R in microglia by lentivirus vectors. (A) Schematic drawing of lentivirus vectors expressing EGFP and shRNAi against P2X₄R (shP2X₄R). A shRNA sequence targeted for firefly luciferase (shControl) was used as a control. (B) Protein expression of P2X₄R and P2X₇R in the microglia transduced with the shRNAi lentivirus vectors. EGFP-positive (+) and -negative (-) cells were sorted with a flow cytometer and lysed in SDS sample buffer. Protein expression of P2X₄R, P2X₇R, EGFP, and actin in the cell lysates was detected by Western blot analysis. Actin served as an internal control. (C) Gene transcript analysis of microglia transduced with the shControl or shP2X₄R vector. RNA was isolated from the sorted cells. Gene transcripts for P2Y₁₂R and β -actin, which served as an internal control, were analyzed by RT-PCR. The relative intensity of the bands for P2Y₁₂R was quantified by densitometry and normalized to the β -actin products. Similar results were obtained from at least three independent experiments.

blocked microglial chemotaxis towards ATP (Fig. 1). However, the PI3K inhibitors had no effect on membrane ruffling (Fig. 2), suggesting that the initial actin reorganization induced by ATP is not dependent on PI3K activation, whereas the ATP gradient-dependent cell migration requires PI3K activation. PI3Ks phosphorylate phosphoinositides at the 3-hydroxyl of the inositol

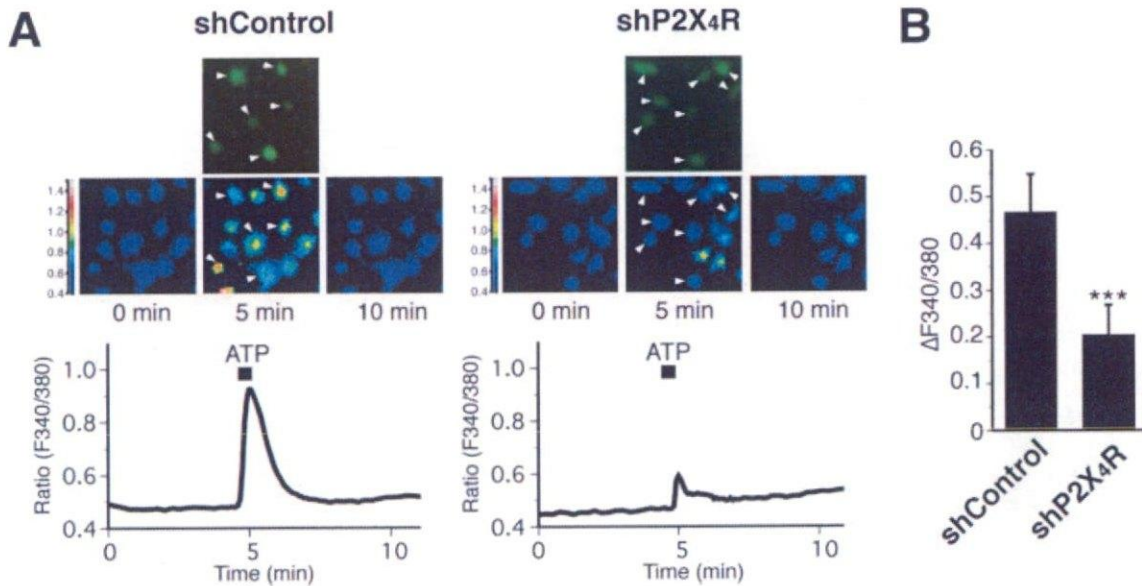


Fig. 8. Effect of P2X₄R downregulation on the ATP-evoked increase in $[Ca^{2+}]_i$ in microglia. (A) Microglia transduced with the shControl (left panel) or shP2X₄R (right panel) vector were loaded with fura-2/AM. $[Ca^{2+}]_i$ was expressed as the ratio of the fluorescence intensity at 340 nm to the fluorescence intensity at 380 nm (F340/380). The pseudocolor image shows three frames (0, 5, and 10 min) of fura2-loaded microglia stimulated with 50 μ M ATP for 30 s. Arrowheads point to

EGFP-positive cells. The traces show the mean increase in F340/380 emission ratio of 14 EGFP-positive cells from each culture. (B) The graphs show the relative increase in ratio ($\Delta F_{340/380}$; mean \pm SD, $n = 14$ cells) from the basal level of the EGFP-positive cells shown in Fig. 8A. *** $P < 0.001$; Student's t -test. Similar results were obtained from three independent experiments.

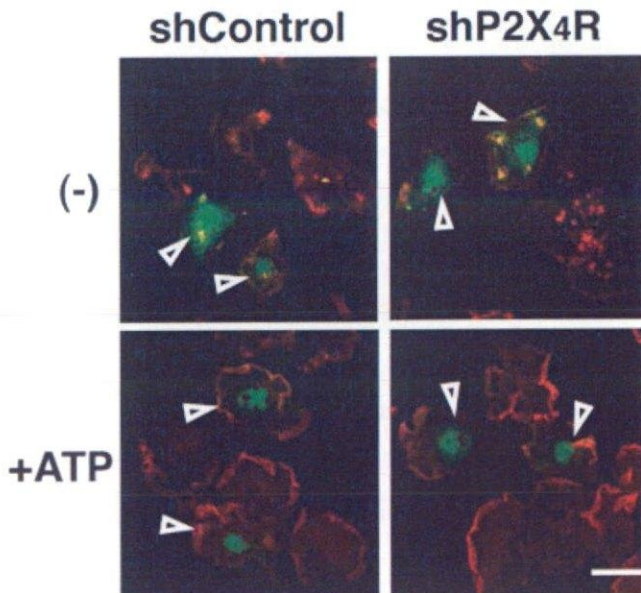


Fig. 9. Effect of P2X₄R downregulation on ATP-induced membrane ruffling of microglia. Microglia transduced with the lentivirus vectors were stimulated with 50 μ M ATP for 5 min. After fixation, the cells were stained with Texas Red-conjugated phalloidin. Arrowheads indicate EGFP-positive cells. ATP-stimulated membrane ruffling of EGFP-positive cells was observed in three independent experiments. Scale bar, 20 μ m.

ring (Vanhaesebroeck et al., 2001). When cells are placed in a chemoattractant gradient, the phosphorylated phospholipids selectively accumulate at the leading edge and act as a membrane anchor for many PI3K downstream effector proteins with pleckstrin homology

(PH) regions, which may regulate directional sensing during chemotaxis (Procko and McColl, 2005; Van Haasterter and Devreotes, 2004). PI3K will play a crucial role in both sensing the ATP gradient and determining the cell polarity of the microglia.

Akt is activated through binding of its PH domains to lipid products of PI3K on the plasma membrane (Scheid and Woodgett, 2003). In this study ATP-induced increase in Akt phosphorylation was suppressed by pretreatment with a P2Y₁₂R antagonist or PI3K inhibitors (Fig. 3). These findings indicated that Akt is phosphorylated following PI3K activation downstream of P2Y₁₂R in microglia. Interestingly, the increase in the Akt phosphorylation was suppressed by chelation of extracellular calcium with EGTA (Fig. 4), and depletion of intracellular calcium by BAPTA-AM also blocked the Akt phosphorylation (data not shown). These results indicate that activation of the PI3K-Akt signal pathway is regulated by an increase in $[Ca^{2+}]_i$. Previous studies have shown that in some cells an increase in $[Ca^{2+}]_i$ can activate Akt through PI3K-dependent or independent pathways. An increase in $[Ca^{2+}]_i$ can also activate Src or proline-rich/ Ca^{2+} -activated tyrosine kinase Pyk2, thereby directly or indirectly regulating the PI3K activation (Chen et al., 2001; Gendron et al., 2003; Okuda et al., 1999). Protein kinase C (PKC) or Ca^{2+} /calmodulin-dependent protein kinase which is activated by calcium, lies upstream of Akt or directly phosphorylates Akt (Bauer et al., 2003; Glikli et al., 2002; Tanaka et al., 2003; Yano et al., 1998). Further investigation is needed to determine how the calcium signaling regulates the PI3K/Akt activation in microglia.

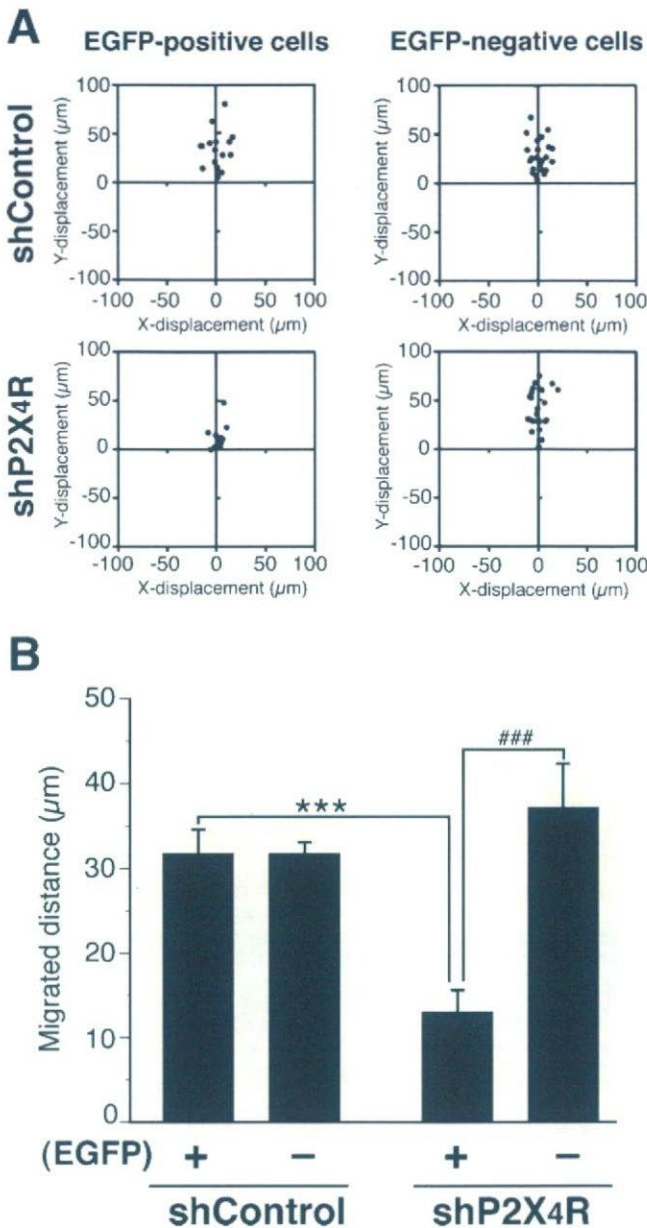


Fig. 10. Inhibitory effect of P2X₄R downregulation on ATP-induced microglial chemotaxis. (A) Microglia were transduced with the lentiviral vectors and microglial migration towards 50 μM ATP was observed in the Dunn chemotaxis chamber. The distance and direction of movement by individual cells are shown as *x* and *y* coordinates on scatter diagrams. (B) Each chemotaxis was quantified by measuring the (*x*, *y*) distance migrated from the starting position of cells. Data are means ± SD of three independent experiments. ****P* < 0.001, Student's *t*-test, compared with shControl EGFP-positive cells; ###*P* < 0.001, compared with shP2X₄R EGFP-negative cells.

The ATP-induced increase in [Ca²⁺]_i in microglia has been shown to be suppressed by chelation of extracellular calcium or pretreatment with TNP-ATP, but not by PPADS or BBG (Tsuda et al., 2003). The present study showed that shRNA-mediated downregulation of P2X₄R in microglia suppressed the ATP-induced increase in [Ca²⁺]_i (Fig. 8). These observations suggest that the increase in [Ca²⁺]_i is mainly caused by the influx of

extracellular calcium through P2X₄R. ATP-induced PI3K/Akt activation was inhibited by pretreatment with TNP-ATP, but not with PPADS or BBG (Fig. 6). Interference with P2X₄R expression markedly inhibited the ATP-induced microglial chemotaxis (Fig. 10) without affecting membrane ruffling (Fig. 9), the same as the effects of PI3K inhibitors (Figs. 1 and 2). We therefore suspect that the P2X₄R-mediated calcium signaling may be involved in PI3K/Akt activation and regulate microglial chemotaxis. Local Ca²⁺ mobilization through P2X₄R at membrane ruffles may be necessary for maintenance or enhancement of the local P2Y₁₂R-activated PI3K signals.

Membrane ruffling is generated by dynamic remodeling of the actin cytoskeleton at the plasma membrane and is thought to be a crucial process for cell migration (Small et al., 2002). P2Y₁₂R activation is essential for ATP-induced membrane ruffling and triggers intracellular signaling events that lead to microglial chemotaxis toward ATP. Ca²⁺ imaging showed that shP2X₄R did not completely suppress the ATP-evoked increase in [Ca²⁺]_i (Fig. 9), suggesting that other subtypes of ATP receptor are involved in the Ca²⁺ response. P2Y receptors are generally linked to activation of phospholipase C (PLC) that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to the intracellular messenger inositol 1,4,5, triphosphate (IP₃) and diacylglycerol (Communi et al., 2000). ATP-stimulated P2Y₁₂R will induce PLC activation, leading to IP₃-mediated Ca²⁺ release from intracellular calcium stores in microglia. P2Y receptors modulate G-protein coupled or voltage-dependent ion channels that affect the Ca²⁺ current (Van Kolen and Slegers, 2006). Vial et al. (2002) reported that coactivation of P2X₄R and P2Y₁R in platelets synergistically enhances the Ca²⁺ response and suggested that P2X₁ may have a priming role in the activation of P2Y₁R during platelet activation. Further study is needed to analyze cross-talk between P2X₄R and other signal pathways downstream of P2Y₁₂R, such as the adenylate cyclase pathway or PLC pathway.

P2Y₁₂R is constitutively expressed in microglia in the normal brain (Sasaki et al., 2003). A recent report by Haynes et al. (2006) shows that P2Y₁₂R is essential for early microglial responses towards either a local ATP injection or a focal laser injury in brain slices. P2Y₁₂R play crucial roles in regulating the morphological changes in ramified microglia and cell migration by activated microglia in response to ATP released by surrounding cells and its hydrolysis product ADP. By contrast, P2X₄R expression in microglia is lower in the normal brain and spinal cord, and it is significantly upregulated in activated microglia within 24 h after ischemia or nerve injury (Cavaliere et al., 2003; Schwab et al., 2005; Zhang et al., 2006). Tsuda et al. (2003) recently reported that the P2X₄R expression is induced in spinal microglia during the tactile allodynia observed after nerve injury. These observations together with our own findings suggest that P2X₄R activation may modulate or enhance the microglial cell migration in pathological conditions. Although further study is needed to clarify the molecular mechanisms underlying the microglial cell migration

mediated by P2Y₁₂R and P2X₄R, the findings in the present study may contribute to understanding the ATP-induced changes in microglial dynamics in the brain in normal and pathological states.

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