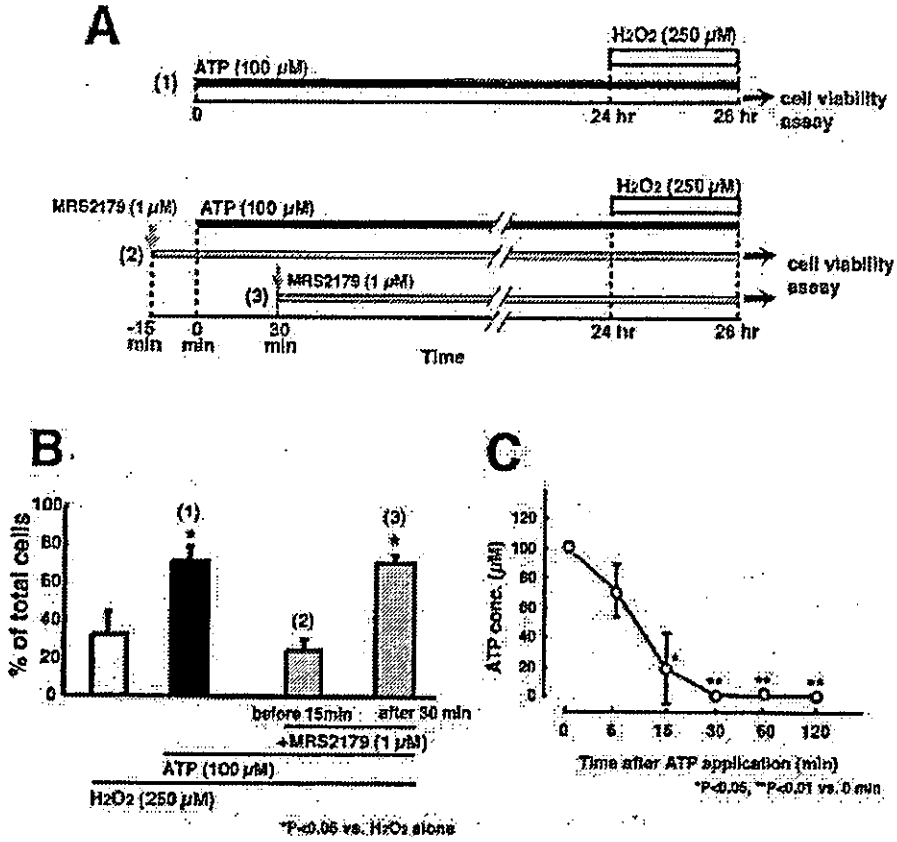


Fig. 4. ATP activates $P2Y_1$ receptors in a short time window. A: (1)–(3) depict the stimulus regime. B: MRS2179 (1 μM) was applied to the cells 15 min before and during ATP application (2), or 30 min after ATP stimulation (3). MRS2179 did not antagonize the effect of ATP when it was applied to the cells 30 min after ATP stimulation (3). Each histogram shows a typical experiment with each data point being mean ± SEM of triplicate measurements. At least three such experiments were performed. Values were normalized to total cell number. Asterisks show significant differences from the response evoked by H_2O_2 alone ($^*P < 0.05$, Student's *t*-test). C: Exogenously applied ATP is metabolized rapidly on astrocytes. Extracellular ATP concentration was measured using a luciferin-luciferase method. ATP (100 μM) was added to the cells, and then supernatant was collected at the time indicated. Exogenously applied ATP was soon metabolized and almost disappeared 30 min after the stimulation. The extracellular ATP concentrations at 5, 15, 30, 60, and 120 min were 76.0 ± 17.8, 18.9 ± 23.7, 1.2 ± 1.0, 0.3 ± 0.36, and 0.02 ± 0.03 μM, respectively. At least three such experiments were performed. Asterisks show significant differences from the ATP concentration at 0 min after 100 μM ATP application ($^*P < 0.05$, $^{**}P < 0.01$, Student's *t*-test).

sured the increases in $[Ca^{2+}]_i$ in astrocytes (Fig. 7). Single RT-PCR analysis revealed that astrocytes express $P2Y_1$ receptor mRNA (Fig. 7A). The $[Ca^{2+}]_i$ analysis showed that ATP (100 μM) evoked an increase in $[Ca^{2+}]_i$ in about 90% of the astrocytes [Fig. 7B(1)], which was independent of the extracellular Ca^{2+} ($0Ca^{2+}$), but was inhibited by the P2 receptor antagonists PPADS (300 μM), reactive blue 2 (RB2) (10 μM), suramin (100 μM), and the $P2Y_1$ receptor antagonist MRS2179 (1 μM) [Fig. 7B(1)]. Similar to ATP, the $P2Y_1$ agonists ADP (100 μM) [Fig. 7B(2)] and 2MeSADP (1 μM) [Fig. 7B(3)] evoked $[Ca^{2+}]_i$ elevations, which were again inhibited by PPADS and MRS2179. Another $P2Y_1$ receptor agonist, ADPβS (1 μM), also produced an increase in $[Ca^{2+}]_i$ (responder, 97 ± 1%; mean amplitude, 0.53 ± 0.03, n = 63). These results suggest that the metabotropic $P2Y_1$ receptor has a dominant role in the Ca^{2+} responses to extracellular nucleotides in astrocytes. UTP, an agonist of UTP-preferring $P_2Y_2/4$ receptors, also evoked an increase in $[Ca^{2+}]_i$ in a concentration-dependent fashion (100–1,000 μM) (Fig. 7C, gray columns) and at 1,000 μM almost all astrocytes responded to UTP (Fig. 7C, open circles). The mean amplitude of the $[Ca^{2+}]_i$ elevation evoked by UTP, however, was less than that evoked by 100 μM ATP (ATP, 0.92 ± 0.04, n = 103 vs. UTP, 100 μM, 0.46 ± 0.03, n = 182; 1,000 μM, 0.69 ± 0.03, n = 167). Neither adenosine nor α,βmeATP, an agonist of P_2X_1 and P_2X_3 receptors, evoked the $[Ca^{2+}]_i$ elevation in astrocytes (Fig. 7C).

Since glutamate and gap junction are the most probable factors that may affect increases in $[Ca^{2+}]_i$ in astrocytes (Chen et al., 1997; Finkbeiner, 1992; Glaum et al., 1990), the effects of glutamate antagonists and a gap junction inhibitor on the Ca^{2+} responses to ATP were investigated. As shown in Figure 7D, neither the amplitude of the $[Ca^{2+}]_i$ elevations evoked by 100 μM ATP (columns) or the fraction of ATP-responders (open circles) was affected by the gap junction inhibitor 1-octanol (500 μM), the NMDA receptor antagonist AP-V (100 μM), the AMPA receptor antagonist CNQX (30 μM) or the metabotropic glutamate receptor antagonist MCPG (300 μM). All inhibitors were applied to the cells 15 min before and during ATP application.

Gene Expression Changes by ATP

To show the effect of ATP on the gene expression of astrocytes, we investigated the differential gene expression induced by ATP in astrocytes using Affymetrix GeneChip. We analyzed ATP-induced genes based on the information obtained from Genbank, Unigene, Locuslink, and PubMed at NCBI. As expected

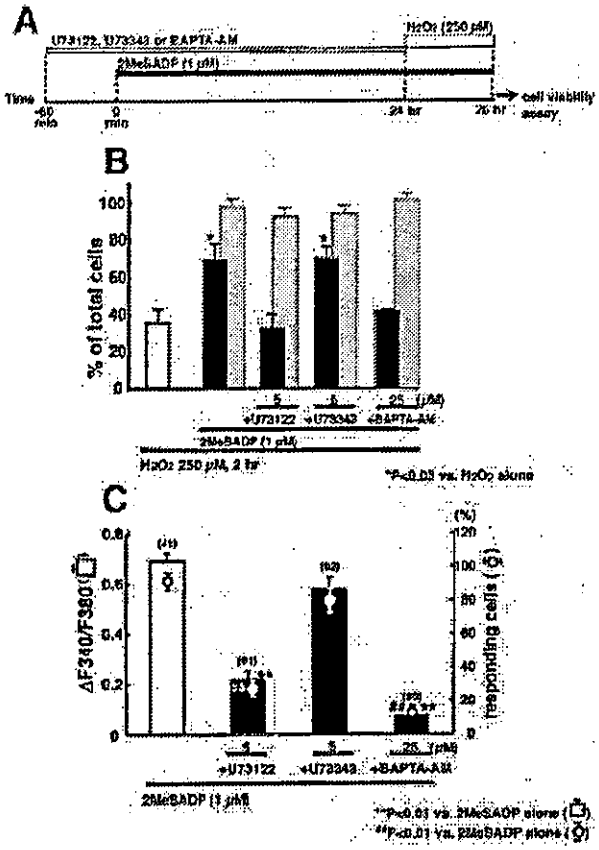


Fig. 5. Intracellular signaling cascades associated with $P2Y_1$ receptor-mediated cytoprotective action. A: Stimulus regime. B: When cells were pretreated with U73122 (5 μM) or BAPTA-AM (25 μM), the 2MeSADP (1 μM)-induced protective action against H_2O_2 disappeared, whereas U73343 (5 μM), a much less active PLC inhibitor, had no effect on it. 2MeSADP was added to the cells 24 h before and during H_2O_2 application, and each inhibitor was added 1 h before and during 2MeSADP application. These inhibitors were washed out just before H_2O_2 application. Gray columns show the effects of 2MeSADP alone or inhibitors alone on the cell viability in the normal condition. Each histogram shows a typical experiment with each data point being mean ± SEM of triplicate measurements. At least three such experiments were performed. Values were normalized to total cell number. Asterisks show significant differences from the response evoked by H_2O_2 alone ($^*P < 0.05$, $^{**} < P < 0.01$, Student's *t*-test). C: Increases in $[Ca^{2+}]_i$ evoked by 2MeSADP, showing the effects of the blockers listed in B. The increase in $[Ca^{2+}]_i$ evoked by 2MeSADP (1 μM) was inhibited by U73122 (5 μM) or BAPTA-AM (5 μM) but not by U73343 (5 μM). These inhibitors were added to the cells 15 min before and during 2MeSADP application. The increases in $[Ca^{2+}]_i$ (Δ340/F380) and fraction of responders are shown as columns and open circles, respectively. The number of cells tested is shown in parentheses. Asterisks show significant difference from the amplitude of $[Ca^{2+}]_i$ and the number of responders evoked by 2MeSADP alone, respectively ($[Ca^{2+}]_i$, $^*P < 0.05$, $^{**} < P < 0.01$ vs. 2MeSADP alone; numbers of responders, $\#P < 0.05$, $\#\#P < 0.01$ vs. 2MeSADP alone; Student's *t*-test).

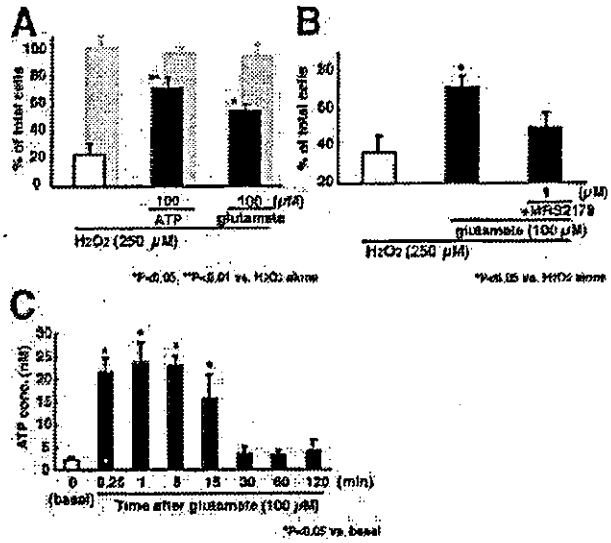


Fig. 6. Effects of glutamate on the H_2O_2-induced cell death in astrocytes. A: Preincubation with glutamate protected astrocytes against H_2O_2-evoked cell death. Cells were incubated with glutamate (100 μM) for 24 h, and then was exposed to H_2O_2. Glutamate showed significant protection against H_2O_2-induced cell death. Gray columns show the cell viability after a 24-h incubation with agonists alone. Glutamate itself had no effect on the cell viability. Asterisks show significant differences from the response evoked by H_2O_2 alone ($^*P < 0.05$, $^{**}P < 0.01$. Student's *t*-test). B: Selective $P2Y_1$ receptor antagonist MRS2179 inhibited the protective effect by glutamate. MRS2179 (1 μM) was added to the cells 15 min before and during glutamate application. Asterisks show significant differences from the response induced by H_2O_2 alone ($^*P < 0.05$, Student's *t*-test). C: Glutamate (100 μM) produced release of ATP from astrocytes. Cells were incubated with 100 μM glutamate for the time indicated, the supernatants were collected, and then extracellular ATP concentrations were measured using a luciferin-luciferase method. Significantly higher ATP concentration above basal was observed from 0.25 to 15 min after glutamate (100 μM) stimulation. Asterisks show significant differences from the basal extracellular ATP concentration ($^*P < 0.05$, Student's *t*-test).

from the previous results, ATP induced a dramatic upregulation of oxidoreductase genes such as TrxR, CBR, and SHL4 (similar to superoxide dismutase SOD-2) (Table 1). These genes were classified on the basis of information from Gene Ontology Consortium (http://www.geneontology.org/). Using a quantitative RT-PCR method, we confirmed that these oxidoreductase genes including TrxR, CBR and SHL4 were upregulated by ATP (100 μM, 2 h). The fold increases are shown in parentheses in Table 1 [i.e., CBR (8.9), SHL4 (17.2), and TrxR (2.9)].

DISCUSSION

The importance of dynamic communication among glial cells in the CNS has been recognized, and astrocytic ATP has a dominant role in such gliotransmission (Koizumi et al., 2003; Newman, 2003; Zhang et al., 2003). In the present study, we demonstrated that such ATP-mediated gliotransmission is important for astrocytic survival because ATP protected astrocytes from H_2O_2-induced cell death. This effect was mediated by the activation of $P2Y_1$ receptors but not by adenosine receptors although adenosine, a metabolite of ATP, is well known to protect neurons from various pathological conditions. After the activation of $P2Y_1$ receptors, it took more than 12 h for the protective action to be revealed, and ATP upregulated several "oxidoreduc-

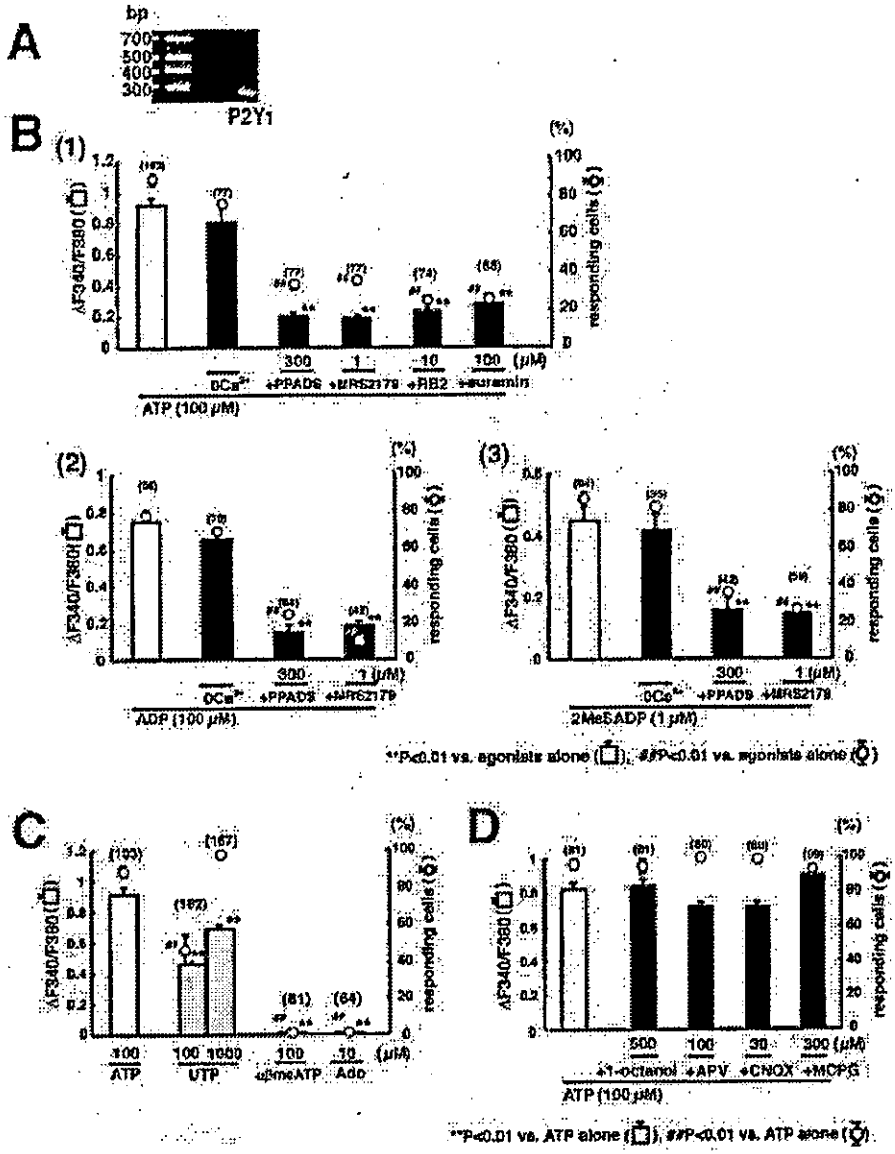


Fig. 7. Expression and function of $P2Y_1$ receptors. A: RT-PCR analysis showing expression of $P2Y_1$ receptor mRNA in astrocytes. Total cellular RNA was subjected to RT-PCR. The resulting cDNA was amplified with specific primers for $P2Y_1$ receptors. PCR product was resolved on agarose gel stained by 2% ethidium bromide and visualized under UV light. B: Functional $P2Y_1$ receptors are present in astrocytes. (1) ATP (100 μM) produced a rise in $[Ca^{2+}]_i$, which was independent of extracellular Ca^{2+} ($0Ca^{2+}$), but was inhibited by PPADS (300 μM), MRS2179 (1 μM), reactive blue 2(RB2) (10 μM), or suramin (100 μM). Similar to ATP, (2) ADP (100 μM) and (3) 2MeSADP (1 μM) induced rapid $[Ca^{2+}]_i$ increases, which were independent of extracellular Ca^{2+} but were sensitive to PPADS and MRS2179. C: $P2Y_{2/4}$ receptor agonist UTP also evoked elevations in $[Ca^{2+}]_i$ in a concentration-dependent fashion and at 1,000 μM produced $[Ca^{2+}]_i$ increases in almost all astrocytes (97 ± 2%, $n = 167$), although the amplitude of $[Ca^{2+}]_i$ elevation was significantly lower than that evoked by 100 μM ATP. Neither α, βmeATP (100 μM) nor adenosine (10 μM) produced any $[Ca^{2+}]_i$ elevations. D: Effects of glutamatergic receptors and gap junctions on the ATP-evoked increase in $[Ca^{2+}]_i$ in astrocytes. 1-Octanol (500 μM), AP-V (100 μM), CNQX (30 μM) and MCPG (300 μM) were added to the cells 15 min before and during ATP-application. All these antagonists had no effect on the amplitude of $[Ca^{2+}]_i$ or the number of responders. The number of cells examined was shown in parentheses. Columns and circles show the mean amplitude of $[Ca^{2+}]_i$ elevations and percentage of responders, respectively. * and # show significant difference from the amplitude of $[Ca^{2+}]_i$ and the number of responders evoked by agonist alone, respectively ($[Ca^{2+}]_i$, $^*P < 0.05$, $^{**}P < 0.01$ vs. agonist alone; numbers of responders, $\#\#P < 0.01$ vs. agonist alone; Student's *t*-test).

tase genes." Thus, astrocytes use $P2Y_1$ receptor- but not adenosine P1 receptor-mediated signals to upregulate self-protection genes, thereby leading to resistance to oxidative stress.

$P2Y_1$ receptors are expressed in various tissues (Tokuyama et al., 1995; Akbar et al., 1996), including the CNS (Tokuyama et al., 1995; Ayyanathan et al., 1996; Webb et al., 1998; Moore et al., 2000). Astrocytes ex-

TABLE 1. List of Genes Upregulated by ATP in Astrocytes

Identifer	Title	Fold increase (RT-PCR)	Oxidoreductase activity[a]
D89069	Inducible carbonyl reductase	6.5 (8.9)	GO:0016616; oxidoreductase activity, acting on the CH—OH group of donors, NAD, or NADP as acceptor
D89070	Noninducible carbonyl reductase	6.8	GO:0016616; oxidoreductase activity, acting on the CH—OH group of donors, NAD or NADP as acceptor
X95986	Carbonyl reductase	5.9	GO:0016616; oxidoreductase activity, acting on the CH—OH group of donors, NAD, or NADP as acceptor
AA926129	Schlafen-4 (similar to SOD-2)	3.1 (17.2)	GO:0016721; oxidoreductase activity, acting on superoxide radicals as acceptor
U63923	Tissue type liver thioredoxin reductase	2.3 (2.9)	GO:0016654; oxidoreductase activity, acting on NADH or NADPH, disulfide as acceptor

SOD-2, superoxide dismutase-2; RT-PCR, reverse transcription-polymerase chain reaction.
[a]GO ontology defined by Gene Ontology Consortium (http://www.godatabase.org/htdocs.html).

press several types of metabotropic P2Y receptors such as $P2Y_1$ (Ho et al., 1995; Fam et al., 2000) and $P2Y_{2,4,6,12,14}$ (Idestrup and Salter, 1998; Lenz et al., 2000; Fumagalli et al., 2003) as well as ionotropic P2X receptors ($P2X_{1,2,3,4,5,7}$). Our present findings showed that the protective effect by ATP against H_2O_2-induced cell death was dependent on both PLC activation and stored Ca^{2+}, suggesting that the protective action of ATP works via metabotropic PLC-linked P2Y receptors in astrocytes (Fig. 5B). The pharmacological analysis revealed that the responsible receptors for the protective action were $P2Y_1$ receptor (Fig. 3). In addition to $P2Y_1$ receptors, $P2Y_2$ receptors, another type of PLC-linked P2Y receptor, are also expressed in astrocytes. UTP, however, failed to protect astrocytes from H_2O_2-induced cell death (Fig. 3C) in spite of the fact that UTP produced increases in $[Ca^{2+}]_i$ via a PLC-linked mechanism (Shahidullah and Wilson, 1997; Idestrup and Salter, 1998; Viana et al., 1998). Both ATP and UTP activate $P2Y_2$ receptors almost equally (Lustig et al., 1993), whereas ATP activates $P2Y_1$ receptors more potently than UTP, and the ED_{50} for ATP to evoke a $[Ca^{2+}]_i$ elevation is almost 10-fold smaller than that of UTP in astrocytes (Koizumi et al., 2002). Thus, this discrepancy might be explained by the lower affinity of $P2Y_2$ receptors to ATP in astrocytes. In addition, although ATP and the selective $P2Y_1$ agonist 2MeSADP evoked increases in $[Ca^{2+}]_i$ in almost all of the astrocytes, UTP (100 μM) produced the $[Ca^{2+}]_i$ elevation in a smaller population of cells (Fig. 7C). The discrepancy may also be explained by the functional heterogeneity of $P2Y_2$ receptor expression among astrocytes. However, when the UTP concentration was raised up to 1,000 μM, it produced elevations in $[Ca^{2+}]_i$ in almost all astrocytes (Fig. 7C) but still failed to protect against cell death in astrocytes (Fig. 3C). These results suggest that the PLC-linked Ca^{2+} mobilization is required for the ATP-induced cytoprotection but is not sufficient to reveal its protective action. Other than PLC-linked Ca^{2+} mobilization, the $P2Y_1$ receptor might stimulate other pathways closely involved in the cytoprotective action. The finding that, although glutamate could mobilize Ca^{2+} and protect against H_2O_2 induced cell death in astrocytes, the glutamate-induced cytoprotection also involved the activation of $P2Y_1$ receptors (Fig. 6) may support this idea.

Since Servitja et al. (2000) showed that H_2O_2 activates PLC in astrocytes, previous exposure of ATP might reduce the amount of PLC available during the application of H_2O_2, thereby leading to the decrease in H_2O_2-induced cell death in astrocytes. Although we cannot exclude this possibility completely, such a PLC reduction, if it occurs, does not seem to be involved in the protective action by ATP for the following reasons. Firstly, activation of $P2Y_1$ receptors by 2MeSADP results in an increase in $[Ca^{2+}]_i$ via PLC-mediated mechanisms. The 2MeSADP-evoked increases in $[Ca^{2+}]_i$ in ATP-treated (24 h) and ATP-untreated control cells were almost identical (ATP treated cells: 0.64 ± 0.05, n = 65; ATP untreated cells: 0.65 ± 0.04, n = 70), suggesting that the $P2Y_1$/PLC-mediated pathway(s) is not affected by ATP pretreatment. Secondly, H_2O_2-induced cell death was unaffected by the PLC blocker U73122, suggesting that PLC itself is not involved in the H_2O_2-induced cell death (H_2O_2 alone; 36 ± 2%, and H_2O^{2+}U73122; 36 ± 1% of control). Judging from these findings, it is unlikely that a reduction of PLC is involved in the ATP-evoked protection against H_2O_2 in astrocytes.

Cells in the CNS have many chances to be exposed to ATP because ATP is released or leaked from both neurons and astrocytes in physiological and pathological conditions. Extracellular ATP, however, is soon metabolized into adenosine by ectonucleotidases (Zimmermann, 1996), and some ectonucleotidases are upregulated after brain ischemia (Braun et al., 1998) especially in glial cells (Braun et al., 1997). Adenosine therefore is considered one of the major molecules that show neuroprotective effects against several types of neuronal damage in the CNS, such as ischemic/hypoxic brain damage or post-hypoxic repurfusion-evoked neuronal injury (Behan and Stone, 2002; Jones et al., 1998), and Parkinson's disease (Schwarzschild et al., 2003). The main mechanism underlying the adenosine-induced neuroprotection appears to be the inhibition of excess excitability of neurons (Fredholm and Dunwiddie, 1988). In the present study, however, adenosine showed no protective effect against H_2O_2-evoked cell death in astrocytes. Although some groups already reported that adenosine protected astrocytes from glucose deprivation-evoked cell death, this protection appeared to be independent of adenosine receptor

activation since the protective action was mimicked by other ATP metabolites, such as AMP, ADP, and inosine, and antagonists to adenosine receptors did not inhibit the effect of adenosine (Schubert et al., 1997; Jurkowitz et al., 1998). This nucleotide/nucleoside-induced protection seems to be due to an inhibition of the decrease in the intracellular ATP levels evoked by glucose deprivation. Instead, it has been reported that adenosine rather induce the cell death of astrocytes via adenosine receptors (Abbracchio et al., 1995; Appel et al., 2001; Di Iorio et al., 2002) without affecting the neuronal cell survival (Ceruti et al., 2000). In addition, adenosine acting on adenosine A3 receptors causes apoptosis in astrocytes (Ceruti et al., 2000; Di Iorio et al., 2002). In contrast, ATP is well known to show trophic effects in astrocytes such as proliferation/gliosis (Brambilla et al., 1999; Neary et al., 1999; Franke et al., 2001b), induce trophic factors such as leukemia inhibitory factor (Yamakuni et al., 2002) and MCP-1 (Panenka et al., 2001) and protect astrocytes against TNF-α-induced cell death (Kim et al., 2003a, b). Thus, unlike neurons, astrocytic survival appears to be mainly controlled by ATP/P2 receptor-mediated but not by adenosine/P1 receptor-mediated pathways. As described above, the responsible receptors for the ATP-induced protective action in astrocytes were $P2Y_1$ receptors. Astrocytes express P2Y receptors (Ho et al., 1995; Idestrup and Salter, 1998; Fumagalli et al., 2003), P2X receptors (Franke et al., 2001a; Fumagalli et al., 2003) and several adenosine receptors as well (Peakman and Hill, 1994; Porter and McCarthy, 1995; Ciccarelli et al., 2001). It appears that ATP and its metabolites have functionally distinct roles in astrocytes.

We demonstrated that the ATP-induced protection of astrocytes required a preincubation period (12–36 h). This may involve two possibilities, namely that prolonged activation of $P2Y_1$ receptors is needed for the protection, or short-time exposure of ATP is enough to trigger the protection but longer periods (>12 h) are required to reveal the protective action. The $P2Y_1$ agonist MRS2179 could not reverse the effect of ATP when it was applied 30 min after ATP stimulation, and exogenously applied ATP was soon metabolized and almost disappeared 30 min after ATP the application (Fig. 4). These findings suggest that exogenously applied ATP should work only for limited periods (30 min), and therefore the short-time effect of ATP should be sufficient to trigger the protective action against H_2O_2 in astrocytes.

After the activation of $P2Y_1$ receptors, it took more than 12 h (12–36 h) for the onset of the ATP-induced protective action in astrocytes (Fig. 2C), and the protection was inhibited by the protein synthesis inhibitor CHX (Fig. 2D). These findings suggest that the protection by ATP is mediated by the upregulation of some proteins that are involved in anti-oxidative functions. In fact, DNA microarray analysis and quantitative RT-PCR analysis demonstrated that ATP upregulated oxidoreductase genes such as TrxR, CBR, and superoxide dismutase-like gene (SHL4, SOD-2 like gene) (Table 1). TrxR reduces Trx and is known to be involved in various important antioxidant functions (Eftekharpour et al., 2000). CBR belongs to a class of oxidoreductase proteins that are part of the family of short-chain dehydrogenase reductase (Inazu et al., 1992; Wirth and Wermuth, 1992), and it detoxifies toxic carbonyl compounds. SOD-2 is the mitochondrial form of superoxide dismutase and reduces superoxide anion (O_2) to H_2O_2 (Furuta et al., 1995). All these upregulated genes are expressed in both neurons and astrocytes, are somehow involved in the protective action against oxidative stress (Rozell et al., 1985; Hansson et al., 1989; Wirth and Wermuth, 1992; Eftekharpour et al., 2000; Forrest and Gonzalez, 2000), and are also known to be increased in some pathological conditions such as Alzheimer's disease (Lovell et al., 2000; Balcz et al., 2001; Kim et al., 2001; Butterfield et al., 2003) and Down syndrome (Balcz et al., 2001; Kim et al., 2001). Interestingly, such an upregulation is observed rather in astrocytes in some pathological conditions or by chemical treatment. For example, the antioxidant response element activator t-butylhydroquinone increases TrxR in astrocytes, but not in neurons (Eftekharpour et al., 2000), and upregulation of SOD-2 in reactive astrocytes is more predominant than that in neurons in Alzheimer's disease brain (Furuta et al., 1995). Astrocytes greatly promote the survival of neurons (Desagher et al., 1996), and also affect neuronal functions (Haydon, 2001). H_2O_2 generation is observed in many pathological conditions and can be a trigger of some brain disorders, including ischemic brain damage (Agardh et al., 1991; Lei et al., 1997), Alzheimer's disease (Cuajungco et al., 2000; Huang et al., 2000; Tabner et al., 2001; Tamagno et al., 2003), and Parkinson's disease (Tabner et al., 2001). Thus, the ATP-induced upregulation of oxidoreductase genes and the protection against cell death in astrocytes seen in the present study might be a key event for even neuronal survival, and possibly be involved in these diseases. However, the direct interaction between the upregulation of these oxidoreductase genes and the ATP/$P2Y_1$ receptor-mediated protection of cell death in astrocytes remains to be clarified.

In conclusion, we demonstrated that ATP protected astrocytes from H_2O_2-induced cell death via $P2Y_1$ receptor-mediated pathways and that the ATP-induced protection of astrocytes required upregulation of oxidoreductase genes. Unlike neurons, adenosine had no such effect in astrocytes. The precise target genes or mechanisms underlying the $P2Y_1$ receptor-mediated protective actions in astrocytes remain to be clarified. Our present findings suggest that one important role of ATP-mediated gliotransmission would be such a protective effect in astrocytes since ATP is released or leaked when cells in the CNS are damaged in several pathological conditions (Dubyak and el-Moatassim, 1993; Lutz and Kabler, 1997; Ahmed et al., 2000; Zhang et al., 2000; Parkinson et al., 2002).

ACKNOWLEDGMENTS

The authors thank Tomoko Obama for technical assistance.

REFERENCES

Abbracchio MP, Burnstock G. 1994. Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64:445–475.
Abbracchio MP, Ceruti S, Barbieri D, Franceschi C, Malorni W, Biondo L, Burnstock G, Cattabeni F. 1995. A novel action for adenosine: apoptosis of astroglial cells in rat brain primary cultures. Biochem Biophys Res Commun 213:908–915.
Agardh CD, Zhang H, Smith ML, Siesjo BK. 1991. Free radical production and ischemic brain damage: influence of postischemic oxygen tension. Int J Dev Neurosci 9:127–138.
Ahmed SM, Rzigalinski BA, Willoughby KA, Sitterding HA, Ellis EF. 2000. Stretch-induced injury alters mitochondrial membrane potential and cellular ATP in cultured astrocytes and neurons. J Neurochem 74:1951–1960.
Aihara H, Fujiwara S, Mizuta I, Tada H, Kanno T, Tozaki H, Nagai K, Yajima Y, Inoue K, Kondoh T, Motooka Y, Nishizaki T. 2002. Adenosine triphosphate accelerates recovery from hypoxic/hypoglycemic perturbation of guinea pig hippocampal neurotransmission via a P_2 receptor. Brain Res 952:31–37.
Akbar GK, Dasari VR, Webb TE, Ayyanathan K, Pillarisetti K, Sandhu AK, Athwal RS, Daniel JL, Ashby B, Barnard EA, Kunapulis SP. 1996. Molecular cloning of a novel P2 purinoceptor from human erythroleukemia cells. J Biol Chem 271:18363–18367.
Altman FP. 1976. Tetrazolium salts: a consumer's guide. Histochem J 8:471–485.
Appel E, Kazimirsky G, Ashkenazi E, Kim SC, Jacobson KA, Brodie C. 2001. Roles of BCL-2 and caspase 8 in the adenosine A3 receptor-induced apoptosis. J Mol Neurosci 17:285–292.
Araque A, Parpura V, Sanzgiri RP, Haydon PG. 1999a. Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 22: 208–215.
Araque A, Sanzgiri RP, Parpura V, Haydon PG. 1999b. Astrocyte-induced modulation of synaptic transmission. Can J Physiol Pharmacol 77:699–706.
Araque A, Carmignoto G, Haydon PG. 2001. Dynamic signaling between astrocytes and neurons. Annu Rev Physiol 63:795–813.
Ayyanathan K, Webbs TE, Sandhu AK, Athwal RS, Barnard EA, Kunapuli SP. 1996. Cloning and chromosomal localization of the human $P2Y_1$ purinoceptor. Biochem Biophys Res Commun 218: 783–788.
Balcz B, Kirchner L, Cairns N, Fountoulakis M, Lubec G. 2001. Increased brain protein levels of carbonyl reductase and alcohol dehydrogenase in Down syndrome and Alzheimer's disease. J Neural Transm Suppl;(61):193–201.
Behan WM, Stone TW. 2002. Enhanced neuronal damage by co-administration of quinolinic acid and free radicals, and protection by adenosine A2A receptor antagonists. Br J Pharmacol 135:1435–1442.
Brambilla R, Burnstock G, Bonazzi A, Ceruti S, Cattabeni F, Abbracchio MP. 1999. Cyclo-oxygenase-2 mediates P2Y receptor-induced reactive astrogliosis. Br J Pharmacol 126:563–567.
Braun N, Lenz C, Gillardon F, Zimmermann M, Zimmermann H. 1997. Focal cerebral ischemia enhances glial expression of ecto-5′-nucleotidase. Brain Res 766:213–226.
Braun N, Zhu Y, Krieglstein J, Culmsee C, Zimmermann H. 1998. Upregulation of the enzyme chain hydrolyzing extracellular ATP after transient forebrain ischemia in the rat. J Neurosci 18:4891–4900.
Butterfield DA, Boyd-Kimball D, Castegna A. 2003. Proteomics in Alzheimer's disease: insights into potential mechanisms of neurodegeneration. J Neurochem 86:1313–1327.
Ceruti S, Franceschi C, Barbieri D, Malorni W, Camurri A, Giammarioli AM, Ambrosini A, Racagni G, Cattabeni F, Abbracchio MP. 2000. Apoptosis induced by 2-chloro-adenosine and 2-chloro-2′-deoxy-adenosine in a human astrocytoma cell line: differential mechanisms and possible clinical relevance. J Neurosci Res 60:388–400.
Charles AC, Merrill JE, Dirksen ER, Sanderson MJ. 1991. Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. Neuron 6:983–992.
Chen J, Backus KH, Deitmer JW. 1997. Intracellular calcium transients and potassium current oscillations evoked by glutamate in cultured rat astrocytes. J Neurosci 17:7278–7287.
Ciccarelli R, Ballerini P, Sabatino G, Rathbone MP, D'Onofrio M, Caciagli F, Di Iorio P. 2001. Involvement of astrocytes in purine-mediated reparative processes in the brain. Int J Dev Neurosci 19:395–414.
Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ. 1990. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science 247:470–473.
Cuajungco MP, Goldstein LE, Nunomura A, Smith MA, Lim JT, Atwood CS, Huang X, Farrag YW, Perry G, Bush AI. 2000. Evidence that the beta-amyloid plaques of Alzheimer's disease represent the redox-silencing and entombment of abeta by zinc. J Biol Chem 275:19439–19442.
Desagher S, Glowinski J, Premont J. 1996. Astrocytes protect neurons from hydrogen peroxide toxicity. J Neurosci 16:2553–2562.
Di Iorio P, Kleywegt S, Ciccarelli R, Traversa U, Andrew CM, Crocker CE, Werstiuk ES, Rathbone MP. 2002. Mechanisms of apoptosis induced by purine nucleosides in astrocytes. Glia 38:179–190.
Dubyak GR, el-Moatassim C. 1993. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. Am J Physiol 265(3 Pt 1):C577–606.
Eftekharpour E, Holmgren A, Jourlink BH. 2000. Thioredoxin reductase and glutathione synthesis is upregulated by t-butylhydroquinone in cortical astrocytes but not in cortical neurons. Glia 31:241–248.
Fam SR, Gallagher CJ, Salter MW. 2000. $P2Y_1$ purinoceptor-mediated Ca^{2+} signaling and Ca^{2+} wave propagation in dorsal spinal cord astrocytes. J Neurosci 20:2800–2808.
Finkbeiner S. 1992. Calcium waves in astrocytes-filling in the gaps. Neuron 8:1101–1108.
Forrest GL, Gonzalez B. 2000. Carbonyl reductase. Chem Biol Interact 129:21–40.
Franke H, Grosche J, Schadlich H, Krugel U, Allgaier C, Illes P. 2001a. P2X receptor expression on astrocytes in the nucleus accumbens of rats. Neuroscience 108:421–429.
Franke H, Krugel U, Schmidt R, Grosche J, Reichenbach A, Illes P. 2001b. P2 receptor-types involved in astrogliosis in vivo. Br J Pharmacol 134:1180–1189.
Fredholm BB, Dunwiddie TV. 1988. How does adenosine inhibit transmitter release? Trends Pharmacol Sci 9:130–134.
Fumagalli M, Brambilla R, D'Ambrosi N, Volonte C, Matteoli M, Verderio C, Abbracchio MP. 2003. Nucleotide-mediated calcium signaling in rat cortical astrocytes: role of P2X and P2Y receptors. Glia 43:218–230.
Furuta A, Price DL, Pardo CA, Troncoso JC, Xu ZS, Taniguchi N, Martin LJ. 1995. Localization of superoxide dismutases in Alzheimer's disease and Down's syndrome neocortex and hippocampus. Am J Pathol 146:357–367.
Gallagher CJ, Salter MW. 2003. Differential properties of astrocyte calcium waves mediated by $P2Y_1$ and $P2Y_2$ receptors. J Neurosci 23:6728–6739.
Glaum SR, Holzwarth JA, Miller RJ. 1990. Glutamate receptors activate Ca^{2+} mobilization and Ca^{2+} influx into astrocytes. Proc Natl Acad Sci USA 87:3454–3458.
Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB. 1999. ATP released from astrocytes mediates glial calcium waves. J Neurosci 19:520–528.
Hansson HA, Holmgren A, Nordstedt G, Rozell B. 1989. Changes in the distribution of insulin-like growth factor I, thioredoxin, thioredoxin reductase and ribonucleotide reductase during the development of the retina. Exp Eye Res 48:411–420.
Haydon PG. 2001. Glia: listening and talking to the synapse. Nat Rev Neurosci 2:185–193.
Hentschel S, Lewerenz A, Nieber K. 2003. Activation of A_3 receptors by endogenous adenosine inhibits synaptic transmission during hypoxia in rat cortical neurons. Restor Neurol Neurosci 21:55–63.
Ho C, Hicks J, Salter MW. 1995. A novel P2-purinoceptor expressed by a subpopulation of astrocytes from the dorsal spinal cord of the rat. Br J Pharmacol 116:2909–2918.
Huang X, Cuajungco MP, Atwood CS, Moir RD, Tanzi RE, Bush AI. 2000. Alzheimer's disease, beta-amyloid protein and zinc. J Nutr 130(5 suppl):1488S–1492S.
Idestrup CP, Salter MW. 1998. P2Y and P2U receptors differentially release intracellular Ca^{2+} via the phospholipase c/inositol 1,4,5-triphosphate pathway in astrocytes from the dorsal spinal cord. Neuroscience 86:913–923.
Inazu N, Ruepp B, Wirth H, Wermuth B. 1992. Carbonyl reductase from human testis: purification and comparison with carbonyl reductase from human brain and rat testis. Biochim Biophys Acta 1116:50–56.

Innocenti B, Parpura V, Haydon PG. 2000. Imaging extracellular waves of glutamate during calcium signaling in cultured astrocytes. J Neurosci 20:1800–1808.
Inoue K, Koizumi S, Nakazawa K. 1995. Glutamate-evoked release of adenosine 5′-triphosphate causing an increase in intracellular calcium in hippocampal neurons. NeuroReport 6:437–440.
James G, Butt AM. 2002. P2Y and P2X purinoceptor mediated Ca^{2+} signalling in glial cell pathology in the central nervous system. Eur J Pharmacol 447:247–260.
Jones PA, Smith RA, Stone TW. 1998. Protection against hippocampal kainate excitotoxicity by intracerebral administration of an adenosine A2A receptor antagonist. Brain Res 800:328–335.
Jurkowitz MS, Litsky ML, Browning MJ, Hohl CM. 1998. Adenosine, inosine, and guanosine protect glial cells during glucose deprivation and mitochondrial inhibition: correlation between protection and ATP preservation. J Neurochem 71:535–548.
Kim SG, Gao ZG, Soltysiak KA, Chang TS, Brodie C, Jacobson KA. 2003a. P2Y6 nucleotide receptor activates PKC to protect 1321N1 astrocytoma cells against tumor necrosis factor-induced apoptosis. Cell Mol Neurobiol 23:401–418.
Kim SG, Soltysiak KA, Gao ZG, Chang TS, Chung E, Jacobson KA. 2003b. Tumor necrosis factor alpha-induced apoptosis in astrocytes is prevented by the activation of P2Y6, but not $P2Y_4$ nucleotide receptors. Biochem Pharmacol 65:923–931.
Kim SH, Vlkolinsky R, Cairns N, Fountoulakis M, Lubec G. 2001. The reduction of NADH ubiquinone oxidoreductase 24- and 75-kDa subunits in brains of patients with Down syndrome and Alzheimer's disease. Life Sci 68:2741–2750.
Koizumi S, Inoue K. 1997. Inhibition by ATP of calcium oscillations in rat cultured hippocampal neurones. Br J Pharmacol 122:51–58.
Koizumi S, Saito Y, Nakazawa K, Nakajima K, Sawada JI, Kohsaka S, Illes P, Inoue K. 2002. Spatial and temporal aspects of Ca^{2+} signaling mediated by P2Y receptors in cultured rat hippocampal astrocytes. Life Sci 72:431–442.
Koizumi S, Fujishita K, Tsuda M, Shigemoto-Mogami Y, Inoue K. 2003. Dynamic inhibition of excitatory synaptic transmission by astrocyte-derived ATP in hippocampal cultures. Proc Natl Acad Sci USA 100:11023–11028.
Lei B, Adachi N, Arai T. 1997. The effect of hypothermia on H_2O_2 production during ischemia and reperfusion: a microdialysis study in the gerbil hippocampus. Neurosci Lett 222:91–94.
Lenz G, Gottfried C, Luo Z, Avruch J, Rodnight R, Nie WJ, Kang Y, Neary JT. 2000. P_{2Y} purinoceptor subtypes recruit different mek activators in astrocytes. Br J Pharmacol 129:927–936.
Lovell MA, Xie C, Gabbita SP, Markesbery WR. 2000. Decreased thioredoxin and increased thioredoxin reductase levels in Alzheimer's disease brain. Free Radic Biol Med 28:418–427.
Lustig KD, Shiau AK, Brake AJ, Julius D. 1993. Expression cloning of an ATP receptor from mouse neuroblastoma cells. Proc Natl Acad Sci USA 90:5113–5117.
Lutz PL, Kabler S. 1997. Release of adenosine and ATP in the brain of the freshwater turtle (*Trachemys scripta*) during long-term anoxia. Brain Res 769:281–286.
Moore D, Iritani S, Chambers J, Emson P. 2000. Immunohistochemical localization of the $P2Y_1$ purinergic receptor in Alzheimer's disease. NeuroReport 11:3799–3803.
Neary JT, Kang Y, Bu Y, Yu E, Akong K, Peters CM. 1999. Mitogenic signaling by ATP/P2Y purinergic receptors in astrocytes: involvement of a calcium-independent protein kinase C, extracellular signal-regulated protein kinase pathway distinct from the phosphatidylinositol-specific phospholipase C/calcium pathway. J Neurosci 19:4211–4220.
Newman EA. 2003. Glial cell inhibition of neurons by release of ATP. J Neurosci 23:1659–1666.
Panenka W, Jijon H, Herx LM, Armstrong JN, Feighan D, Wei T, Yong VW, Ransohoff RM, MacVicar BA. 2001. P2X7-like receptor activation in astrocytes increases chemokine monocyte chemoattractant protein-1 expression via mitogen-activated protein kinase. J Neurosci 21:7135–7142.
Parkinson FE, Sinclair CJ, Othman T, Haughey NJ, Geiger JD. 2002. Differences between rat primary cortical neurons and astrocytes in purine release evoked by ischemic conditions. Neuropharmacology 43:836–846.
Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG. 1994. Glutamate-mediated astrocyte-neuron signalling. Nature 369:744–747.
Pasti L, Volterra A, Pozzan T, Carmignoto G. 1997. Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. J Neurosci 17:7817–7830.
Peakman MC, Hill SJ. 1994. Adenosine A2B-receptor-mediated cyclic AMP accumulation in primary rat astrocytes. Br J Pharmacol 111: 191–198.
Porter JT, McCarthy KD. 1995. Adenosine receptors modulate $[Ca^{2+}]_i$ in hippocampal astrocytes in situ. J Neurochem 65:1515–1523.
Porter JT, McCarthy KD. 1996. Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J Neurosci 16:5073–5081.
Queiroz G, Meyer DK, Meyer A, Starke K, von Kugelgen I. 1999. A study of the mechanism of the release of ATP from rat cortical astroglial cells evoked by activation of glutamate receptors. Neuroscience 91:1171–1181.
Rozell B, Hansson HA, Luthman M, Holmgren A. 1985. Immunohistochemical localization of thioredoxin and thioredoxin reductase in adult rats. Eur J Cell Biol 38:79–86.
Schipke CG, Boucsein C, Ohlemeyer C, Kirchhoff F, Kettenmann H. 2002. Astrocyte Ca^{2+} waves trigger responses in microglial cells in brain slices. FASEB J 16:255–257.
Schubert P, Ogata T, Marchini C, Ferroni S, Rudolphi K. 1997. Protective mechanisms of adenosine in neurons and glial cells. Ann NY Acad Sci 825:1–10.
Schwarzschild MA, Xu K, Oztas E, Petzer JP, Castagnoli K, Castagnoli N Jr, Chen JF. 2003. Neuroprotection by caffeine and more specific A2A receptor antagonists in animal models of Parkinson's disease. Neurology 61(11 suppl 6):S55–61.
Servitja JM, Masgrau R, Pardo R, Sarri E, Picatoste F. 2000. Effects of oxidative stress on phospholipid signaling in rat cultured astrocytes and brain slices. J Neurochem 75:788–794.
Shahidullah M, Wilson WS. 1997. Mobilisation of intracellular calcium by $P2Y_2$ receptors in cultured, non-transformed bovine ciliary epithelial cells. Curr Eye Res 16:1006–1016.
Shin CY, Choi JW, Ryu JR, Ko KH, Choi JJ, Kim HS, Lee JC, Lee SJ, Kim HC, Kim WK. 2002. Glucose deprivation decreases nitric oxide production via NADPH depletion in immunostimulated rat primary astrocytes. Glia 37:268–274.
Tabner BJ, Turnbull S, El-Agnaf O, Allsop D. 2001. Production of reactive oxygen species from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. Curr Top Med Chem 1:507–517.
Tamagno E, Robino G, Obbili A, Bardini P, Aragno M, Parola M, Danni O. 2003. H_2O_2 and 4-hydroxynonenal mediate amyloid beta-induced neuronal apoptosis by activating JNKs and p38MAPK. Exp Neurol 180:144–155.
Tokuyama Y, Hara M, Jones EM, Fan Z, Bell GI. 1995. Cloning of rat and mouse P2Y purinoceptors. Biochem Biophys Res Commun 211: 211–218.
Twentyman PR, Luscombe M. 1987. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 56:279–285.
Verderio C, Matteoli M. 2001. ATP mediates calcium signaling between astrocytes and microglial cells: modulation by IFN-gamma. J Immunol 166:6383–6391.
Viana F, de Smedt H, Droogmans G, Nilius B. 1998. Calcium signalling through nucleotide receptor $P2Y_2$ in cultured human vascular endothelium. Cell Calcium 24:117–127.
Webb TE, Simon J, Barnard EA. 1998. Regional distribution of [35S]2′-deoxy 5′-O-(1-thio) ATP binding sites and the $P2Y_1$ messenger RNA within the chick brain. Neuroscience 84:825–837.
Wieraszko A, Goldsmith G, Seyfried TN. 1989. Stimulation-dependent release of adenosine triphosphate from hippocampal slices. Brain Res 485:244–250.
Wirth H, Wermuth B. 1992. Immunohistochemical localization of carbonyl reductase in human tissues. J Histochem Cytochem 40: 1857–1863.
Yamakuni H, Kawaguchi N, Ohtani Y, Nakamura J, Katayama T, Nakagawa T, Minami M, Satoh M. 2002. ATP induces leukemia inhibitory factor mRNA in cultured rat astrocytes. J Neuroimmunol 129:43–50.
Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S. 2003. ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron 40: 971–982.
Zhang M, Zhong H, Vollmer C, Nurse CA. 2000. Co-release of ATP and ACh mediates hypoxic signalling at rat carotid body chemoreceptors. J Physiol 525(Pt 1):143–158.
Zimmermann H. 1996. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Prog Neurobiol 49: 589–618.

GLIA 51:000–000 (2005)

Involvement of β1 Integrin in Microglial Chemotaxis and Proliferation on Fibronectin: Different Regulations by ADP Through PKA

KAORU NASU-TADA,[1] SCHUICHI KOIZUMI,[1] AND KAZUHIDE INOUE[2,3]*
[1]*Division of Pharmacology, National Institute of Health Sciences, Setagaya, Tokyo, Japan*
[2]*Division of Biosignaling, National Institute of Health Sciences, Setagaya, Tokyo, Japan*
[3]*Graduate School of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan*

KEY WORDS
PKA; ATP; ADP; purinergic; glia; extracellular matrix; migration

ABSTRACT
Microglia are immune cells in the brain; their activation, migration, and proliferation have pivotal roles in brain injuries and diseases. Microglia are known to attach firmly to fibronectin, the upregulation of which is associated with several pathological conditions in the CNS, through β1 integrin and become activated. Extracellular nucleotides can serve as potent signaling molecules. Recently, ATP and ADP were revealed to possess chemoattractive properties to microglia via Gi-coupled P2Y receptors. In the present study, we report that the ADP-induced chemotaxis of microglia is mediated by P2Y12/13 receptors and is β1 integrin-dependent in the presence of fibronectin. Signals from P2Y12/13 receptors also cause β1 integrin translocation to the membrane ruffle regions, but this redistribution was lost when the intracellular cyclic AMP (cAMP) was increased by forskolin or dibutyryl cAMP. This inhibitory effect of cAMP-elevating agents did not appear when microglia were co-incubated with a protein kinase A (PKA) inhibitor, KT-5720, suggesting that PKA is a negative regulator of the β1 integrin translocation. We also show that the engagement of β1 integrin enhanced microglial proliferation. Signals from P2Y12/13 receptors attenuated the proliferation, whereas ADP itself had no effect on microglial growth. Furthermore, β1 integrin-induced proliferation is positively regulated by the cAMP-dependent PKA. Together, these results indicate the involvement of β1 integrin in microglial proliferation and chemotaxis, both of which have clinical importance. The data also suggest that PKA is inversely involved in these two cellular functions.

INTRODUCTION

Microglia are considered to act as brain macrophages. They participate in brain injuries and diseases (Nakajima and Kohsaka, 1993), in which their motility, aberrant activation, and proliferation are known to play crucial roles. Microglia quickly respond to numerous inflammatory mediators by migrating to the source of the mediators, where they become activated and exert their neuroprotective effects (Hanisch, 2002; Streit, 2002). Unfortunately, their hyperactivation often leads to neurotoxicity instead, and several pathological conditions in the CNS are, in fact, accompanied by an excess proliferation of microglia (Gehrmann et al., 1995). Thus, better understanding of the regulation of microglial chemotaxis and proliferation may have important therapeutic implications.

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits; they mediate cell–cell and cell–extracellular matrix (ECM) interactions. At present, 16 α and 8 β chains have been identified, and at least 22 different complexes are known. β1 integrin, the most ubiquitous β subunit, pairs with at least 10 different α chains to comprise receptors for a wide variety of ECM proteins. Within the CNS, β1 integrin is expressed on many different cell types, including neurons, glial cells, and endothelial cells (Pinkstaff et al., 1999). As shown previously by other investigators, microglia express several integrin receptors of β1 and β2 families that are upregulated following microglial activation in vitro (Hailer et al., 1996; Yu et al., 1998; Kloss et al., 2001; Milner and Campbell, 2003). In vivo, integrin expression is found to be increased on activated microglia in Alzheimer's disease (Akiyama and McGeer, 1990), after nerve injuries (Coyle, 1998; Kloss et al., 1999; Tsuda et al., 2003) and in multiple sclerosis lesions (Bo et al., 1996). Integrins serve not only as adhesive molecules but also as signaling receptors, and they regulate numerous cellular functions (Hemler, 1998) including cell migration and proliferation. β1 integrin is closely associated with the regulation of cell motility and growth in many cell types (Hynes, 1992; Jones and Watt, 1993; Howlett et al., 1995), but its role in microglial chemotaxis and proliferation remains unclear.

Fibronectin is one of the ECM molecules; it is a large, multidomain glycoprotein that exists both as a cell surface protein and in plasma. The expression of ECM molecules is regionally and developmentally regulated in the brain, and their presence is relatively minor in the

*Correspondence to: Kazuhide Inoue, Department of Molecular and System, Pharmacology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail: inoue@phar.kyushu-u.ac.jp

Received 16 December 2004; Accepted 24 March 2005

DOI 10.1002/glia.20224

Published online 00 Month 2005 in Wiley InterScience (www.interscience.wiley.com).

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

normal CNS. Some ECM molecules, including fibronectin, however, are upregulated after adult CNS injury. Fibronectin also exists at high concentrations in the blood plasma and a breakdown of the blood-brain barrier should result in an increase in its local concentration in the CNS. Fibronectin induces firm adhesion and activation of microglia (Milner and Campbell, 2002, 2003). It is a major ligand of the β1 integrin family, but recently Mac-1 was also reported to play a role in the adhesion of leukocytes to fibronectin (Lishko et al., 2003).

Previously, Honda et al. (2001) demonstrated that extracellular ATP and ADP could induce the chemotactic migration of microglia. Several lines of evidence so far have indicated that extracellular nucleotides serve as signaling molecules (Bodin and Burnstock, 2001). ATP, and possibly other nucleotides, are released from damaged cells or secreted via nonlytic mechanisms and activate microglia. In the work by Honda et al. (2001), extracellular ATP and ADP induced chemotaxis as well as membrane ruffling, which was possibly mediated by Gi-coupled P2Y receptors. The P2Y12 receptor is a recently cloned Gi-coupled P2 receptor (Hollopeter et al., 2001), and expressed on platelets and exclusively in microglia in the brain (Sasaki et al., 2003). The P2Y13 receptor is another Gi-coupled P2 receptor that was recently identified (Communi et al., 2001; Zhang et al., 2002). Its messenger RNA is expressed at highest levels in the brain and immune tissues, particularly the spleen (Zhang et al., 2002), suggesting its roles in neuron and in immune systems. P2Y12 and P2Y13 receptors present a very similar pharmacological profile. Both receptors show high affinities for ADP and 2MeSADP (Hollopeter et al., 2001; Zhang et al., 2002), and are selectively blocked by ARC-67085 and ARC-69931 (Ingall et al., 1999). No specific agonists/antagonists are known currently to distinguish these two receptors pharmacologically.

The involvement of β1 integrin in chemotaxis and proliferation is already well characterized. Its role in microglial chemotaxis and proliferation, however, has not been well studied, and its correlation with purinoceptors such as P2Y12/13 receptor is still unclear. We provide new evidence that (1) ADP-induced chemotaxis through P2Y12/13 receptors involves β1 integrin in the presence of fibronectin, (2) ADP induces β1 integrin redistribution which colocalizes with membrane ruffling on microglia, and PKA functions as a negative regulator of this translocation, (3) β1 integrin mediates microglial proliferation through positive regulation of PKA, and (4) signals from P2Y12/13 receptors abrogate the proliferative effect of β1 integrin. Taken together, these results indicate that β1 integrin is crucially involved in both the proliferation and chemotaxis of microglia, which are under the inverse regulation of PKA.

Abbreviations

ATP	adenosine 5′-triphosphate
ADP	adenosine 5′-diphosphate
cAMP	adenosine 2′:3′-cyclic monophosphate
CNS	central nervous system
CCR	CC chemokine receptor
CXCR	CXC chemokine receptor
CX_3CR	CX_3C chemokine receptor
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
GPCR	G-protein-coupled receptor
IL-8	interleukin-8
MAP	mitogen-activated protein
2MeSADP	dimethylthioadenosine 5′-diphosphate
PKA	protein kinase A
VLA	very late antigen

MATERIALS AND METHODS

Isolation of Microglia

Rat primary cultures were derived from the cerebral cortex of neonatal Wistar rats. In brief, the rat cortices were separated from the meninges, minced, treated with trypsin and DNase, and then centrifuged to remove dead cells. The pellet was resuspended in DMEM, filtrated and cultured in medium with 10% fetal bovine serum for 12–23 days. Microglia were isolated on day 10 and day 15 by gently shaking of the flasks for 2 min and were attached to appropriate dishes or coverslips. One flask (75 cm^2) yielded 1–2 × 10^6 microglial cells by this preparation, and the cultures were of >98% purity. The purity of microglial culture was determined by immunostaining for Ox-42 and Eva-1.

Immunofluorescence Staining of Cell Surfaces

Microglia were washed once with ice-cold staining buffer (phosphate-buffered saline [PBS] 1% fetal calf serum [FCS], 0.1% NaN_3) and then Fc blocked for 15 min on ice. After washing, they were incubated with Ha2/5 (PharMingen) for 30 min on ice. They were washed once, incubated with mouse anti-hamster IgM (PharMingen) for 30 min on ice, and then washed again. Finally, cells were incubated with Alexa-Fluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) for 30 min on ice in the dark, washed twice, resuspended in the staining buffer, and the fluorescence intensity of the labeled microglia was analyzed with FACScan (Becton Dickinson).

Immunocytochemistry

Coverslips were briefly treated with hydrochloric acid and extensively washed with PBS. They were then coated with fibronectin (Sigma, St. Louis, MO) at 10 μg/ml overnight and washed with PBS immediately before use. Microglia were plated on coverslips and kept at 37°C for 1 h, and unattached cells were washed off gently with warm DMEM. After 1-h serum starvation, the cells were stimulated with ADP (50 μM) for 5 min at 37°C. The attached cells were then fixed in 3.7% formaldehyde in PBS for 5 min and then washed with PBS. The cells were permeabilized with 0.1% Triton-X in PBS for 5 min, washed again with PBS, and then blocked for 30 min with ACE blockase (Yukijirushi, Ltd.) with 3% goat serum at room temperature. To visualize β1 integ-

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

rin and membrane ruffling, the cells were stained with Ha2/5 mAb, mouse anti-hamster IgM Ab, Alexa-Fluor 488-conjugated goat anti-mouse IgG, or Texas Red-X phalloidin (Molecular Probes) and observed under a fluorescent microscope.

Defining Membrane Rufflings

To the best of our knowledge, there are no methods to quantify the degree of membrane ruffling. Therefore, we tried to define membrane ruffling by analyzing the pictures from immunocytochemistry. First we pictured cells that were stained with Texas Red-X phalloidin, and then a line was drawn across the cell and the intensity of the Texas Red-X phalloidin labeling along the line was analyzed using computer software. Membrane ruffling is characterized by the sharp, strong labeling of Texas Red-X phalloidin, which reaches >200 a.u. (out of 256) of intensity, whereas cells with no ruffling show less intense, blurred, and dispersed staining. We defined membrane ruffling as a wave-like structure that is stained with phalloidin and the labeling intensity of which reaches >200 a.u.. Cells satisfying these criteria were construed as bearing membrane ruffling morphology.

Chemotaxis Assay

Chemotaxis assays were performed using a direct-viewing Dunn chemotaxis chamber (Weber Scientific International, Teddington, UK). The details concerning this apparatus and its use are given in Webb et al. (1996) and Zicha et al. (1991). In brief, microglia were attached to coverslips that had been coated with fibronectin. After 1-h incubation, the cells were washed with warm DMEM and cultured in the absence of FCS for an additional 1 h. The coverslips were then inverted onto the slide, the inner and the outer wells of which were filled with DMEM. The edges of the coverslip were tightly sealed with adhesive tape except for one on which a thin filling slit was left. Using a needle and a syringe, the medium in the outer well was gently replaced with DMEM containing 100 μM ADP. The filling slit was quickly sealed with adhesive tape and the chamber was carefully set on the stage of a microscope equipped with a 10× phase-contrast objective. Microglia adhered to the coverslip were exposed to the ADP gradient and then monitored in the Dunn chemotaxis chamber for a period of 1 h. One region of the bridge was viewed directly via a CCD video camera and the data were recorded every 30 s during the 1-h observation using image software. After recording, cells were randomly selected from a set area of the field and the straight distance they migrated was plotted against x,y coordinates on scatter diagrams. The x-axis was parallel to the outer ring while the y-axis was vertical. Recording of the cell migration usually started within 30 min of assembling the chamber, by which time a linear diffusion gradient had been established (Webb et al., 1996).

Proliferation Assay

In this study, 96-well plates (Corning) were incubated with fibronectin (10 μg/ml), anti-β1 integrin Ab (10 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit serum (Sigma) overnight at 4°C and washed withPBS before use. For anti-β1 integrin Ab and normal rabbit serum coatings, the wells were pre-coated with goat anti-rabbit IgG (Sigma) for 2 h at 37°C and washed with PBS. Microglia were attached to the wells and cultured in DMEM 4% FCS for 24 h at 37°C. After the incubation, cell survival and proliferation were assayed using an MTT cell growth kit (Chemicon) according to the manufacturer's instructions and also by direct cell counting.

RESULTS

ADP-Induced Chemotaxis of Microglia Is Mediated by P2Y12/13 Receptor and Is Blocked by an Elevation of cAMP

In the previous study by Honda et al. (2001), extracellular ADP induced microglial chemotaxis through Gi-coupled P2Y receptors and the ADP-induced membrane ruffling was inhibited by a P2Y12- and P2Y13-selective antagonist, ARC-69931. First, to make the system closer to pathological conditions in the CNS, we performed the chemotaxis assay on fibronectin substrates. To examine whether microglial chemotaxis toward ADP on fibronectin substrate is also mediated by the P2Y12/13 receptor, ARC-69931 was used in the chemotaxis experiment (Fig. 1). Cultured microglia were adhered to the fibronectin-coated coverslips and their chemotaxis against the ADP concentration gradient was studied with the Dunn chemotaxis chamber (see materials and methods for details). In the absence of the ADP gradient, microglia did not move on the fibronectin-coated coverslips (Fig. 1b). When ADP 100 μM was applied to the system, however, the cells migrated toward the stimulant (Fig. 1a,c). Pretreatment of microglia with ARC-69931 at 10 μM totally blocked the ADP-induced microglial chemotaxis (Fig. 1d), suggesting that the P2Y12/13 receptor is responsible for the microglial chemotaxis toward ADP. Since the activation of P2Y12/13 receptors inhibits adenylate cyclase, the effect of changes in the intracellular cAMP level on microglial chemotaxis was tested. Forskolin is a potent adenylate cyclase activating agent and dibutyryl cAMP is a membrane permeable AMP analogue that activates cAMP protein kinases. When microglia were pretreated with forskolin (Fig. 1e) or dibutyryl cAMP (Fig. 1f), their chemotaxis toward ADP was greatly attenuated. F1

β1 Integrin Is Involved in the Microglial Migration Toward ADP on Fibronectin Substrate

To study the function of β1 integrin in the P2Y12/13-receptor mediated migration of microglia, we first examined its expression on cultured microglia. Flow cyto-

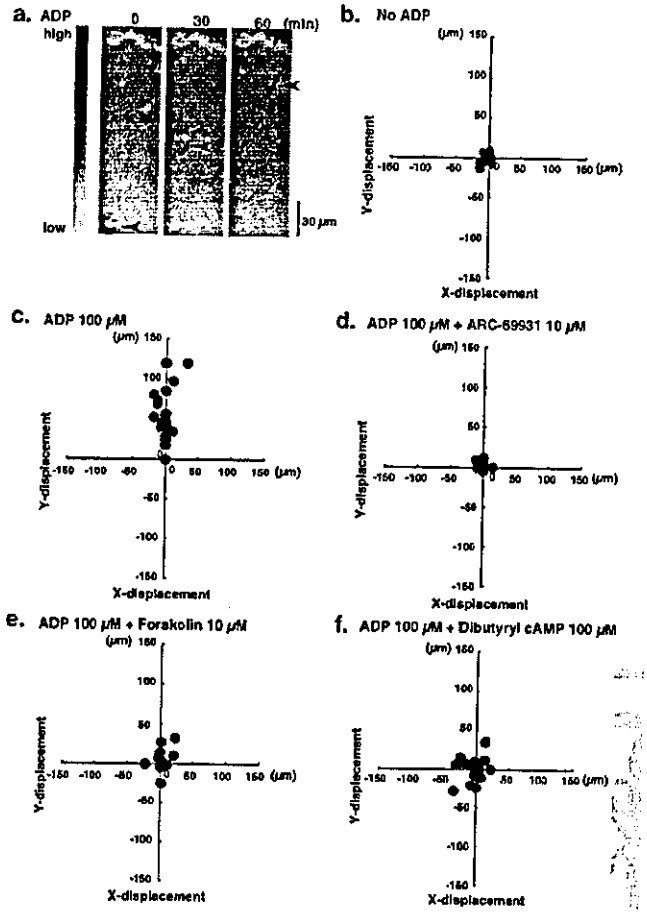


Fig. 1. ADP-induced chemotaxis of microglia was mediated through P2Y12/13 receptors and blocked by an elevation of intracellular cAMP. Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were assayed for migration toward ADP in the Dunn chemotaxis chamber. a: Typical chemotactic responses of microglia toward ADP. Arrowheads depict the position of a single microglial cell at the indicated time, showing the kinetics of chemotaxis. Microglia were almost static on fibronectin in the absence of the stimulant (b). Microglia, however, showed chemotactic responsiveness to ADP (100 μM) (c), which was completely blocked by the P2Y12/13 receptor antagonist ARC-69931 (d), confirming that the chemotaxis to ADP is mediated by P2Y12/13 receptor. Forskolin pretreatment (e) and dibutyryl cAMP pretreatment (f) attenuated the ADP-induced chemotaxis of microglia. The data represent three independent experiments.

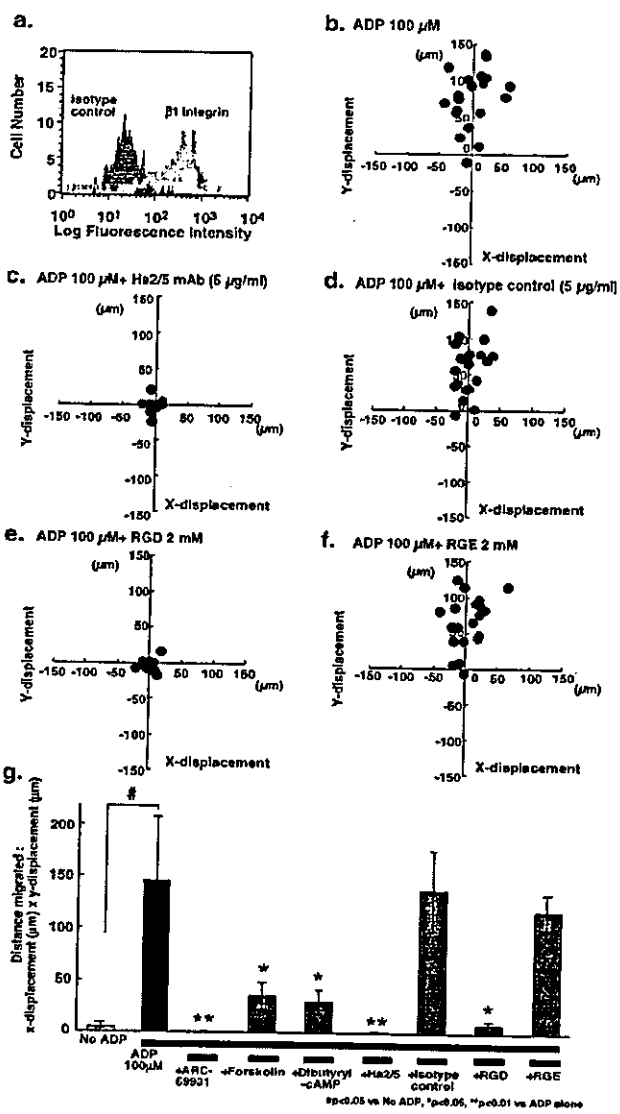


Fig. 2. β1 integrin is highly expressed on cultured microglia, and it is involved in the ADP-induced chemotaxis of microglia on the fibronectin substrate. Cultured microglia were labeled with anti-β1 integrin antibody (Ha2/5) (gray) or with isotype-matched control antibody (black). The labeling was detected by Alexa-Fluor 488-conjugated antibody and the cells were subjected to flow cytometry analysis (a). b–f: Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were assayed for migration toward ADP in the Dunn chemotaxis chamber. The microglia migration toward ADP (100 μM) (b) was totally inhibited by Ha2/5 (c) and RGD peptide (e). In contrast, the migration was not affected by isotype-matched control (d) and RGE peptide (f). Data (a–f) represent three independent experiments. Each chemotaxis was quantified by calculating the x displacement (μm) multiplied by the y-displacement (μm) (g). Data (g) are mean ± SE of three separate experiments. #Greater than No ADP ($P < 0.05$, Student's t-test); *Smaller than ADP 100 μM ($P < 0.05$, Student's t-test); **Smaller than ADP 100 μM ($P < 0.01$, Student's t-test).

metric analysis confirmed that the cultured microglia expressed significant amounts of the β1 integrin subunit (Fig. 2a). Next, to assess the role of β1 integrin in the cell migration toward ADP, a chemotaxis assay was performed with the Dunn chemotaxis chamber in the presence of a monoclonal antibody specific for the β1 integrin subunit (Ha2/5) and RGD peptide (Fig. 2b–f). Treatment of microglia with Ha2/5 antibody at 5 μg/ml suppressed the ADP-induced chemotaxis (Fig. 2c), indicating that β1 integrin is required for this process. An isotype-matched control antibody did not interfere with the microglial chemotaxis toward ADP (Fig. 2d). Treatment with RGD peptide at 2 mM also perturbed the microglial migration toward ADP (Fig. 2e), whereas control RGE peptide did not inhibit the migration (Fig. 2f), suggesting that the RGD sequence is important. The RGD (Arg-Gly-Asp) sequence is present in several extra-

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

cellular matrix components including fibronectin, and many integrins show RGD sequence-dependent binding to their ligands. Therefore, these results demonstrate that β1 integrin was responsible for the motility of microglia on the fibronectin substrate and its interaction with fibronectin involves the RGD sequence. Each chemotaxis was quantified by calculating the x displacement multiplied by the y displacement (Fig. 2g), and thus the dot-plotted data in Figures 1 and 2 were all evaluated for statistical significance.

Stimulation of P2Y12/13 Receptor Causes β1 Integrin Redistribution That Colocalizes With Membrane Ruffling

The involvement of β1 integrin in the ADP-induced chemotaxis was further studied using immunofluorescence (Fig. 3). Extracellular ATP and ADP have been reported to induce a membrane structure called membrane ruffling (Honda et al., 2001). Membrane ruffling is a unique, wave-like structure of the plasma membrane. It is the actin polymerization seen as a projection from the cell membrane, and the most conventional way of visualizing this unique structure is to stain the cells with phalloidin. In our experiments, membrane ruffling was construed as labeling intensity that reached >200 a.u. (see Materials and Methods). Microglia were attached to fibronectin-coated coverslips, stimulated with ADP at 50 μM for 5 min, fixed and permeabilized, and then the appearance of membrane ruffling and the cellular localization of β1 integrin were studied using Texas Red-X phalloidin and Ha2/5 antibody, respectively. Without ADP stimulation, microglia showed no membrane ruffling and the β1 integrin subunit was dispersed over the plasma membrane of the microglia (Fig. 3a–c). In response to ADP stimulation at 50 μM, membrane ruffling appeared within 5 min after stimulation (Fig. 3e) and β1 integrin was redistributed to the cell edge (Fig. 3d). A merged image revealed that β1 integrin colocalized with the membrane ruffling (Fig. 3f). Treatment of microglia with ARC-69931 abrogated the membrane ruffling formation (Fig. 3h) (Honda et al., 2001), β1 integrin redistribution (Fig. 3g) and thus the colocalization of these two (Fig. 3i), suggesting that these responses were mediated by P2Y12/13 receptors. The numbers of cells with membrane ruffling were directly counted among control, ADP, and ADP with ARC-69931 populations (Fig. 3B). The result again indicated that ADP induces membrane ruffling which is inhibited by ARC-69931.

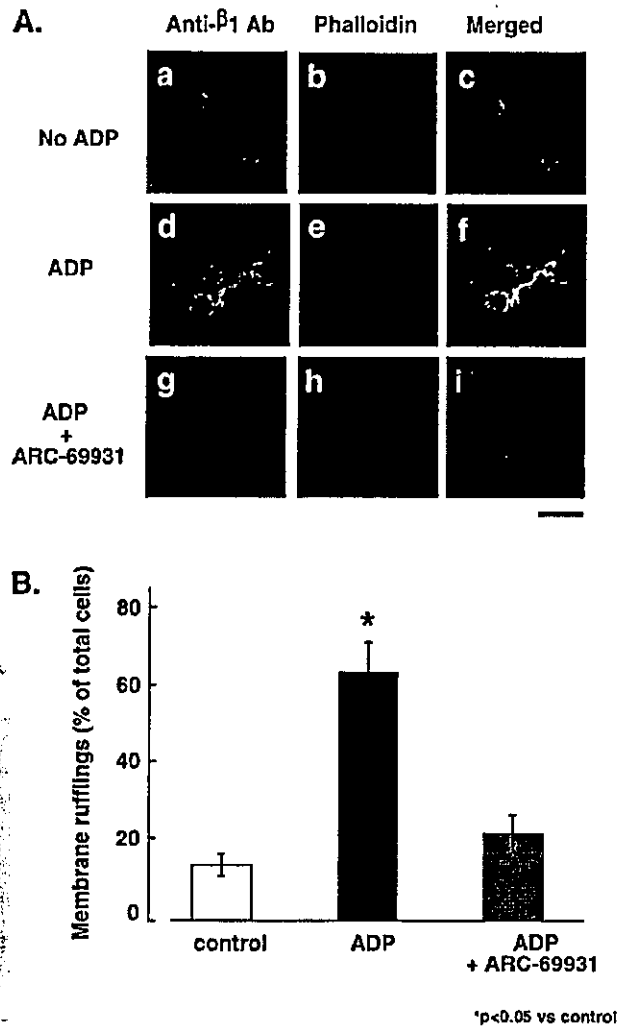
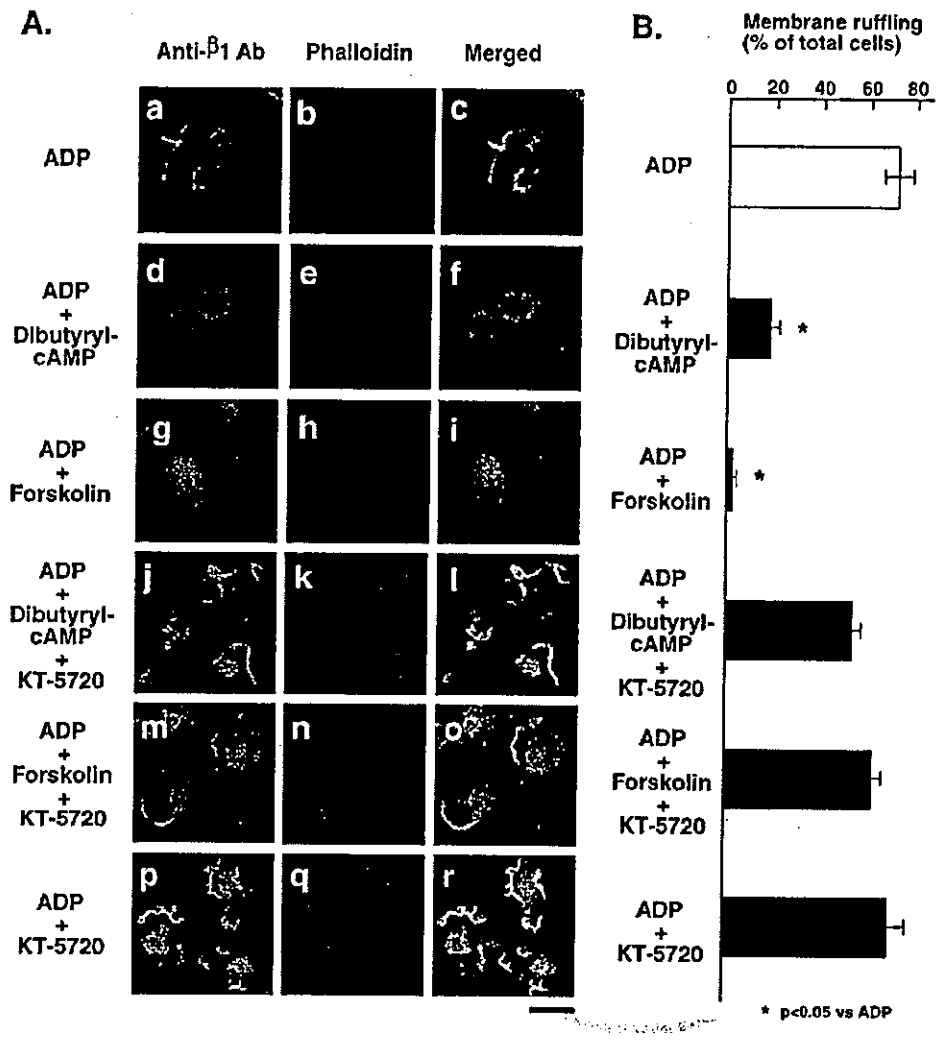


Fig. 3. P2Y12/13 receptor-mediated microglial β1 integrin redistribution that colocalized with membrane ruffling in response to ADP stimulation. A: Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were stimulated with ADP (50 μM) for 5 min, fixed and permeabilized. Then, for the immunofluorescence studies, the cells were incubated with anti-β1 antibody Ha2/5 (a,d,g) and phalloidin (b,e,h). a–c: Controls, i.e., PBS alone. β1 integrin redistribution and membrane ruffling occur in response to ADP stimulation (d,e), but they disappear in the presence of ARC-69931 (g,h). Merged image (f) shows that β1 integrin redistributes and colocalizes with membrane ruffling in response to ADP (50 μM). B: Cultured microglia on fibronectin-coated coverslips were stimulated with ADP (50 μM) for 5 min, fixed and permeabilized. The cells were labeled with Texas Red-X phallodin to visualize membrane rufflings. Total cells and cells undergoing membrane ruffling were counted respectively, and the percentage was calculated. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test). Scale bar = 10 μm in A.

An Increase in the Intracellular cAMP Level Abrogated Colocalization of Membrane Ruffling and β1 Integrin, and the PKA Inhibitor KT-5720 Restored the β1 Integrin Redistribution and Membrane Ruffling

We next investigated whether an increase in the intracellular concentration of cAMP in microglia would have any effect on the appearance of the membrane ruffling and redistribution of β1 integrin. Microglia were adhered to fibronectin-coated coverslips and pretreated either with forskolin or dibutyryl cAMP. The cells were then stimulated with ADP at 50 μM for 5 min and stu-

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I



COLOR

Fig. 4. Elevation of intracellular cAMP abrogated the β1 integrin redistribution and membrane ruffling in response to ADP stimulation. The PKA inhibitor KT-5720 restored the ADP-induced β1 integrin redistribution and membrane ruffling on microglia. A: Cultured microglia were adhered to fibronectin-coated coverslips. After serum starvation, the cells were stimulated with ADP (50 μM) for 5 min in the presence of dibutyryl cAMP (d–f) or forskolin (g–i) to study the involvement of the intracellular cAMP concentration. The elevation of intracellular cAMP abrogated the β1 integrin redistribution (d,g) and membrane ruffling (e,h) in response to ADP (50 μM). PKA involvement was examined by pretreating the cells with KT-5720 at 5 μM (j–o). KT-5720 treatment itself did not affect the morphology of the cells (data not shown) and KT-5720 pretreated cells responded normally to ADP at 50 μM (p–r). Blocking PKA by pretreatment with KT-5720 restored β1 integrin clustering and increased membrane ruffling even in the presence of dibutyryl cAMP (j–l) or forskolin (m–o). B: Cultured microglia on fibronectin-coated coverslips were stimulated as in A for 5 min, fixed and permeabilized. The cells were labeled with Texas Red-X phallodin to visualize membrane rufflings. Total cells and cells undergoing membrane ruffling were counted respectively, and the percentage was calculated. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test).

F4 died by immunofluorescence. As seen in Figure 4Ad–i, (Fig. 4Aj–o) The number of cells with membrane ruffling was directly counted for each population (Fig. 4B), and the result confirmed the effect of dibutyryl cAMP, forskolin, and KT-5720 on membrane ruffling.

neither the β1 integrin redistribution nor the membrane ruffling appeared on the surface of microglia when the intracellular cAMP level was elevated. These results indicate that an elevation of intracellular cAMP by dibutyryl cAMP or by forskolin inhibited the β1 integrin accumulation and membrane ruffling in response to ADP. PKA is located downstream of the cAMP elevation, and its negative regulation of the β2 integrin avidity and the integrin-mediated adhesion of lymphocytes has been described in other studies (Laudanna et al., 1997; Jones, 2002). Thus, since the PKA activity appears to regulate the integrin function, we next investigated whether PKA activation is responsible for the loss of the β1 integrin redistribution and the attenuation of the ADP-induced chemotaxis of microglia. Microglia were pretreated with forskolin or with dibutyryl cAMP, both of which were in the presence of the PKA inhibitor KT-5720. When these cells were stimulated with ADP at 50 μM, the integrin redistribution was restored

β1 Integrin Mediates Microglial Proliferation

β1 integrin regulates cellular proliferation, migration, survival, and differentiation via outside-in signaling. β1 integrin is involved in the proliferation of many cell types (Jones and Watt, 1993; Howlett et al., 1995), but its effect on microglial proliferation has not been clarified. To study the function of β1 integrin in microglial proliferation, an MTT assay and direct cell counting were used in this study. Figure 5 shows that microglia cultured on fibronectin substrate proliferate more than 1.5-fold after 24-h incubation as compared to the control. Microglia plated on anti-β1 integrin antibody showed similar results, suggesting that cross-linking of β1 integ- F5

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

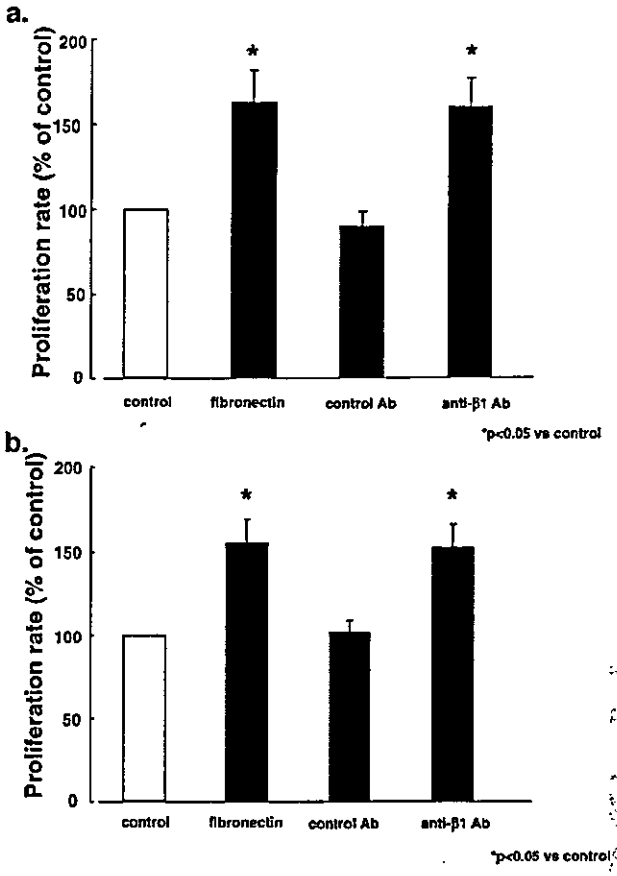


Fig. 5. β1 integrin mediates microglial proliferation. Cultured microglia were adhered to uncoated, fibronectin-coated, anti-β1 antibody-coated or control antibody-coated plates and cultured for 24 h at 37°C. After the incubation, the cell's viability was measured by (a) MTT assay or (b) direct cell counting. The data are shown as percentage of proliferation of microglia on uncoated culture plates after 24-h incubation. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test).

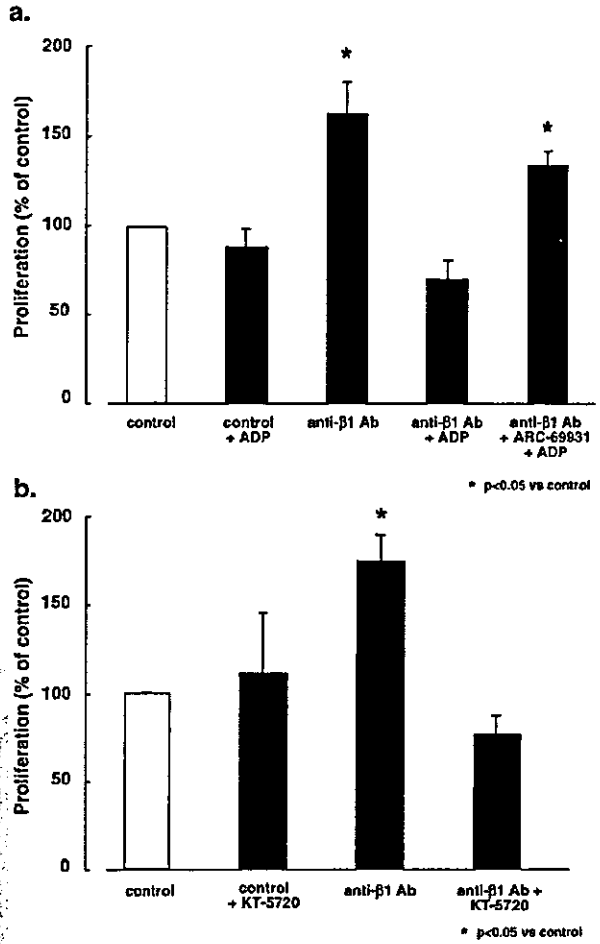


Fig. 6. β1 integrin-mediated proliferation of microglia is inhibited by ADP. PKA is a positive regulator of β1 integrin-mediated proliferation. Cultured microglia were adhered to uncoated or anti-β1 integrin antibody-coated plate in the presence of ADP (100 μM) and ARC-69931 (10 μM) (a) or KT-5720 (10 μM) (b) and incubated for 24 h at 37°C. The cells' viability was then tested by MTT assay. Data are mean ± SE of three separate experiments. *Greater than control ($P < 0.05$, Student's t-test).

rins leads to microglial proliferation. Recent reports have shown that integrin engagements, either with ligands or with antibodies, are capable of transducing signals (Miyamoto et al., 1995). An isotype-matched control antibody did not induce microglial proliferation. In this way, it is clear from these results that microglial β1 integrin mediates the outside-in signal, which promotes the proliferation of the cell.

ADP Inhibits β1 Integrin-Mediated Proliferation of Microglia and the Mechanism Involves PKA Activation

Chemokines act through Gi-coupled GPCRs to attract the target cells (Neptune and Bourne, 1997; Rollins, 1997), and several of them are known to induce the proliferation of cells. Several chemokine receptors including IL-8R, CXCR1-4, CCR2, CCR3, CCR5, and CX_3CR1 are detected on microglia (Hanisch, 2002; Abbadie et al., 2003) and most of them are reported to induce microglial proliferation as well. Since P2Y12/13 is also a Gi-coupled receptor, we studied its effect on cell growth. When microglia were stimulated with ADP alone at 100 μM, they did not increase in number (Fig. 6a), suggesting that ADP itself does not cause microglial proliferation. As mentioned earlier, microglia with β1 integrin cross-linking showed marked proliferation, but the integrin-induced high proliferation rate was no longer observed in the presence of ADP (Fig. 6a). The addition of ARC-69931 restored the high proliferation rate, suggesting that the ADP signal from the P2Y12/13 receptor did not synergize the proliferative signal from β1 integrin, but rather counteracted it.

F6

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

We next investigated whether PKA, a major intracellular target for cAMP in mammalian cells, is involved in this process. Microglial proliferation was studied in the presence of the PKA inhibitor KT-5720 (Fig. 6b). As seen in the control, the addition of KT-5720 at 10 μM did not affect the cell's viability after 24-h incubation. When KT-5720 was added to microglia with anti-β1 integrin antibody, however, the proliferation rate was significantly reduced. These results indicate that the activation of PKA positively regulates the microglial proliferation signal from β1 integrin, in sharp contrast to its effect on P2Y12/13-induced β1 integrin translocation.

DISCUSSION

Microglia are resident tissue macrophages in the brain, exhibiting ramified morphologies in the quiescent state. Once activated by numerous soluble molecules, including cytokines, growth mediators, and nucleotides, however, they quickly respond by transforming into an amoeboid phenotype, migrating to the source of mediators, proliferating and upregulating the expression of various inflammatory cytokines (Nakajima and Kohsaka, 1993; Hanisch, 2002; Inoue, 2002). In the study described herein, we investigated the role of β1 integrin in microglial chemotaxis and proliferation, and characterized its regulation by signals delivered from P2Y12/13 receptors.

Our observation that P2Y12/13 receptors mediate the chemotaxis of microglia on the fibronectin substrate was compatible with the earlier finding by Honda et al. that membrane ruffling is cause by ADP stimulation (Honda et al., 2001). ADP is a potent agonist of P2Y1, P2Y12, and P2Y13. The purine and pyrimidine receptors known to be expressed in microglia include P2X4, P2X7, P2Y2, and P2Y12 (Inoue, 2002; Sasaki et al., 2003; Tsuda et al., 2003), and possibly P2Y13, due to its abundant mRNA in the brain and the immune system (Zhang et al., 2002). P2Y12 and P2Y13 are recently identified Gi-coupled receptors that share the same agonists and antagonists, making it very difficult to pharmacologically differentiate between these two receptors. Whether the P2Y12 and/or P2Y13 receptor was/were truly responsible for the chemotaxis and/or inhibition of proliferation awaits the development of a selective agonist or antagonist.

To mimic damages in the CNS, we performed chemotaxis assays on coverslips coated with an ECM molecule that is known to be expressed in the real pathological situation and thus chose fibronectin, which is upregulated following adult CNS injury (Jones, 1996). In the present study, we demonstrated that β1 integrin is highly expressed in microglia and is crucially involved in the ADP-induced chemotaxis of microglia on fibronectin, and that signals from P2Y12/13 receptors recruit β1 integrin to the membrane ruffle regions. When non-coated coverslips that were pretreated with hydrochloric acid were used in the assays, microglia neither adhered well to the coverslips nor migrated against the ADP gradient (data not shown). These results also indicate that β1 integrin is crucially involved in the adhesion to fibronectin and ADP-induced chemotaxis in the presence of fibronectin in the CNS. β1 integrin couples with α1 integrin through α6 integrin to form VLA-1 through VLA-6, respectively, and with αv integrin to form αvβ1. Among these pairs, VLA-3, VLA-4, VLA-5, and αvβ1 have fibronectin as their ligand, and microglia are known to express α4, α5, and αv chains (Kloss et al., 2001; Milner and Campbell, 2003). The ADP-induced translocation of β1 integrin may contribute to the subsequent formation of focal complexes and then focal adhesions, both of which are important for cell migration.

We have shown that ADP induced β1 integrin translocation to the membrane ruffles by lowering the intracellular cAMP via the P2Y12/13 receptor, and that β1 integrin is important for microglial chemotaxis mediated by the same receptor. Chemotaxis is an integrated process consisting of multiple steps (Lauffenburger and Horwitz, 1996). Integrins are essential for cell migration not only because they mediate adhesion directly, but also because they regulate intracellular signaling pathways required for the cell locomotion (Hood and Cheresh, 2002). When fibroblasts migrate, integrins and other molecules form focal complexes and assemble at the leading edge of the cell. These complexes evolve into highly organized focal adhesions (Laukaitis et al., 2001), which in turn generate a signal to cause actin cytoskeletal reorganization resulting in cell motility (Lauffenburger and Horwitz, 1996). However, details of the signaling pathway(s) generated by β1 integrin translocation at the membrane ruffles and the effect of this outside-in signaling on microglial chemotaxis remain unclear. Although the P2Y12/13 receptor-mediated decrease in cAMP is an important step for microglial migration, it is interesting to note that excess inhibition as well as hyperactivation of cAMP/PKA pathways also inhibits cell migration (Edin et al., 2001; O'Connor and Mercurio, 2001). Thus, an integrin-mediated outside-in signaling may function as a fine and local tuning device to control such signaling cascades for more efficient cell migration.

In the present study, we showed that cross-linking of β1 integrin resulted in a dramatic increase of microglia. CNS injuries and diseases are often accompanied by microglial proliferation (Gehrmann et al., 1995) and our results indicate that this may be due to the β1 integrin engagement by newly upregulated ECM molecules such as fibronectin. The MAP kinase cascade is an essential component of pathways that regulate cell proliferation (Widmann, 1999). In microglia, ERKs are involved in the proliferative response to granulocyte-macrophage colony-stimulating factor (Liva et al., 1999) or corticotropin-releasing hormone (Wang et al., 2003), and the importance of Jak/STAT (Liva et al., 1999) and p38 (Tikka et al., 2001) cascades in proliferation has also been suggested. Integrin engagement also causes the activation of MAP kinases (Chen et al., 1994; Zhu and Assoian, 1995; Schlaepfer and Hunter, 1998) and, therefore, it is likely that the cross-linking of β1 integrin trig-

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

gered the proliferation signal of MAP kinases within the cell in our experiments. In view of other evidence that integrin engagement also mediates cytokine production (Miyake et al., 1993; Walzog et al., 1999) and that the proliferation of adherent cells usually requires coordinated signals from growth factor receptors and integrin, which anchors the cell to the ECM (Renshaw, 1997; Aplin et al., 1999), we cannot exclude the possibility that β1 integrin mediated an upregulation of cytokines, which then stimulated the cell in an autocrine fashion during the 24-h incubation.

We demonstrated that ADP canceled out the proliferative effect of β1 integrin engagement, although ADP alone did not influence the cell's viability. The suppression by ADP disappeared when ARC-69931 was added, suggesting that signals from P2Y12/13 receptors are responsible. β1 integrin-mediated proliferation seems to involve PKA, since the proliferation rate was markedly reduced by the downregulation of the cAMP/PKA signaling pathway by ADP or KT-5720. Taken together, we concluded that cAMP positively regulates the β1 integrin-mediated proliferation of microglia through the activation of PKA. In microglia, PKA is known to activate MEKK1/MEK/JNK (Delgado, 2002). Moreover, PKA activity favors the nuclear translocation of ERK1/2 in PC12 and hippocampal neurons, as well as presynaptic sensory neurons from *Aplysia* (Impey et al., 1998; Martin et al., 1998, Yao et al., 1998). These reports support our hypothesis that the β1 integrin engagement promotes microglial proliferation via PKA/MAP kinase pathways.

The finding that the P2Y12/13 receptor-mediated translocation of β1 integrin and membrane ruffle formation were negatively regulated by PKA was in marked contrast to the role of in microglial proliferation. It is intriguing to hypothesize that signals from P2Y12/13 receptors impaired the proliferation by shifting integrins from the firm adhesion mode that leads to cell proliferation to the mobile mode, which is more suitable for dynamic movement. Further studies will be necessary to address the functional relationship between β1 integrin and the intracellular PKA concentration in proliferation and chemotaxis.

AQ1

In summary, both microglial chemotaxis and proliferation clearly consist of numerous and complicated processes in which integrins are fundamentally involved. The results presented show that both functions are tightly linked, possibly through integrins and PKA, and that PKA likely serves as an opposite regulator between the two cellular functions. Elucidation of such mechanisms might reveal new therapeutic strategies for conditions in which the activation of microglia is detrimental such as some neuropathies of the spinal cord (Tsuda et al., 2003).

REFERENCES

Abbadie C, Lindia JA, Cumiskey AM, Peterson LB, Mudgett JS, Bayne EK, DeMartino JA, MacIntyre DE, Forrest MJ. 2003. Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. Proc Natl Acad Sci USA 100:7947–7952.

Akiyama H, McGeer PL. 1990. Brain microglia constitutively express beta-2 integrins. J Neuroimmunol 30:81–93.

Aplin AE, Juliano RL. 1999. Integrin and cytoskeletal regulation of growth factor signaling to the MAP kinase pathway. J Cell Sci 112:695–706.

Bo L, Peterson JW, Mork S, Hoffman PA, Gallatin WM, Ransohoff RM, Trapp BD. 1996. Distribution of immunoglobulin superfamily members ICAM-1, -2, -3, and the beta 2 integrin LFA-1 in multiple sclerosis lesions. J Neuropathol Exp Neurol 55:1060–1072.

Bodin P, Burnstock G. 2001. Purinergic signalling: ATP release. Neurochem Res 26:959–969.

Chen Q, Kinch MS, Lin TH, Burridge K, Juliano RL. 1994. Integrin-mediated cell adhesion activates mitogen-activated protein kinases. J Biol Chem 269:26602–26605.

Communi D, Gonzalez NS, Detheux M, Brezillon S, Lannoy V, Parmentier M, Boeynaems JM. 2001 Identification of a novel human ADP receptor coupled to G_i. J Biol Chem 276:41479–41485.

Coyle DE. 1998. Partial peripheral nerve injury leads to activation of astroglia and microglia which parallels the development of allodynic behavior. 23:75–83.

Delgado M. 2002. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit the MEKK1/MEK4/JNK signaling pathway in endotoxin-activated microglia. Biochem Biophys Res Commun 293:771–776.

Edin ML, Howe AK, Juliano RL. 2001. Inhibition of PKA blocks fibroblast migration in response to growth factors. Exp Cell Res 270:214–222.

Gehrmann J, Matsumoto Y, Kreutzberg GW. 1995. Microglia: intrinsic immunoeffector cell of the brain. Brain Res Rev 20:269–287.

Hailer NP, Jarhult JD, Nitsch R. 1996. Resting microglial cells in vitro: analysis of morphology and adhesion molecule expression in organotypic hippocampal slice cultures. Glia 18:319–331.

Hanisch U-K. 2002. Microglia as a source and target of cytokines. Glia 40:140–155.

Hemler ME. 1998. Integrin associated proteins. Curr Opin Cell Biol 10:578–585.

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB. 2001. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409:202–207.

Honda S, Sasaki Y, Ohsawa K, Imai Y, Nakamura Y, Inoue K, Kohsaka S. 2001. Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled P2Y receptors. J Neurosci 21:1975–1982.

Hood JD, Cheresh DA. 2002. Role of integrins in cell invasion and migration. Nat Rev Cancer 2:91–100.

Howlett AR, Bailey N, Damsky C, Petersen OW, Bissell MJ. 1995. Cellular growth and survival are mediated by beta 1 integrins in normal human breast epithelium but not in breast carcinoma. J Cell Sci 108:1945–1957.

Hynes RO. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11–25.

Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR. 1998. Cross talk between ERK and PKA is required for Ca^{2+} stimulation of CREB-dependent transcription and ERK nuclear translocation. Neuron 21:869–883.

Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McInally JI, Hunt SF, Kindon ND, Teobald BJ, Willis PA, Humphries RG, Leff P, Clegg JA, Smith JA, Tomlinson W. 1999. Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. J Med Chem 28:213–220.

Inoue K. 2002. Microglial activation by purines and pyrimidines. Glia 40:156–163.

Jones LS. 1996. Integrins: possible functions in the adult CNS. Trends Neurosci 19:68–72.

Jones SL. 2002. Protein kinase A regulates beta2 integrin avidity in neutrophils. J Leukoc Biol 71:1042–1048.

Jones PH, Watt FM. 1993. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 73:713–724.

Kloss CU, Werner A, Klein MA, Shen J, Menuz K, Probst JC, Kreutzberg GW, Raivich G. 1999. Integrin family of cell adhesion molecules in the injured brain: regulation and cellular localization in the normal and regenerating mouse facial motor nucleus. J Comp Neurol 411:162–178.

Kloss CU, Bohatschek M, Kreutzberg GW, Raivich G. 2001. Effect of lipopolysaccharide on the morphology and integrin immunoreactivity of ramified microglia in the mouse brain and in cell culture. Exp Neurol 168:32–46.

Laudanna C, Campbell JJ, Butcher EC. 1997. Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants. J Biol Chem 272:24141–24144.

J_ID: Z76 A_ID: K0335-04 Art: GLIA1191 Date: 4-MAY-05 Stage: I

Laufferburger DA, Hortitz AF. 1996. Cell migration: a physical integrated molecular process. Cell 84:359–369.
Laukaitis CM, Webb DJ, Donais K, Horwitz AF. 2001. Differential dynamics of α5 integrin, paxillin, and α-actinin during formation and disassembly of adhesions in migrating cells. 153:1427–1440.
Lishko VK, Yakubenko VP, Ugarova TP. 2003. The interplay between integrins alphaMbeta2 and alpha5beta1 during cell migration to fibronectin. Exp Cell Res 283:116–126.
Liva SM, Kahn MA, Dopp JM, de Vellis J. 1999. Signal transduction pathways induced by GM-CSF in microglia: significance in the control of proliferation. Glia 26:344–352.
Martin KC, Michael D, Roes JC, Barad M, Casadio A, Zhu H, Kandel ER. 1998. MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in Aplysia. Neuron 18:899–912.
Milner R, Campbell IL. 2002. Cytokines regulate microglial adhesion to laminin and astrocyte extracellular matrix via protein kinase C-dependent activation of the alpha6beta1 integrin. J Neurosci 22: 1562–1572.
Milner R, Campbell IL. 2003. The extracellular matrix and cytokines regulate microglial integrin expression and activation. J Immunol 170:3850–3858.
Miyake S, Yagita H, Maruyama T, Hashimoto H, Miyasaka N, Okumura K. 1993. Beta 1 integrin mediated interaction with extracellular matrix proteins regulates cytokine gene expression in synovial fluid cells of rheumatoid arthritis patients. J Exp Med 177:863–868.
Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK, Yamada KM. 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J Cell Biol 131:791–805.
Nakajima K, Kohsaka S. 1993. Characterization of brain microglia and the biological significance in the central nervous system. Adv Neurol 60:734–743.
Neptune ER, Bourne HR. 1997. Receptors induce chemotaxis by releasing the betagamma subunit of Gi, not by activating Gq or Gs. Proc Natl Acad Sci USA 94:14489–14494.
O'Connor KL, Mercurio AM. 2001. Protein kinase A regulates Rac and is required for the growth factor-stimulated migration of carcinoma cells. J Biol Chem 276:47895–47900.
Pinkstaff JK, Detterich J, Lynch G, Gall C. 1999. Integrin subunit gene expression is regionally differentiated in adult brain. J Neurosci 19:1541–1556.
Renshaw MW, Ren XD, Schwartz MA. 1997. Growth factor activation of MAP kinase requires cell adhesion. EMBO J 16:5592–5599.
Rollins BJ 1997. Chemokines. Blood 90:909–928.
Sasaki Y, Hoshi M, Akazawa C, Nakamura Y, Tsuzuki H, Inoue K, Kohsaka S. 2003. Selective expression of Gi/o-coupled ATP receptor P2Y12 in microglia in rat brain. Glia 44:242–250.
Schlaepfer DD, Hunter T. 1998. Integrin signalling and tyrosine phosphorylation: just the FAKs? Trends Cell Biol 8:151–157.
Simon J, Filippov AK, Goransson S, Wong YH, Frelin C, Michel AD, Brown DA, Barnard EA. 2002. Characterization and channel coupling of the $P2Y_{12}$ nucleotide receptor of brain capillary endothelial cells. J Biol Chem 277:31390–31400.
Streit WJ. 2002. Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 40:133–139.
Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J. 2001. Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. J Neurosci 21:2580–2588.
Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K. 2003. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 424:778–783.
Walzog B, Weinmann P, Jeblonski F, Scharffetter-Kochanek K, Bommert K, Gaehtgens P. 1999. A role for β_2 integrins (CD11/CD18) in the regulation of cytokine gene expression of polymorphonuclear neutrophils during the inflammatory response. FASEB J 13:1855–1865.
Wang W, Ji P, Dow KE. 2003. Corticotropin-releasing hormone induces proliferation and TNF-alpha release in cultured rat microglia via MAP kinase signalling pathways. J Neurochem 84:189–195.
Webb SE, Pollard JW, Jones GE. 1996. Direct observation and quantification of macrophage chemoattraction to the growth factor CSF-1. J Cell Sci 109:793–803.
Widmann C, Gibson S, Jarpe MB, Johnson GL. 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 79:143–180.
Yao H, York RD, Misra-Press A, Carr DW, Stork PJ. 1998. The cyclic adenosine monophosphate-dependent protein kinase (PKA) is required for the sustained activation of mitogen-activated kinases and gene expression by nerve growth factor. J Biol Chem 273:8240–8247.
Yu N, Zhang X, Magistretti PJ, Bloom FE. 1998. IL-1-alpha and TNF-alpha differentially regulate CD4 and Mac-1 expression in mouse microglia. Neuroimmunomodulation 5:42–52.
Zhang FL, Luo L, Gustafson E, Palmer K, Qiao X, Fan X, Yang S, Laz TM, Bayne M, Monsma F Jr. 2002. $P2Y_{13}$: identification and characterization of a novel Galphai-coupled ADP receptor from human and mouse. J Pharmacol Exp Ther 301:705–713.
Zhu X, Assoian RK. 1995. Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation. Mol Biol Cell 6:273–282.
Zicha D, Dunn GA, Brown AF. 1991. A new direct-viewing chemotaxis chamber. J Cell Sci 99:769–775.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Brain Research 1038 (2005) 208–215
BRAIN RESEARCH
www.elsevier.com/locate/brainres

Research report

Brain pericytes contribute to the induction and up-regulation of blood–brain barrier functions through transforming growth factor-β production

Shinya Dohgu[a], Fuyuko Takata[a], Atsushi Yamauchi[a], Shinsuke Nakagawa[b], Takashi Egawa[a], Mikihiko Naito[c], Takashi Tsuruo[c], Yasufumi Sawada[d], Masami Niwa[b], Yasufumi Kataoka[a,*]

[a]*Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan*
[b]*Department of Pharmacology 1, Graduate School of Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*
[c]*Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan*
[d]*Department of Medico-Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

Accepted 10 January 2005

Abstract

The blood–brain barrier (BBB) is a highly organized multicellular complex consisting of an endothelium, brain pericytes and astrocytes. The present study was aimed at evaluating the role of brain pericytes in the induction and maintenance of BBB functions and involvement of transforming growth factor-β (TGF-β) in the functional properties of pericytes. We used an in vitro BBB model established by coculturing immortalized mouse brain capillary endothelial (MBEC4) cells with a primary culture of rat brain pericytes. The coculture with rat pericytes significantly decreased the permeability to sodium fluorescein and the accumulation of rhodamine 123 in MBEC4 cells, suggesting that brain pericytes induce and up-regulate the BBB functions. Rat brain pericytes expressed TGF-β1 mRNA. The pericyte-induced enhancement of BBB functions was significantly inhibited when cells were treated with anti-TGF-β1 antibody (10 μg/ml) or a TGF-β type I receptor antagonist (SB431542) (10 μM) for 12 h. In MBEC4 monolayers, a 12 h exposure to TGF-β1 (1 ng/ml) significantly facilitated the BBB functions, this facilitation being blocked by SB431542. These findings suggest that brain pericytes contribute to the up-regulation of BBB functions through continuous TGF-β production.

Theme: Cellular and molecular biology
Topic: Blood–brain barrier

Keywords: Blood–brain barrier; Pericyte; Transforming growth factor-β; P-glycoprotein; Permeability; Mouse brain endothelial cell

1. Introduction

The blood–brain barrier (BBB) is highly restrictive of the transport of substances between blood and the central nervous system. The BBB is a complex system of different cellular components including brain microvascular endothelial cells, pericytes and astrocytes. Astrocytes induce and maintain the properties of the BBB including the integration of tight junctions and expression of P-glycoprotein (P-gp) through cell-to-cell contact and the secretion of soluble factors [23]. Brain pericytes are important for control of the growth and migration of endothelial cells and the integrity of microvascular capillaries [22]. Such functions are known to be mediated by transforming growth factor-β (TGF-β) [1,21,25], vascular endothelial growth factor (VEGF)

* Corresponding author. Fax: +81 92 862 2696.
E-mail address: ykataoka@cis.fukuoka-u.ac.jp (Y. Kataoka).

0006-8993/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2005.01.027

[14,17] and direct cell-to-cell communications. Pericytes have several apparatuses to directly make contact with endothelial cells: gap junctions, adhesion plaques and peg-and-socket junctions [24]. Soluble factors including TGF-β, VEGF and basic fibroblast growth factor (bFGF) were produced by and released from pericytes [2,24]. These growth factors control the permeability of the BBB [8,26,31]. These evidences indicate that pericytes regulate the brain's endothelial barrier by collaborating with astrocytes.

TGF-β, a family of multifunctional peptide growth factors, has several isoforms (TGF-β1, 2, 3, 4 and 5), shares the same structure (65–80% homology) and displays similar biological activity in vitro [11]. TGF-β acts on two highly conserved single transmembrane receptors with an intracellular serine/threonine kinase domain (TGF-β type I and type II receptors) to activate an intracellular signaling system, such as Smad proteins or the p38 mitogen-activated protein kinase (MAPK) and the extracellular signal-regulated kinase pathway [6]. TGF-β is listed as a potent endogenous substance protecting against neurodegenerative diseases of the central nervous system [11].

Recently, brain pericytes were reported to induce occludin and multidrug resistance-associated protein (MRP) 6 mRNA expression in brain endothelial cells [3,15]. As for BBB functions, brain pericytes reduce the endothelial permeability of the brain [13]. However, little is known about the mechanism behind the facilitatory role of brain pericytes in the induction and maintenance of BBB functions. The aim of this study was to clarify whether TGF-β participates in the pericyte-induced regulation of BBB functions. We made an in vitro model of the BBB by coculturing immortalized mouse brain capillary endothelial (MBEC4) cells with rat brain pericytes. MBEC4 cells are known to have the highly specialized characteristics of brain microvascular endothelial cells including the expression of P-gp [28,29]. BBB functions were assessed based on the permeability coefficient of sodium fluorescein (Na-F) and the cellular accumulation of rhodamine 123 in MBEC4 cells as the paracellular permeability of brain endothelial cells and the functional activity of P-gp, respectively.

2. Materials and methods

2.1. Animals

Wistar rats aged 2 weeks old were housed in a room at a temperature of 22 ± 2 °C under a 12-h light/dark schedule (lights on at 7:00 h) and given water and food ad libitum. All the procedures involving experimental animals adhered to the law (No. 105) and notification (No.6) of the Japanese Government and were approved by the Laboratory Animal Care and Use Committee of Fukuoka University.

2.2. MBEC4 cell culture

MBEC4 cells, which were isolated from BALB/c mouse brain cortices and immortalized by SV40-transformation [28], were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C with a humidified atmosphere of 5% CO_2/95% air. They were seeded on 12-well Transwell®-Clear inserts (Costar, MA) and 24-well culture plates (BD FALCON™, BD Biosciences, NJ) at a density of 42,000 cells/insert and 21,000 cells/well, respectively.

2.3. Primary culture of rat pericytes

Rat cerebral pericytes were isolated according to the method of Hayashi et al. [13]. Pure cultures of rat cerebral pericytes were obtained by prolonged culture of isolated brain microvessel fragments under selective culture conditions because microvessel fragments contain 23% pericytes [27]. The cerebral cortices from 2-week-old Wistar rats were cleaned of meninges and minced. The homogenate was digested with collagenase CLS2 (1 mg/ml; Worthington, Lakewood, NJ) and DNase I (37.5 μg/ml; Sigma, St. Louis, MO) in DMEM (Sigma) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 50 mg/ml gentamicin and 2 mM glutamine at 37 °C for 1.5 h. Neurons and glial cells were removed by centrifugation in 20% bovine serum albumin (BSA)-DMEM (1000 × g for 20 min). The microvessels obtained in the pellet were further digested with collagenase/dispase (1 mg/ml; Roche, Mannheim, Germany) and DNase I (16.7 μg/ml) in DMEM at 37 °C for 1 h. Microvessel endothelial cell clusters were separated by 33% Percoll (Amersham Biosciences, Piscataway, NJ) gradient centrifugation (1000 × g for 10 min). The obtained microvessel fragments were washed twice in DMEM (first 1000 × g for 8 min, then, 700 × g for 5 min) and placed in uncoated culture flasks in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C with a humidified atmosphere of 5% CO_2/95% air. After 14 days in culture, rat pericytes overgrew brain endothelial cells and reached typically 80–90% confluency. The cells were used at passages 2–3.

2.4. Preparation of three in vitro BBB models

The preparation of the in vitro BBB models was previously described [7]. In brief, rat pericytes (40,000 cells/cm^2) were first cultured on the outside of the collagen-coated polyester membrane (1.0 cm^2, 0.4 μm pore size) of a Transwell®-Clear insert (12-well type, Costar) directed upside down in the well. Two days later, MBEC4 cells (42,000 cells/cm^2) were seeded on the inside of the insert placed in the well of a 12-well culture plate (Costar) (the

opposite coculture system). In the other (bottom coculture) system, rat pericytes (20,000 cells/cm^2) were first cultured in the wells of the 12-well culture plate. After 2 days, MBEC4 cells were seeded on the inside of a Transwell®-Clear insert placed in the plate containing layers of rat pericytes. A monolayer system was also made with MBEC4 cells alone (MBEC4 monolayer).

2.5. Paracellular transport of Na-F

To initiate the transport experiments, the medium was removed and MBEC4 cells were washed three times with Krebs–Ringer buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 11 mM D-glucose, pH 7.4). Krebs–Ringer buffer (1.5 ml) was added to the outside of the insert (abluminal side). Krebs–Ringer buffer (0.5 ml) containing 100 μg/ml of Na-F (MW 376) (Sigma) was loaded on the luminal side of the insert. Samples (0.5 ml) were removed from the abluminal chamber at 30, 60, 90 and 120 min and immediately replaced with fresh Krebs–Ringer buffer. Aliquots (5 μl) of the abluminal medium were mixed with 200 μl of Krebs–Ringer buffer and then the concentration of Na-F was determined with a CytoFluor Series 4000 fluorescence multiwell plate reader (PerSeptive Biosystems, Framingham, MA) using a fluorescein filter pair (Ex(λ) 485 ± 10 nm; Em(λ) 530 ± 12.5 nm). The permeability coefficient and clearance were calculated according to the method described by Dehouck et al. [5]. Clearance was expressed as microliters (μl) of tracer diffusing from the luminal to abluminal chamber and was calculated from the initial concentration of tracer in the luminal chamber and final concentration in the abluminal chamber: Clearance (μl) = $[C]_A \times V_A / [C]_L$, where $[C]_L$ is the initial luminal tracer concentration, $[C]_A$ is the abluminal tracer concentration and V_A is the volume of the abluminal chamber. During a 120-min period of the experiment, the clearance volume increased linearly with time. The average volume cleared was plotted versus time, and the slope was estimated by linear regression analysis. The slope of clearance curves for the MBEC4 monolayer or coculture systems was denoted by PS_{app}, where PS is the permeability-surface area product (in μl/min). The slope of the clearance curve with a control membrane was denoted by $PS_{membrane}$. In the rat pericyte opposite coculture system, the control membrane is the rat pericyte-layered membrane. The real PS value for the MBEC4 monolayer and the coculture system (PS_{trans}) was calculated from $1/PS_{app} = 1/PS_{membrane} + 1/PS_{trans}$. The PS_{trans} values were divided by the surface area of the Transwell inserts to generate the permeability coefficient (P_{trans}, in cm/min).

2.6. Functional activity of P-gp

The functional activity of P-gp was determined by measuring the cellular accumulation of rhodamine 123 (Sigma) according to the method of Fontaine et al. [12]. MBEC4 cells were washed three times with assay buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES and 11 mM D-glucose, pH 7.4). In rat pericyte coculture systems, rat pericytes on the outside of the membrane were removed with a cell scraper. MBEC4 cells were incubated with 0.5 ml of assay buffer containing 5 μM of rhodamine 123 for 60 min. Then, the solution was removed and the cells were washed three times with ice-cold phosphate-buffered saline and solubilized in 1 M NaOH (0.2 ml). Aliquots (5 μl) of the cell solution were removed for measurement of cellular protein according to the method of Bradford [4] using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The remaining solution was neutralized with 1 M HCl and the rhodamine 123 content was determined with a CytoFluor Series 4000 fluorescence multiwell plate reader (PerSeptive Biosystems) using a fluorescein filter pair (Ex(λ) 485 ± 10 nm; Em(λ) 530 ± 12.5 nm).

2.7. Detection of TGF-β1 mRNA

Total RNA from rat pericytes was extracted using TRIzol™ reagent (Invitrogen). The primer pair used in the reverse transcription-polymerase chain reaction (RT-PCR) was designed based on the nucleotide sequence of the rat TGF-β1 and rat GAPDH. The sequences of primers were as follows: the upper primer 5′-ATACGCCTGAGTGGCTGTCT-3′ and the lower primer 5′-TGGGACTGATCCCATTGATT-3′ for TGF-β1; the upper primer 5′-CTACCCACGGCAAGTTCAAT-3′ and the lower primer 5′-GGATGCAGGGATGATGTTCT-3′ for GAPDH. The expected sizes of the RT-PCR products, predicted from the positions of the primers, were 153 bp for TGF-β1 and 479 bp for GAPDH. A SuperScript One-Step RT-PCR system (Invitrogen) was used for reverse transcription of RNA, and TGF-β1 cDNA was amplified by PCR. Amplification was performed in a DNA thermal cycler (PC707; ASTEC, Fukuoka, Japan) according to the following protocol: cDNA synthesis for 30 min at 50 °C, pre-denaturation for 5 min at 94 °C; 25 cycles of denaturation for 30 s at 94 °C, primer annealing for 30 s at 57 °C and polymerization for 30 s at 72 °C; and a final extension for 5 min at 72 °C. Each 10 μl of PCR product was analyzed by electrophoresis on a 3% agarose (Sigma) gel with ethidium bromide staining. The gels were visualized on a UV light transilluminator and photographed using a DC290 Zoom digital camera (Kodak, Rochester, New York).

2.8. Effects of the modulation of TGF-β1 signaling on BBB functions

A TGF-β type I receptor antagonist, SB431542 (TOCRIS, Bristol, UK), and human TGF-β1 (Sigma) were first dissolved in dimethylsulfoxide (DMSO) and 4 mM HCl