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Cytoprotection Against Oxidative Stress-Induced Damage of Astrocytes by Extracellular ATP Via $P2Y_1$ Receptors

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KEY WORDS ATP; $P2Y_1$ receptors; astrocytes; oxidative stress

ABSTRACT Oxidative stress is the main cause of neuronal damage in traumatic brain injury, hypoxia/reperfusion injury, and neurodegenerative disorders. Although extracellular nucleosides, especially adenosine, are well known to protect against neuronal damage in such pathological conditions, the effects of these nucleosides or nucleotides on glial cell damage remain largely unknown. We report that ATP but not adenosine protects against the cell death of cultured astrocytes induced by hydrogen peroxide (H_2O_2). ATP ameliorated the H_2O_2-induced decrease in cell viability of astrocytes in an incubation time- and concentration-dependent fashion. Protection by ATP was inhibited by P2 receptor antagonists and was mimicked by $P2Y_1$ receptor agonists but not by adenosine. The expressions of $P2Y_1$ mRNAs and functional $P2Y_1$ receptors in astrocytes were confirmed. Thus, ATP, acting on $P2Y_1$ receptors in astrocytes, showed a protective action against H_2O_2. The astrocytic protection by the $P2Y_1$ receptor agonist 2-methylthio-ADP was inhibited by an intracellular Ca^{2+} chelator and a blocker of phospholipase C, indicating the involvement of intracellular signals mediated by Gq/11-coupled $P2Y_1$ receptors. The ATP-induced protection was inhibited by cycloheximide, a protein synthesis inhibitor, and it took more than 12 h for the onset of the protective action. In the DNA microarray analysis, ATP induced a dramatic upregulation of various oxidoreductase genes. Taken together, ATP acts on $P2Y_1$ receptors coupled to Gq/11, resulting in the upregulation of oxidoreductase genes, leading to the protection of astrocytes against H_2O_2.

INTRODUCTION

Astrocytes are much more than merely support cells for neurons in the central nervous system (CNS). They can receive inputs, assimilate information, and send instructive chemical signals to neighboring glial cells as well as neurons (Araque et al., 1999a, b, 2001; Haydon, 2001). Thus, communication among astrocytes would play an important role in brain function. Initially, so-called gliotransmission, a glia-to-glia communication or even neuron-to-glia communication, was reported to be mediated by glutamate (Cornell-Bell et al., 1990; Charles et al., 1991; Parpura et al., 1994; Innocenti et al., 2000) because astrocytes express glutamate receptors and release glutamate. However, re-

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cent accumulating evidence has shown that extracellular ATP released from astrocytes has a central role in astrocyte-to-astrocyte (Guthrie et al., 1999), astrocyte-to-microglia (Verderio and Matteoli, 2001; Schipke et al., 2002), and even astrocyte-to-neuron communication (Koizumi et al., 2003; Newman, 2003; Zhang et al., 2003).

ATP is an endogenous ligand for P2 receptors that are classified into ligand-gated P2X and G-protein-coupled metabotropic P2Y receptors (Abbracchio and Burnstock, 1994). Astrocytes express both types of P2 receptors (James and Butt, 2002; Fumagalli et al., 2003) and can release ATP in response to various stimuli (Guthrie et al., 1999; Queiroz et al., 1999; Koizumi et al., 2003). Astrocytic ATP acting on these P2 receptors forms intercellular Ca^{2+} waves that mediate long-range communications in astrocytes (Fam et al., 2000; Gallagher and Salter, 2003). However, the physiological or pathological significance of such an ATP/P2 receptor-mediated response in astrocytes remains largely unknown.

It has been reported that ATP inhibits excess neuronal excitations by inhibiting the release of glutamate (Koizumi and Inoue, 1997; Zhang et al., 2003) or by facilitating inhibitory γ-aminobutyric acid (GABA) release in the hippocampus (Aihara et al., 2002) and is therefore presumably involved in protecting neurons against excitotoxicity. With regard to neuroprotective actions, however, adenosine, a metabolite of ATP, has received much attention as an important inhibitory molecule because it is formed by the immediate degradation of ATP by ectonucleotidases, potently inhibiting the excitability of neurons and protecting them against various neurodegenerative disorders including excitatory neuronal death (Jones et al., 1998; Behan and Stone, 2002; Hentschel et al., 2003; Schwarzschild et al., 2003). This might be why the functional role of ATP in relation to neuroprotection has received only limited attention. Interestingly, however, adenosine does not show any protective action in astrocytes, rather it induces the cell death of astrocytes (Abbracchio et al., 1995; Appel et al., 2001; Di Iorio et al., 2002). It has been reported that ATP protects astrocytes against glucose deprivation-induced cell death, although this protection appears to be independent of P2 receptors (Shin et al., 2002). ATP is released from both neurons (Wieraszko et al., 1989; Inoue et al., 1995) and astrocytes (Guthrie et al., 1999; Ahmed et al., 2000) in physiological and pathological conditions, and astrocytes could receive the ATP signal via various P2 receptors, including a high-affinity $P2Y_1$ receptor (Koizumi et al., 2002). These findings raise the possibility that, unlike neurons, astrocytes mainly use ATP/P2 receptor-mediated pathway(s) for their own survival.

We report that ATP acting on $P2Y_1$ receptors protects astrocytes from cell death induced by hydrogen peroxide (H_2O_2), one of the main reactive oxygen species (ROS) generated by traumatic brain injury, hypoxia/reperfusion, and various neurodegenerative disorders (Agardh et al., 1991; Lei et al., 1997; Cuajungco et al., 2000; Huang et al., 2000; Tabner et al., 2001; Tamagno et al., 2003). We further demonstrate by using differential gene expression analysis that ATP induces the upregulation of oxidoreductase genes, suggesting the involvement of these genes in the protective action.

MATERIALS AND METHODS

Chemicals

Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), uridine 5′-triphosphate (UTP), adenosine, 2-methylthio-adenosine diphosphate (2MeSADP), adenosine 5-o-(2-thiodiphophate) (ADPβS), α,β-methylene-adenosine triphosphate (α,βmeATP), suramin, reactive blue 2 (RB2), pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), MRS2179, U73122, U73343, glutamate, 1-octanol, DL-2-amino-5-phosphonopentanoic acid (AP-V), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and (RS)-α-methyl-4-carboxyphenylglycine (MCPG) were purchased from Sigma Chemical Co. (St Louis, MO). The sources of other chemicals are shown in parentheses as follows; trypsin-EDTA, M-MLV reverse transcriptase, 100 mM dNTP set, recombinant ribonuclease (RNase) inhibitor and deoxyribonuclease (DNase) I (GIBCO/Invitrogen, Tokyo, Japan), RNA STAT 60 (Tel-Test, Friendswood, TX), hydrogen peroxide (H_2O_2) (Wako Pure Chemicals, Osaka, Japan), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Chemicon International, Temecula, CA), GeneAmp PCR Reagent Kit and AmpliTaq DNA polymerase (Perkin-Elmer, (Foster City, CA) Roche Molecular Systems, (Pleasanton, CA), O,O′-Bis (2-aminophenyl) ethyleneglycol-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) (Calbiochem Biosciences, San Diego, CA).

Cells and Cell Culture

Astrocytes were prepared from neonatal rat forebrain. The cells were cultured in Dulbecco's modified essential medium (DMEM, GIBCO/Invitrogen) supplemented with 10% fetal bovine serum (FBS; GIBCO/Invitrogen). After 3 weeks with changing of the medium every 3 days, the medium was changed to DMEM with 5% horse serum (HS; GIBCO/Invitrogen) and 5% FBS; the cells were shaken for 15 h at 100 rpm. Then, the cells were washed 3 times with phosphate-buffered saline (PBS) (10 ml each) and supplemented with 0.025% trypsine-EDTA (diluted with PBS), and incubated for 2 min under 10% CO_2 /90% air at 37°C. After the cells were harvested, 2×10^5 cells were seeded on 60 × 15-mm dishes (Falcon/Becton Dickinson, San Jose, CA) and cultured in DMEM with 5% HS and 5% FBS. Total RNA was collected from five dishes. For the cell viability assay, cells were seeded on 96-well plates (NUNC, Roskilde, Denmark) at a density of 1.25×10^4 cells/well. At 24 h after the seeding, the medium was

changed. The cells were used for experiments 72 h after the medium exchange.

Experimental Design of Hydrogen Peroxide (H_2O_2)-Evoked Cell Death

Astrocytes were exposed to various concentrations of H_2O_2 (75–300 μM) for 1–24 h, and then the cell viability was investigated. In the present study, we chose a H_2O_2 concentration of 250 μM and an incubation period of 2 h to assess the effect of ATP.

Cell Viability Assay

For the cell viability assay, we used an MTT assay. MTT is a yellow tetrazolium salt that is reduced to purple formazan (Altman, 1976). The MTT assay assesses cell viability by measuring the mitochondrial function (Twentyman and Luscombe, 1987). After incubation with H_2O_2 for 2 h, a 1/10 volume of MTT solution (5 mg/ml in PBS) was added and incubated for 4 h under 10% CO_2/90% air at 37°C. Then an equal volume of isopropanol (with 0.04 N HCl) was added to the cells, and the MTT formazan was dissolved by pipetting. The absorbance was measured on an enzyme-linked immunosorbent assay (ELISA) plate reader (ASYS Hitech, Eugendorf, Austria) with a test and reference wavelength of 570 and 630 nm, respectively.

Expression of $P2Y_1$ Receptors in Astrocytes

The expression of $P2Y_1$ receptor mRNA was analyzed by single reverse transcription-polymerase chain reaction (RT-PCR). For RT-PCR analysis, astrocytes were directly lysed with 0.5 ml of RNA STAT-60 (Tel-Test B) and total RNA was isolated; 1 μg of RNA was reverse-transcribed with M-MLV transcriptase. Aliquots (1 μl) of the RT product were added to the reaction mixture containing 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of *Taq* polymerase and $P2Y_1$ receptors specific primers according to the nucleotide sequences as follows; forward, 5′-ctgatcttgggctgttatgg-3′ and reverse, 5′-gctgttgagacttgctagac-3′. Amplification was performed in a Gene Amp PCR System 2400-R (Perkin-Elmer/Roche Molecular Systems) thermal cycler for 30–40 cycles, after an initial denaturation at 94°C for 2 min by utilizing sense and antisense primers specifically designed for $P2Y_1$ receptors. The PCR product was resolved on agarose gel stained by 2% ethidium bromide and visualized under ultraviolet (UV) light.

Measurement of Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$) in Single Cells

The increase in $[Ca^{2+}]_i$ in single cells was measured by the fura-2 method as described by Grynkiewicz et al. (1985) with minor modifications (Koizumi et al., 2002). In brief, the cells were washed with a balanced salt solution (BSS) of the following composition (in mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.2, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) 25, and D-glucose 10 (pH = 7.4). Cells were then loaded with 5 μM fura-2 acetoxymethylester (fura-2 AM) at room temperature in BSS for 45 min, followed by a BSS wash and a further 30-min incubation to allow deesterification of the loaded dye. For the Ca^{2+}-free experiment, Ca^{2+} was removed from the BSS (Ca^{2+}-free BSS). The coverslips were mounted on an inverted epifluorescence microscope (TE-2000-U, Nikon, Tokyo, Japan). Fluorescent images were obtained by alternate excitation at 340 nm (F340) and 380 nm (F380). The emission signal at 510 nm was collected by a charge-coupled device camera (C-6790, Hamamatsu Photonics, Hamamatsu, Japan) coupled with an image intensifier (GaAsP, C8600-03, Hamamatsu Photonics); digitized signals were stored and processed using an image processing system (Aquacosmos, Hamamatsu Photonics). Drugs were dissolved in BSS and applied by superfusion.

Measurement of Extracellular ATP Concentration

The extracellular ATP concentration in astrocytes was detected with a luciferin-luciferase bioluminescence assay. After glutamate stimulation or exogenous ATP application, supernatants were collected at different time points and were mixed with luciferase reagents (ATP bioluminescence assay kit CLS II; Roche Diagnostics, Mannheim, Germany). ATP bioluminescence was detected by a luminometer (Lumiphotometer TD-4000, Labo Science, Tokyo, Japan). The absolute ATP concentration was estimated using a standard ATP solution (0.001–1 μM).

Total RNA Preparation

After washing the cells twice with PBS, total RNA was prepared with RNeasy Mini total RNA Preparation Kit (Qiagen GmbH, Tokyo, Japan) according to the manufacturer's instructions.

DNA Microarray Analysis

Converting total RNA to the targets for Affymetrix GeneChip DNA microarray hybridization was done according to the manufacturer's instructions. The targets were hybridized to rat genome U34A Gene Chip microarray (Affymetrix) for 16–24 h at 45°C. After the hybridization, the DNA microarrays were washed and stained on Fluidics Station (Affymetrix) according to the protocol provided by Affymetrix. Then, the DNA microarrays were scanned, and the images obtained

were analyzed by Microarray Suite Expression Analysis Software (version 5.0; Affymetrix). To analyze the gene expressions in astrocytes, differences in the mean level of the gene expression index between the control group and drug-treated group were assessed using the Student's *t*-test for each probeset.

Astrocytes were incubated for 2 h with ATP at a final concentration of 100 μM. Total RNA was prepared at the end of incubation and converted to the target for GeneChip hybridization. The gene expression was analyzed in duplicate by Rat Genome U34A GeneChip using these targets. The addition of ATP and the preparation of total RNA was done four times independently.

Selection of Differentially Expressed Genes

The first step was selecting genes whose expression levels were increased 2-fold by treatment with ATP. The second step was selecting genes whose *P*-values were $P < 0.05$ using Student's *t*-test. The last step was selecting genes whose expression levels of the drug treated group were 1,000.

Quantitative RT-PCR of Oxidoreductase Genes

RT-PCR amplifications were performed using Taqman One-step RT-PCR Master Mix Reagents and, 200 nM oxidoreductase-specific primers. Using the computer software Primer Express (Applied Biosystems), clone-specific primers were designed to recognize rat oxidoreductase genes, i.e., rat carbonyl reductase (CBR, Taqman Probe, 5′-cctcctgaatgcctgccctg-3′; forward, 5′-tgaggagaggagagaggacaaga-3′; reverse, 5′-cctgccatgtcggttctga-3′), schlafen-4 (SHL4, Taqman probe, 5′-aggccttatcgaggccagatggtttg-3′; forward, 5′-tcttgtttttcctagaactgttgtg-3′; reverse, 5′-ggtgaggtagcctggctatagc-3′), and thioredoxin reductase (TrxR, Taqman probe, 5′-attgaagcagggacaccaggccg-3′; forward, 5′-gtgccgacgaaaattgaaca-3′; reverse, 5′-gtggatttagcggtcacctga-3′). RT-PCR was performed by 30 min reverse transcription at 48°C, 10 min Amplitaq Gold activation at 95°C, then 15-s denaturation at 95°C, 1 min annealing and elongation at 60°C for 40 cycles in a PRISM7700 (Applied Biosystems). To exclude contamination by nonspecific PCR products such as primer dimmers, melting curve analysis was applied to all final PCR protocols after the cycling protocol. Each experiment was performed in triplicate.

RESULTS

Protection by ATP Against Oxidative Stress-Induced Cell Death in Astrocytes

Using an MTT assay, we tested the effect of hydrogen peroxide (H_2O_2) on cell viability in astrocytes. We found that H_2O_2 caused a time- (Fig. 1A) and concentration-dependent (Fig. 1B) decrease in the cell viability of the astrocytes, i.e., cell death of the astrocytes. When incubated for 1 h at 250 μM, the cell viability was almost halved and then was gradually decreased to ~20% of the non-treated control level by a further incubation (2–24 h, Fig. 1A). When the H_2O_2 concentrations were varied, the cell viability was decreased in a concentration-dependent fashion and reached the minimum at 250 μM. We therefore chose an H_2O_2 concentration of 250 μM and an incubation period of 2 h for the following experiments.

We tested the effect of exogenously applied ATP on the H_2O_2-induced astrocytic cell death. ATP was applied to the cells 24 h before and during H_2O_2 applica-

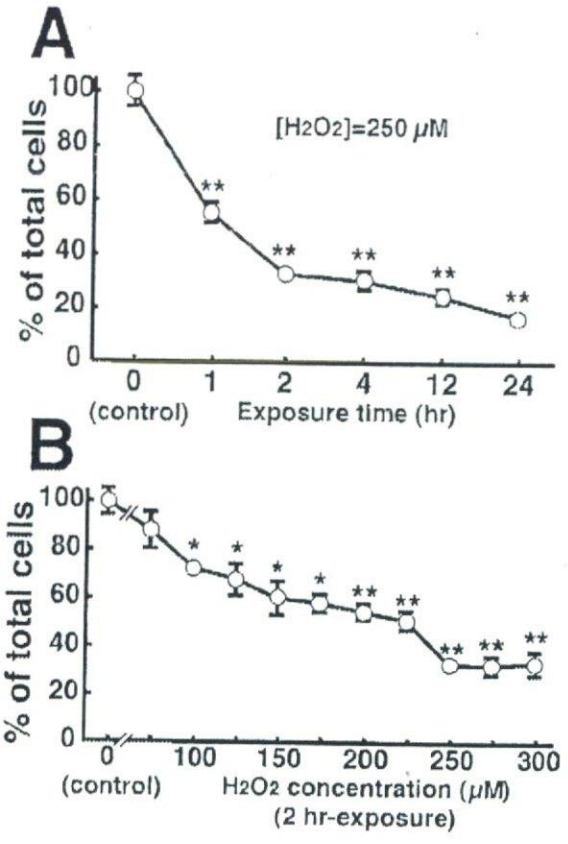

Fig. 1. Changes in cell viability of astrocytes by H_2O_2. **A:** Cells were incubated with 250 μM H_2O_2 for various periods before the cell viability test. The cell viability was evaluated by the MTT assay as described in Materials and Methods. H_2O_2 induced a decrease in cell viability in an exposure time-dependent fashion. **B:** Cells were stimulated with various concentrations of H_2O_2 for 2 h; cell viability was then examined. H_2O_2 evoked cell death in a concentration-dependent fashion. Sequential plots show mean ± SEM of triplicate measurements, depicting a representative experiment (n = 3). Values were normalized to total cell number (control) and the cell viability was expressed as percentage of total cell. Asterisks show significant differences from the control response (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

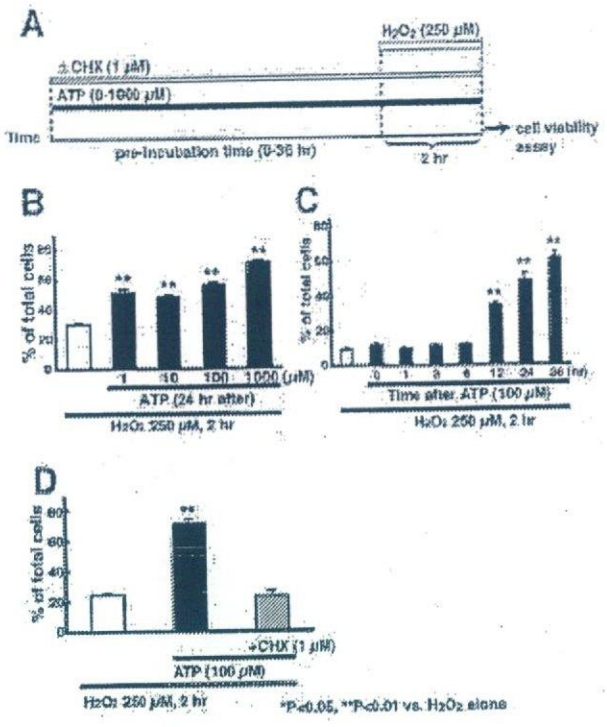

Fig. 2. Protection by ATP of H_2O_2-evoked cell death in astrocytes. A: Stimulus regime. B: ATP protected against H_2O_2-evoked cell death in a concentration-dependent fashion. Cells were incubated with ATP (1–1,000 μM) 24 h before and during H_2O_2 application. C: Protective effect of ATP was dependent on the duration of preincubation. Cells were incubated with ATP (100 μM) for various periods (from 0 to 36 h), and then exposed to H_2O_2 together with ATP. Without any preincubation periods, ATP did not show any significant protective effect. D: Inhibition by CHX of the ATP-induced protection. Incubation of cells with CHX (1 μM for 24 h) abolished the protective effect of ATP. 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 and the cell viability was expressed as percentage of total cell number. Asterisks show significant difference from the response evoked by H_2O_2 alone (*$P < 0.05$, **$P < 0.01$, Student's t-test).

tion. Pretreatment with ATP significantly inhibited the H_2O_2-induced cell death in a concentration-dependent manner over a concentration range of 1–1,000 μM (Fig. 2B). When pretreated for 12–36 h, the H_2O_2-induced cell death in astrocytes was significantly reduced to about 60% of control (Fig. 2C). However, ATP did not show any cytoprotective action when the exposure time of ATP was less than 12 h. When astrocytes were pretreated with ATP plus cycloheximide (CHX, 1 μM), a protein synthesis inhibitor, the protection by ATP (100 μM for 24 h) disappeared (Fig. 2D). CHX alone had no effect on the viability of astrocytes (control; 100 ± 6%, CHX 1 μM; 90 ± 6%, n = 6).

Protection by ATP against the H_2O_2-induced cell death of astrocytes was evaluated pharmacologically. As shown in Figure 3B, when the P2 receptor antagonists suramin (100 μM), PPADS (300 μM), and RB2 (10 μM) were added to the cells 15 min before and during ATP (100 μM) application, ATP protection was almost abolished, indicating the involvement of P2 receptors. UTP (100 and 1,000 μM), an agonist of $P2Y_2$ and $P2Y_4$ receptors, α,βmeATP (100 μM), an agonist of $P2X_1$ and $P2X_3$ receptors, had no effect on the H_2O_2-evoked cell death (Fig. 3C). Adenosine (10 μM) did not show any protection against the cell death. The $P2Y_1$ receptor agonists 2MeSADP (1 μM) and ADPβS (1 μM) provided significant protection against cell death (Fig. 3D) and the ATP-induced protection was inhibited by the $P2Y_1$ receptor antagonist MRS2179 in a concentration-dependent manner (Fig. 3E). Thus, ATP appears to show its protective action mainly via a $P2Y_1$ receptor-mediated pathway(s) in astrocytes. None of the agonists and antagonists alone had any effect on the cell viability of astrocytes (Fig. 3B–E, gray columns).

We tested whether prolonged ATP is required or a brief exposure of ATP is enough to trigger its protective action in astrocytes. Since the ATP-induced protection is mediated by $P2Y_1$ receptors (Fig. 3), we added the $P2Y_1$ receptor antagonist MRS2179 (1 μM) to the culture medium 15 min before or 30 min after ATP stimulation, and then further incubated for 24 h prior to H_2O_2 exposure. MRS2179 reversed the ATP-induced protection only when it was added to the cells 15 min before and during ATP stimulation (MRS2179 15 min before ATP, 33.6 ± 5.9% of total cells, n = 3, $P = 0.91$ vs. H_2O_2 alone; MRS2179 30 min after ATP, 62.9 ± 3.5 of total cells, n = 3, $P < 0.05$ vs. H_2O_2 alone; Fig. 4B). Furthermore, we analyzed the time-course of ATP degradation in astrocytes. ATP was exogenously applied to astrocytes, and the supernatants were collected at different incubation periods. Exogenously applied ATP (100 μM) was soon metabolized; the 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 (Fig. 4C). Although longer periods (>12 h, see Fig. 2C) were required for the onset of the cytoprotective action, prolonged exposure of ATP was not necessarily required for the protection in astrocytes.

Intracellular Signaling Cascades Involved in $P2Y_1$ Receptor-Mediated Protection

We investigated the involvement of $P2Y_1$ receptor-mediated intracellular signaling cascades in the protection against the H_2O_2-induced cell death in astrocytes. Both the PLC inhibitor U73122 (5 μM) and the rapid intracellular Ca^{2+} chelator BAPTA-AM (25 μM) inhibited the protection by 1 μM 2MeSADP (Fig. 5). The much less active PLC inhibitor U73343 (5 μM) had no effect on the ATP-evoked protection. These chemicals were added to the cells 1 h before and during 2MeSADP-application and were washed away before H_2O_2 application. These blockers themselves had no effect on the cell viability under the normal condition (control, 100 ± 3%; U73122, 90 ± 5%; U73343, 92 ± 6%; and BAPTA-AM, 105 ± 3%, n = 6) (Fig. 5B, gray columns) nor affected the H_2O_2-induced cell death in

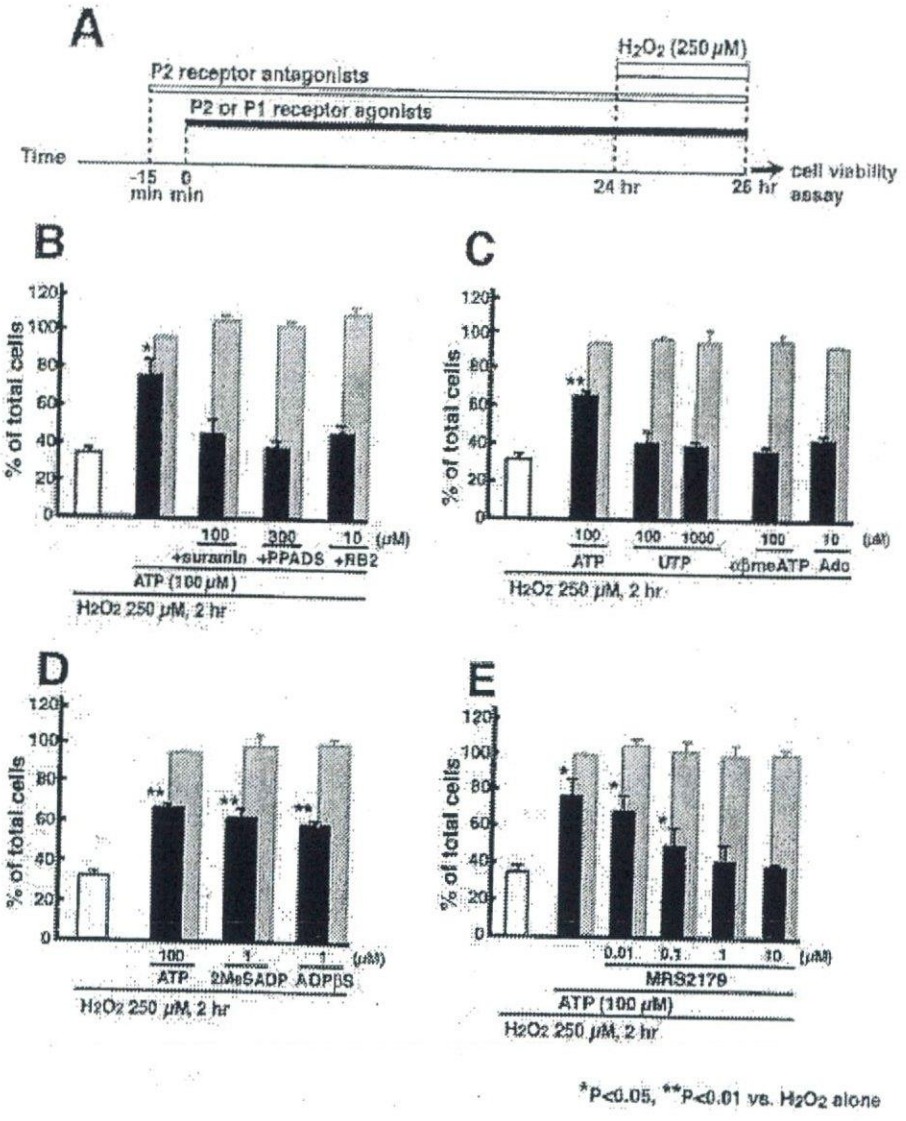

Fig. 3. Effect of P2 receptor agonists and antagonists on the H_2O_2-evoked cell death of astrocytes. **A**: Stimulus regime. **B**: Inhibition by P2 receptor antagonists of ATP-induced protection. Suramin (100 μM), PPADS (300 μM) and RB2 (10 μM) reversed the effect of ATP. Cells were incubated with ATP (100 μM) for 24 h before H_2O_2 application. Each antagonist was added to the cells 15 min before and during ATP application. **C**: Effect of P1 and P2 receptor agonists. UTP (100 and 1,000 μM), α,βmeATP (100 μM) and adenosine (Ado, 10 μM) had no significant protective effect against the H_2O_2-evoked death in astrocytes. **D**: Protection by $P2Y_1$ selective agonists of H_2O_2-evoked cell death in astrocytes. The $P2Y_1$ selective agonists 2MeSADP and ADPβS (1 μM) mimicked the cytoprotective effect of ATP. **E**: Inhibition by $P2Y_1$ selective antagonist of ATP-induced protection. The $P2Y_1$ selective antagonist MRS2179 inhibited the effect of ATP in a concentration-dependent manner. Various concentrations of MRS2179 were added to the cells 15 min before and during ATP application. Gray columns show the effects of agonists or antagonists 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 difference form the response evoked by H_2O_2 alone (*$P < 0.05$. **$P < 0.01$, Student's t-test).

astrocytes (H_2O_2 alone, 36 ± 2%; +U73122, 36 ± 1%; +U73343, 34 ± 4%; and BAPTA-AM, 35 ± 13%, n = 6).

We also studied the effect of these blockers on the 2MeSADP-evoked increase in $[Ca^{2+}]_i$ in astrocytes (Fig. 5C). Both BAPTA-AM (25 μM) and U73122 (5 μM) inhibited the 2MeSADP-evoked increase in $[Ca^{2+}]_i$, whereas U73343 (5 μM) did not. BAPTA-AM and U73122 also reduced 2MeSADP-responders (Fig. 5C, open circles). U73122, U73343 and BAPTA-AM were added to the cells 15 min before and during 2MeSADP application.

Glutamate is another important gliotransmitter that leads to an increase in $[Ca^{2+}]_i$ in astrocytes via PLC-linked metabotropic glutamate receptors (Pasti et al., 1997; Porter and McCarthy, 1996). We therefore tested the effect of pretreatment with glutamate on the H_2O_2-induced cell death in astrocytes. As shown in Figure 6A, pretreatment of glutamate (100 μM for 24 h) significantly protected the H_2O_2-induced cell. Glutamate alone had no effect on cell viability (Fig. 6A, gray column). Interestingly, such protection by glutamate disappeared when the $P2Y_1$ receptor antagonist MRS2179 was added to the cells 15 min before and during glutamate application (Fig. 6B). We further investigated whether exogenously applied glutamate induces the release of ATP from astrocytes and found that it evoked ATP release that lasted for 15 min (Fig. 6C).

Expression and Function of $P2Y_1$ Receptors in Astrocytes

To elucidate whether $P2Y_1$ receptors are actually expressed and functional in astrocytes, we analyzed the expression of $P2Y_1$ receptors by RT-PCR and mea-

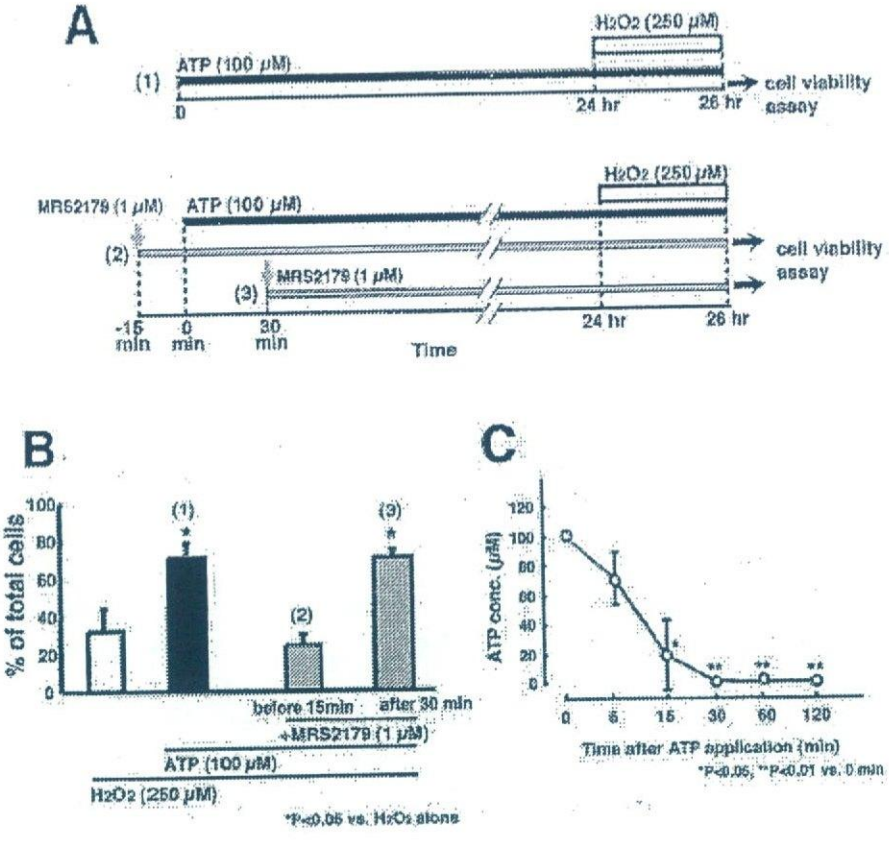

Fig. 4. ATP activates $P2Y_1$ receptors in a short time window. A: (1)–(3) depict the stimulus regime. B: MRS2179 (1 μM) was applied to the cells 15 min before and during ATP application (2), or 30 min after ATP stimulation (3). MRS2179 did not antagonize the effect of ATP when it was applied to the 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

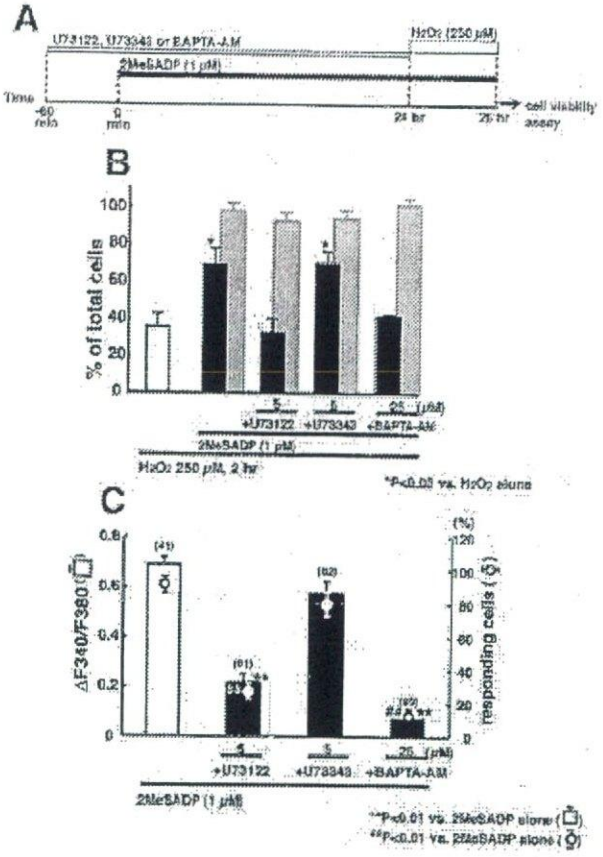

Fig. 5. Intracellular signaling cascades associated with $P2Y_1$ receptor-mediated cytoprotective action. A: Stimulus regime. B: When cells were pretreated with U73122 (5 μM) or BAPTA-AM (25 μM), the 2MeSADP (1 μM)-induced protective action against H_2O_2 disappeared, whereas U73343 (5 μM), a much less 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).

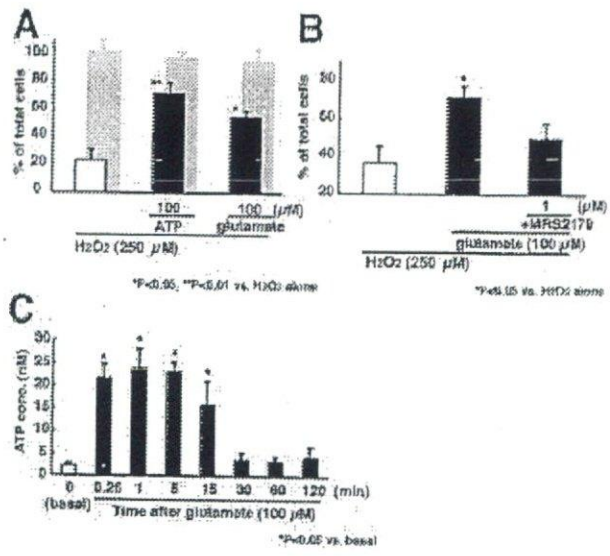

Fig. 6. Effects of glutamate on the H_2O_2-induced cell death in astrocytes. A: Preincubation with glutamate protected astrocytes against H_2O_2-evoked cell death. Cells were incubated with glutamate (100 μM) for 24 h, and then was exposed to H_2O_2. Glutamate showed significant protection against H_2O_2-induced 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-

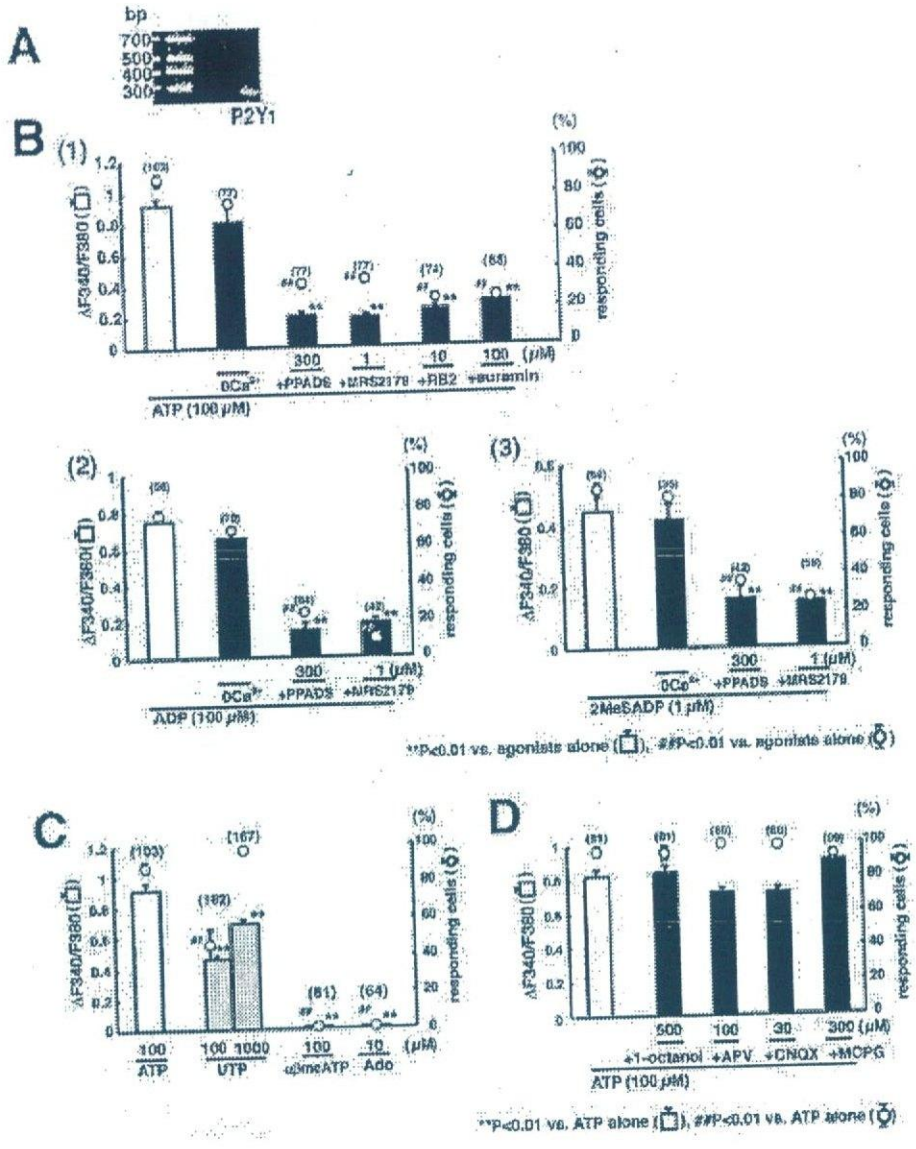

Fig. 7. Expression and function of $P2Y_1$ receptors. **A:** RT-PCR analysis showing expression of $P2Y_1$ receptor mRNA in astrocytes. Total cellular RNA was subjected to RT-PCR. The resulting cDNA was amplified with specific primers for $P2Y_1$ receptors. PCR product was resolved on agarose gel stained 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	Seblafen-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).

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Toxicity of Quinacrine Can Be Reduced By Co-Administration of P-Glycoprotein Inhibitor in Sporadic Creutzfeldt-Jakob Disease

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SUMMARY

1. Recent publication has suggested that quinacrine may be a candidate for treatment of Creutzfeldt-Jakob disease (CJD). But serious toxicity of quinacrine to liver and hematological system has been reported.

2. We disclosed the permeability of quinacrine can be enhanced by presence of p-glycoprotein inhibitor at blood–brain barrier in vitro. Therefore, we tried the protocol of combination of quinacrine and p-glycoprotein inhibitor, verapamil for patients with CJD.

3. When compared clinical effects by quinacrine and the combination therapy, improvement of clinical findings was observed at the same level without any adverse effects. Low-dose quinacrine with verapamil can be used as safe treatment of CJD.

KEY WORDS: quinacrine; sporadic Creutzfeldt-Jakob disease; p-glycoprotein inhibitor.

INTRODUCTION

Although there are number of promising agents to control prion protein in vitro or in vivo, no sufficiently safe agent has yet been discovered for patients with Creutzfeldt-Jakob disease (CJD) (Doh-ura *et al.*, 2000).

Quinacrine, originally used as an anti-malaria agent, was reported as a possible agent useful for treatment of CJD (Korth *et al.*, 2001). Recent report found that quinacine might present serious toxicity to the liver and hematological system (Scoazec *et al.*, 2003).

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Quinacrine inhibited the accumulation of PrP^{Sc} in cultured infected cells, but did not have an apparent effect on PrP^{C} biosynthesis or turnover.

To develop some method of suppression of the adverse effects of quinacrine, we investigated the mechanism of quinacrine transport across the blood–brain barrier (BBB), and found that the permeability of quinacrine could be enhanced at the BBB by the presence of a p-glycoprotein inhibitor such as verapamil or cyclosporine (Dohgu *et al.*, 2003).

Therefore, we administrated a therapy regimen of combination of 200 mg/day of quinacrine and 120 mg/day of oral verapamil and compared it to one of 300–600 mg/day of quinacrine only.

We administrated quinacrine without verapamil for one patient, 64-year-old female who developed dementia and gait disturbance within two months. She was given 300 mg/day for the first two weeks, then the quantity was increased to 600 mg/day without p-glycoprotein inhibitor. Frequency of myoclonus, gaze, and smile were markedly improved. We stopped quinacrine administration due to liver dysfunction after four weeks. Two other sporadic CJD cases were treated by combination of quinacrine (200 mg/day) and verapamil (120 mg/day). The first case treated with combination therapy was a 71-year-old male, who had developed unstable gait, disorientation, and myoclonus. After two weeks administration of quinacrine and verapamil, frequency of myoclonus was dramatically decreased. Before starting medication, his eyes had rolled aimlessly. He began to gaze at his family and his doctor after the combination of quinacrine and verapamil. However, his symptoms returned to the non-medicated state after eight weeks, although he has been receiving medication.

The second case treated with combination therapy was a 65-year-old male. He was bedridden as a result of cerebellar ataxia and progressive dementia. Action myoclonus was observed. We started combination treatment of quinacrine and verapamil on him. After two weeks, his eye movement and myoclonus had improved markedly though the improvement was temporal. These three patients were diagnosed as possible CJD by based on clinical criteria of World Health Organization, diffusion-weighted MRI, and 14-3-3 protein in cerebrospinal fluid (CSF).

To determine whether quinacrine could be sufficiently transported to the brain, we measured the concentration of quinacrine in CSF at 4 weeks after administration of case in 2 and case 3 (Table I). Concentrations of quinacrine in CSF were measured by high-performance liquid chromatography method as described previously (Björkman and Elisson, 1987). The concentration of quinacrine in CSF, supposed to

Table I. Effects and Adverse Effects of Quinacrine in Patients with CJD

Case	Co-administration	Concentration of quinacrine in CSF	AST level[a]	Hematological dysfunction	Skin color change	Clinical effects: Frequency of myoclonus	Clinical effects: Improvement of gaze and smile
1	None	ND	158	—	+	Decreased	+
2	Verapamil	392 nM	24	—	+	Decreased	+
3	Verapamil	226 nM	53	—	+	Decreased	+

Note. Plus symbol shows that each patient has the indicated findings.
[a]ALT; peak data under quinacrine administration.

be approximately equal to the concentration of quinacrine in experimental treatment in vitro approx. 200–400 nM (Korth *et al.*, 2001).

When the clinical effects on the first patient were compared with the other two patients (combination of 200 mg/day of quinacrine and 120 mg/day of verapamil), improvement of the clinical findings in patients receiving a combination of low dose quinacrine and verapamil was observed to be approximately equal to the level improvement seen in the patient receiving quinacrine only. In two patients treated with the combination of low-dose quinacrine and verapamil, no liver dysfunction and hematological toxicity was observed. Although French National Surveillance Network of Prion Diseases recommended to use quinacrine 1000 mg the first day, then 300 mg each day, we conclude that low-dose quinacrine can be used as a safe and effective treatment of CJD when given in combination with a p-glycoprotein inhibitor such as verapamil.

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Nitric oxide mediates cyclosporine-induced impairment of the blood–brain barrier in cocultures of mouse brain endothelial cells and rat astrocytes

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Abstract

The present study was designed to clarify the involvement of nitric oxide (NO) signaling in the adverse effect of cyclosporine on the blood–brain barrier. Cyclosporine increased the permeability of sodium-fluorescein and the cellular accumulation of rhodamine 123, a substrate of P-glycoprotein, in mouse brain endothelial (MBEC4) cells. This effect was markedly enhanced two- to threefold when MBEC4 cells were cocultured with rat astrocytes or C6 glioma cells. Direct and continuous electrochemical measurement of NO demonstrated that cyclosporine dose-dependently increased histamine- and phenylephrine-evoked NO production in MBEC4 cells and astrocytes, respectively. A NO synthase inhibitor (N^G-monomethyl-L-arginine) blocked slightly and markedly cyclosporine-induced impairment of the endothelial barrier in the monolayer and coculture system, respectively. These findings suggest that cyclosporine impairs the brain endothelial barrier function by accelerating NO production in the brain endothelial and astroglial cells. This event may be interpreted as triggering the occurrence of cyclosporine neurotoxicity.

Keywords: Cyclosporine; Neurotoxicity; NO (nitric oxide); Blood–brain barrier; Permeability; P-glycoprotein

1. Introduction

Cyclosporine, a cyclic 11-amino acid peptide, is widely used as a potent immunosuppressant to prevent allograft rejection in solid organ transplantation and in fatal graft-vs.-host disease after bone marrow transplantation, and to treat various autoimmune diseases including rheumatoid arthritis (Kahan, 1989). Despite its high efficacy, cyclosporine has adverse effects including renal dysfunction, cardiovascular disorders, gastrointestinal disorders and neurological complications. These events occur with a relatively high frequency (20–40%) in organ-transplanted patients (Gijtenbeek et al., 1999; Pirsch et al., 1997; U.S. Group, 1994).

The entry of cyclosporine into the brain is prevented by the tight junctions and P-glycoprotein, a multi-drug efflux pump, of the brain microvascular endothelial cells. But the adverse neurological effects of cyclosporine, including tremors, seizures and encephalopathy, strongly suggest the possibility of cyclosporine transport across the blood–brain

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barrier. We previously reported that cyclosporine produced convulsions by inhibiting γ-aminobutyric acid (GABA)-ergic neural activity and binding properties of the $GABA_A$ receptor (Shuto et al., 1999). The inhibition of GABAergic neurotransmission by cyclosporine may lead to an activation of serotonergic neural activity and consequently produce tremors (Shuto et al., 1998). These findings in vivo are considered to be due to a direct action of cyclosporine transported across the blood–brain barrier rather than an indirect effect through the periphery. In fact, we demonstrated that cyclosporine at a high concentration decreased the function and expression of P-glycoprotein in brain capillary endothelial cells (Kochi et al., 1999; 2000). The blood–brain barrier is primarily formed by brain capillary endothelial cells, which are closely sealed by tight junctions (Partridge, 1999). P-glycoprotein is abundantly expressed in the brain endothelial cells and limits the accumulation of many hydrophobic molecules and toxic substances in the brain (Schinkel, 1999). Recently, we demonstrated that nitric oxide (NO) increased the permeability and inhibited the P-glycoprotein efflux pump of brain capillary endothelial cells, suggesting that NO impairs the dynamic regulation of the blood–brain barrier function (Yamauchi et al., in press). Astrocytes and pericytes are cellular components of the blood–brain barrier. Astrocytes surround the cerebral capillaries and regulate blood–brain barrier function through cell-to-cell contact and secretion of soluble factors (Terasaki et al., 2003).

The present study was designed to clarify the involvement of NO signaling in the adverse effect of cyclosporine on the blood–brain barrier. We first evaluated the effect of cyclosporine on the permeability and the P-glycoprotein function of mouse brain endothelial (MBEC4) cells alone and cocultured with rat astrocytes or C6 glioma cells. Second, the effect of cyclosporine on the stimulation-evoked NO production was examined in MBEC4 cells and rat astrocytes using direct electrochemical NO monitoring.

2. Materials and methods

2.1. Materials

Cyclosporine was kindly supplied by Novartis Pharma (Bazel, Switzerland). Sodium fluorescein (Na-F, MW 376), rhodamine 123, phenylephrine hydrochloride, histamine, L-arginine and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma (St. Louis, MO, USA). Culture medium and subculture reagents were obtained from Invitrogen (Carlsbad, CA, USA). All remaining reagents of analytical grade were purchased from Wako (Osaka, Japan).

2.2. Animals

Wistar rats aged 3 days old were used in this study. 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.

2.3. Cell culture

MBEC4 cells, which were isolated from BALB/c mouse brain cortices and immortalized by SV40-transformation (Tatsuta et al., 1992), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. C6 glioma cells (JCRB9096, Health Science Research Resources Bank, Osaka, Japan) were cultured in DMEM supplemented with 10% fetal calf serum, and 50 μg/ml gentamicin. Newborn rat astrocytes were isolated according to the method of McCarthy and de Vellis (1980) and Sastradipura et al. (1998) with a slight modification. Briefly, the cerebral cortex from 3-day-old rats was minced and treated with papain (90 units/ml; Worthington, Lakewood, NJ) and DNase I (2000 units/ml; Sigma) at 37 °C for 15 min. The mechanically dissociated cells were seeded into plastic flasks in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. After 10–14 days in culture, floating cells and weakly attached cells on the mixed primary cultured cell layer were removed by vigorous shaking of the flask. Then, astrocytes on the bottom of the culture flask were trypsinized and seeded into new culture flasks. The primary cultured astrocytes were maintained in DMEM. They were grown in a humidified atmosphere of 5% CO_2/95% air at 37 °C.

The preparation of the in vitro blood–brain barrier models has been described previously (Dohgu et al., 2000). In brief, C6 cells or rat astrocytes (40,000 cells/cm^2) were first cultured on the outside of the collagen-coated polycarbonate membrane (3.0 μm pore size) of the Transwell™ insert (12-well type, Costar, MA, USA) 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 the 12-well culture plate (Costar) (C6 coculture and rat astrocyte coculture). The monolayer system was also made with MBEC4 cells alone (MBEC4 monolayer).

2.4. Treatment with cyclosporine and nitric oxide (NO) synthase inhibitor

Cyclosporine was first dissolved in ethanol and diluted with serum-free culture medium (0.1% as the final ethanol concentration). MBEC4 cells were cultured for 3 days, and these inserts were washed three times with serum-free medium. Then cells were exposed to 1–5 μM cyclosporine injected into the inside of the insert (luminal side) for 12 h. When the effect of NO synthase (NOS) inhibitor was examined, L-NMMA (1 mM) was loaded both inside and outside of the insert (luminal and abluminal side). In

parallel, cells were treated with serum-free medium containing the corresponding amount of ethanol as the vehicle.

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 was loaded on the luminal side of the insert. Samples (0.5 ml) were removed from the abluminal chamber at 10, 20, 30 and 60 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 using a fluorescence multiwell plate reader (Ex(λ) 485 nm; Em(λ) 530 nm) (CytoFluor Series 4000, PerSeptive Biosystems, Framingham, MA, USA). The permeability coefficient and clearance were calculated according to the method described by Dehouck et al. (1992). 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 60-min period of the experiment, the clearance volume increased linearly with time. The average volume cleared was plotted vs. 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 coculture system, the control membrane is the C6 cell- or rat astrocyte-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-glycoprotein

The functional activity of P-glycoprotein was determined by measuring the cellular accumulation of rhodamine 123 (Sigma) according to the method of Fontaine et al. (1996). 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 both coculture systems, C6 cells and rat astrocytes 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). The solution was neutralized with 1 M HCl (0.2 ml) and the rhodamine 123 content was determined using a fluorescence multiwell plate reader (Ex(λ) 485 nm; Em(λ) 530 nm, CytoFluor Series 4000). The cellular protein was measured by the method of Bradford (1976).

2.7. Electrochemical monitoring of NO

Direct and continuous electrochemical measurement of NO was performed with a three-electrode potentiostatic EMS-100 system (BIO-LOGIC, Grenoble, France) as previously described (Ikesue et al., 2000, Trevin et al., 1998). In brief, confluent MBEC4 cells or rat astrocytes in a 2.5 cm^2 dish (BD FALCON™, BD Biosciences, NJ, USA) were washed three times with Mg^{2+}-free Krebs–Ringer solution (143.0 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4 and 11.0 mM D-glucose, pH 7.4). The dish was placed on the stage of an inverted microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) mounted with a NO monitoring system. The NO-biosensor (ASTEC, Fukuoka, Japan) was positioned about 10 μm above the cell surface. Ten minutes after treatment with L-arginine (1 mM), histamine or phenylephrine in a volume of 10 μl was added to the cells in 1 ml of Mg^{2+}-free Krebs–Ringer solution with a transient mixing step to give the final concentration indicated. The level of production of NO in MBEC4 cells or rat astrocytes was monitored for a 15-min period after the addition of histamine or phenylephrine. Cyclosporine was added 20 min before treatment with L-arginine.

2.8. Assessment of cell viability

The effect of cyclosporine on the viability of cells in the MBEC4 monolayer, C6 coculture and rat astrocyte coculture systems was assessed using a WST-8 assay (Cell Counting Kit-8, DOJINDO, Kumamoto, Japan). A highly water-soluble formazan dye (WST-8), reduced by mitochondrial dehydrogenase, was measured by determining the absorbance of each sample with a 450 nm test wavelength and a 700 nm reference wavelength using a microplate reader (Opsys MR, DYNEX technologies, Chantilly, VA, USA).

2.9. Measurement of NO Production using NO-specific dye

An accumulation of NO production during a 12 h period was assessed using a NO-specific fluorescent dye, 4,5-diaminofluorescein diacetate (DAF-2 DA, Sigma) (Nakatsubo et al., 1998). MBEC4 and C6 cells were seeded on wells of the 24-well culture plate. Cells were