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Original Paper

A_{2a} Adenosine Receptor Mediates PKA-Dependent Glutamate Release from Synaptic-like Vesicles and Ca^{2+} Efflux from an IP_3- and Ryanodine-Insensitive Intracellular Calcium Store in Astrocytes

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Key Words
A_{2a} adenosine receptor • Protein kinase A • Glutamate release • Synaptic-like vesicles • Intracellular Ca^{2+} rise

Abstract
Background/Aims: The mechanism underlying transmitter release from astrocytes is not fully understood. The present study examined A_{2a} adenosine receptor-mediated glutamate release and intracellular Ca^{2+} rise in cultured rat hippocampal astrocytes. ***Methods:*** Intracellular amino acids were measured with HPLC. Glutamate release from astrocytes and intracellular Ca^{2+} mobilizations were monitored in the NADH imaging, FM1-43 imaging, and fura-2 imaging. The siRNA to silence the A_{2a} adenosine receptor-targeted gene was constructed and transfected into cells. ***Results:*** Glutamate was condensed in 'synaptic-like vesicle' fractions. In the NADH imaging, CGS21680, an agonist of A_{2a} adenosine receptors, increased NADH fluorescent signals, that reflects glutamate release, and the effect was inhibited by DMPX, an inhibitor of A_{2a} adenosine receptors, H-89, a PKA inhibitor, vesicular transport inhibitors, or botulinum toxin-A, an exocytosis inhibitor. In the FM1-43 imaging to see vesicular recycling, CGS21680 decreased FM1-43 fluorescent signals, that was also prevented by DMPX, H-89, vesicular transport inhibitors, or botulinum toxin-A. CGS21680 increased intracellular Ca^{2+} concentrations both in Ca^{2+}-containing and -free extracellular solution. The Ca^{2+} rise was inhibited by DMPX, H-89, or the vesicular transport inhibitor brefeldin A, but it was not affected by inhibitors for phospholipase C, IP_3 receptor, and ryanodine receptor. CGS21680-induced glutamate release and intracellular Ca^{2+} rise were prevented by knocking-down A_{2a} adenosine receptor. ***Conclusion:*** The results of the present study show that A_{2a} adenosine receptor/PKA promotes glutamate release from synaptic-like vesicles and stimulates Ca^{2+} efflux from an IP_3- and ryanodine-insensitive intracellular calcium store.

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Introduction

Astrocytes release a variety of transmitters in response to transmitters, mechanical stimulation, or depolarization [1], and participate in tripartite synaptic transmission [2-5]. Transmitters released from neurons activate the receptors expressed not only in neurons but astrocytes by being spilt out from the synaptic cleft. Then, some of the activated receptors may cause an intracellular Ca^{2+} rise, which triggers transmitter release from astrocytes, to modulate neuronal activity.

Several avenues of evidence have shown that glutamate is released from astrocytes by Ca^{2+}-dependent vesicular exocytosis, reverse transport by glutamate transporters, and anion transporters [6-8]. Glutamate release from astrocytes is inhibited by cleaving synaptic vesicular proteins, which are expressed in astrocytes too [9-16]. An intracellular Ca^{2+} rise in astrocytes, mainly mediated via G_q protein-coupled receptors, is linked to vesicular transmitter release from astrocytes [17-20]. Amazingly, astrocytes express voltage-dependent calcium channels (VDCCs) [21, 22], and depolarization causes vesicular glutamate release from astrocytes by activating VDCCs and increasing intracellular Ca^{2+} concentrations [23]. Calcium elevation in whole astrocytic cells may not affect neuronal activity [24, 25]. Local calcium dynamics in astrocytic processes, however, is shown to modulate local synaptic function through GTP- and inositol-1,4,5-trisphosphate (IP_3)-dependent signaling [26]. Under the ischemic conditions, on the other hand, glutamate is counter-transported from inside to outside cells by glutamate transporters, independently of Ca^{2+} and vesicular exocytosis [27-29].

In our earlier study, a low concentration (10 nM) of adenosine potentiated hippocampal neuronal activity, that was abolished by 3,7-dimethyl-1-propargylxanthine (DMPX), a relative specific antagonist of A_{2a} adenosine receptor, or H-89, an inhibitor of protein kinase A (PKA), where adenosine stimulated glutamate release from astrocytes in an A_{2a} adenosine receptor- and PKA-dependent manner, while adenosine did not affect presynaptic glutamate release or responses of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors [30]. This indicates that adenosine modulates hippocampal neuronal activity by stimulating glial glutamate release via A_{2a} adenosine receptors linked to G_s protein involving PKA activation. In support of this idea, 5'-*N*-ethylcarboxyladenosine hydrochloride (CGS21680), an agonist of A_{2a} adenosine receptor, but not cyclohexyladenosine (CHA), an agonist of A_1 adenosine receptor, stimulated glutamate release from astrocytes [31]. Moreover, *N*-(4-acetyl-1-piperazinyl)-*p*-fluorobenzamide monohydrate (FK960) enhanced intracellular Ca^{2+} concentrations and stimulated glutamate release from hippocampal astrocytes via a PKA pathway, where the glutamate release was inhibited by the vesicular transport inhibitor bafilomycin A1 [32]. The cAMP analogue, dibutyryl cAMP, alternatively, stimulates glutamate release from astrocytes [33]. Taken together, these findings suggest that PKA is a critical messenger in the regulation of tripartite synaptic transmission.

To gain further insight into the A_{2a} adenosine receptor-mediated glutamate release from astrocytes, we measured glutamate concentrations in each fraction separated according to sucrose density gradients, monitored glutamate mobilizations by detecting NADH fluorescent signals with an NAD^+/NADH enzymatic reaction, assayed vesicular recycling with an FM1-43 dye, and assayed intracellular Ca^{2+} mobilizations in cultured rat hippocampal astrocytes. We show here that A_{2a} adenosine receptor/PKA controls glutamate release from synaptic-like vesicles and intracellular Ca^{2+} mobilizations in astrocytes.

Materials and Methods

Animal care

All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture

The hippocampus was isolated from the brain of embryonic Wistar rats (gestational age 20 days) under ether anesthesia, and dissociated cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum, 2.5 mM glutamine, 50 μM glutamate, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After 3 passages, cells were seeded on poly-L-lysine-coated 6-well culture dishes at a density of 1.2 x 10^6 cells/plate, and grown until being confluent. More than 95% of the cells were positive to glial fibrillary acidic protein (GFAP), an astrocytic marker.

Fractionation according to sucrose density gradients

Cultured rat hippocampal astrocytes were lysed in a HEPES buffer (pH 7.5) containing 50 mM sucrose and 1% (v/v) protease inhibitor cocktail. Cell lysates were centrifuged at 800 x g for 10 min, and the supernatant was centrifuged at 10,000 x g for 30 min. Then, the supernatant was layered onto a stepwise gradient from 100 mM to 800 mM sucrose in a centrifuge tube followed by 65,000 x g for 5 h, and then 11 fractions (0.5 ml) were collected from the top to the bottom of the centrifuge tube and numbered from 1 to 11.

Assay of amino acids

Each fractionated sample labeled with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was injected onto the column (TSKgel ODS-80TM, TOSOH Co., Tokyo, Japan) and loaded on a high performance liquid chromatography (HPLC) system (LC-10ATvp; Shimadzu Co., Kyoto, Japan)[23]. Concentrations of each amino acid were calculated from the peak area/ concentration calibration curve made using a standard solution for each amino acid.

Western blotting

Each fractionated sample was loaded on 12% (w/v) sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with Tris-buffered salt solution (TBS-T)[150 mM NaCl, 0.05% (v/v) Tween20, and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently reacted with antibodies against synaptophysin (Chemicon, Temecula, CA, USA), VGLUT1 (Chemicon), VGLUT2 (Chemicon), and VGLUT3 (Chemicon), followed by a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody. In a different set of experiments, Western blotting was carried out in cultured rat hippocampal astrocytes transfected with the NC siRNA or the A_{2a}R siRNA using an anti-A_{2a} adenosine receptor antibody (Oncogene, San Diego, CA, USA). For SNARE proteins, Western blotting was carried out using antibodies against SNAP-25 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), syntaxin-1 (Santa Cruz), synaptobrevin-2 (Chemicon), and β-actin (Sigma, San Diego, MO, USA) in lysates from the rat intact whole brain and cultured rat hippocampal astrocytes. Immunoreactivity was detected with an ECL kit (GE Healthcare, NJ, USA) and visualized using a chemiluminescene detection system (FUJIFILM, Tokyo, Japan).

NADH imaging

Glutamate released from cultured rat hippocampal astrocytes was visualized by detecting NADH fluorescent signals [23]. Cells were incubated with L-glutamic dehydrogenase (GDH)(20 U/ml) and β-nicotinamide adenine dinucleotide (NAD^+)(1 mM). In the presence of glutamate, GDH reduces NAD^+ to NADH, a product that fluoresces when excited with an ultraviolet (UV) light, and therefore, extracellularly released glutamate can be detected as an increase in NADH fluorescent signals. NADH was excited with an UV laser of 365 nm, and the emitted fluorescent signal was detected by a digital camera (ORCA-ER, Hamamatsu Photonics, Inc., Japan) through a cut-off filter (cut-off below 400 nm). Data were expressed as percentage of basal NADH fluorescent signals of the optical field before stimulation.

FM1-43 imaging

Vesicular recycling in cultured rat hippocampal astrocytes was visualized with an FM1-43 dye [23]. Cells were loaded with FM1-43 (8 μM) for 90 s followed by extracellular high K^+ (70 mM) for 90 s at room temperature. Then, cells were washed with the standard extracellular solution containing β-cyclodextrin

(ADVASEP-7)(1 mM). Cells were placed into a recording chamber onto the stage of a Nikon DIAPHOTO 300 microscope (Nikon, Tokyo, Japan). FM1-43 was excited with an UV laser of 470 nm and the fluorescent signal was filtered through a band-pass filter transmitting 505-520 nm, and detected by a digital camera (ORCA-ER, Hamamatsu Photonics, Inc., Japan). Data were expressed as percentage of basal FM1-43 fluorescent signals before stimulation.

Monitoring of intracellular free Ca^{2+} mobilizations

Cultured rat hippocampal astrocytes were incubated with fura-2/AM (4 μM) at 37 °C for 1 h in the standard extracellular solution containing 0.02% (v/v) of the cell-permeant AM esters pluorinic F-127. Fura-2-loaded cells were placed into a recording chamber onto the stage of a Nikon DIAPHOTO 300 microscope and bathed in the standard Ca^{2+}-containing or -free extracellular solution. Cells were viewed using a fluorescence 20x dry objective lens and a 20x dry phase-contrast objective. Fura-2 was excited at 340 and 380 nm alternatively switched every 250 ms. The fluorescent signal was filtered through a bandpass filter transmitting 500-511nm and detected by a digital camera (ORCA-ER, Hamamatsu Photonics, Inc., Japan). Ratio images were calculated in real time, stored on hard disk, and analyzed using an AQUACOSMOS software (Version 2.0, Hamamatsu Photonics, Inc., Japan).

Construction of siRNA

The siRNA to silence A_{2a} adenosine receptor-targeted gene (A_{2a}R siRNA) and the negative control siRNA (NC siRNA) were obtained from Ambion (Austin, TX, USA). The sequences of siRNA used to silence the rat A_{2a} receptor gene were 5'-GACGGGAACUCCACGAAGATT-3' and 5'-UCUUCGUGGAGUUCCCGUCTT-3'. The non-specific siRNA with the scrambled sequence, the same GC content and nucleic acid composition was used as a negative control (NC siRNA).

The A_{2a} siRNA and the NC siRNA were transfected into cultured rat hippocampal astrocytes using a Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were used for experiments 48 h later after transfection.

In situ PKA assay

PKA activity in cultured rat hippocampal astrocytes was assayed by the method described previously [34]. Astrocytes were treated with CGS21680 (1 μM) in the presence and absence of DMPX (1 μM) or H-89 (1 μM) at 37 °C for 5 min in an extracellular solution [137 mM NaCl, 5.4 mM KCl, 10 mM $MgCl_2$, 5 mM ethylene glycol tetraacetic acid (EGTA), 0.3 mM Na_2HPO_4, 0.4 mM K_2HPO_4, and 20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethansulfonic acid (HEPES), pH 7.2]. Then, cells were added with the extracellular solution containing 100 μg/ml digitonin, 50 mM glycerol 2-phosphate, 400 μM ATP, and 200 μM Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly; MW, 771.9), a synthetic substrate peptide for PKA (Calbiochem, San Diego, CA, USA), and incubated at 30 °C for 5 min. The supernatants were collected and boiled at 100 °C for 5 min to terminate the reaction. An aliquot of the solution (20 μl) was loaded onto the reverse-phase HPLC system (LC-10ATvp; Shimadzu Co.). A substrate peptide peak and a new product peak were detected at an absorbance of 214 nm (SPD-10Avp UV-VIS detector; Shimadzu). It was confirmed that each peak corresponds to nonphosphorylated and phosphorylated substrate peptide in an analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Voyager DE-STR; PE Biosystems, Inc., Foster City, CA). Molecular weights were calibrated from the two standard spectrums, bradykinin (MW, 1,060.2) and neurotensin (MW, 1,672.9). Areas for nonphosphorylated and phosphorylated PKA substrate peptide were measured (total area corresponds to the concentration of PKA substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/min/cell protein weight) was used as an index of PKA activity.

Statistical analysis

Statistical analysis was carried out using analysis of variance (ANOVA), unpaired *t*-test, and Dunnett's test.

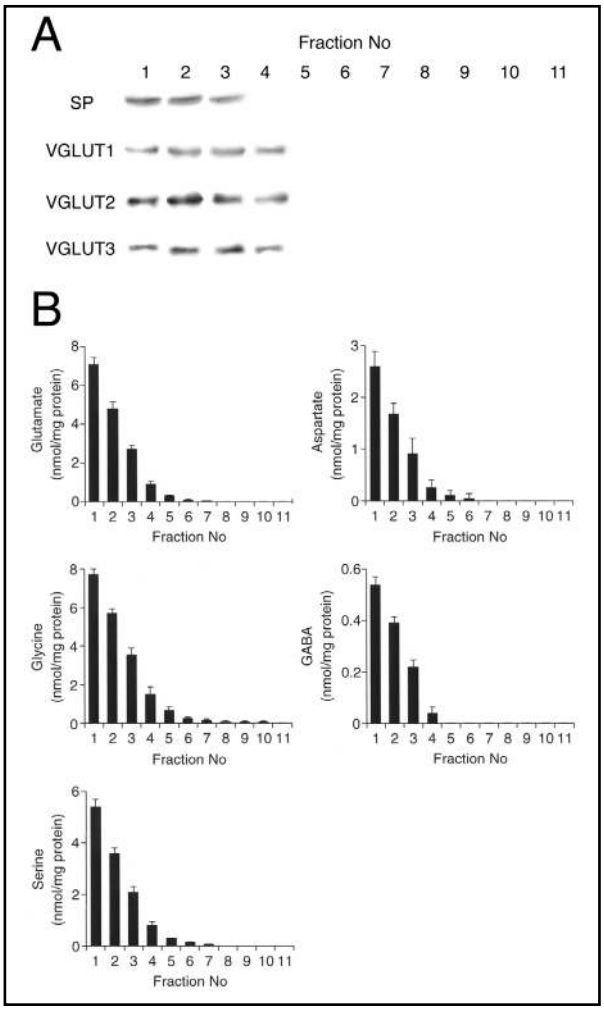

Fig. 1. Intracellular distribution of amino acids in cultured rat hippocampal astrocytes. Cells were separated into 11 fractions according to sucrose density gradients. (A) Western blotting using antibodies against synaptophysin (SP), VGLUT1, VGLUT2, and VGLUT3 in each fraction. Note that a similar result was obtained with 3 independent experiments. (B) The amount of glutamate, glycine, serine, aspartate, and GABA in each fraction was assayed with an HPLC. In the graphs, each column represents the mean (± SEM) concentration (n=4 independent experiments).

Results

Glutamate is condensed in sucrose density fractions containing synaptic-like vesicles

Synaptophysin and vesicular glutamate transporters such as VGLUT1, VGLUT2, and VGLUT3 can be used as a synaptic vesicle marker. Cultured rat hippocampal astrocytes were separated into 11 fractions according to sucrose density gradients and Western blotting was carried out using antibodies against synaptophysin, VGLUT1, VGLUT2, and VGLUT3. Immunoreactive signals for synaptophysin were detected in fraction number 1-3 and the signals for VGLUT1, VGLUT2, and VGLUT3 were in fraction number 1-4 (Fig. 1A). This, in the light of the fact that more than 95% of cultured cells examined here were GFAP-positive cells, i.e., astrocytes, indicates that astrocytes express 'synaptic-like' vesicles containing glutamate, with the restricted localization in fraction number 1-4, no matter how much neuronal material reactive to an anti-synaptophysin antibody is contained in the cultured material.

We subsequently assayed the amount of amino acids such as glutamate, aspartate, glycine, γ-aminobutyric acid (GABA), and serine in each fraction. Higher concentrations of amino acids were consistently found with fraction number 1-4, with the highest concentration in fraction number 1; 7-8 nmol/mg protein for glutamate and glycine, 5 nmol/mg protein for serine, 3 nmol/mg protein for aspartate, and 0.5 nmol/mg protein for GABA, followed by a step-wise decrease (Fig. 1B). This suggests that glutamate is condensed in synaptic-like vesicles. The result also suggests that synaptic-like vesicles contain not only glutamate but glycine, serine, aspartate, and GABA.

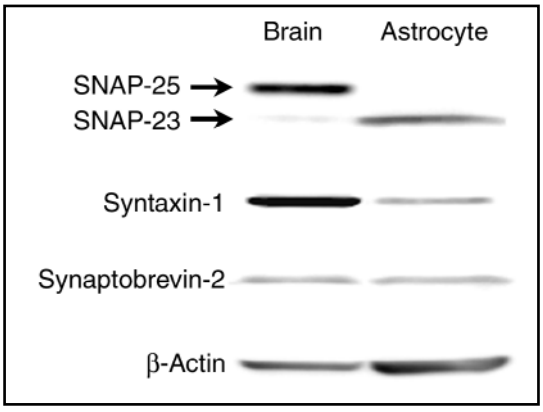

Fig. 2. Expression of SNARE proteins in cultured rat hippocampal astrocytes. Western blotting was carried out using antibodies against SNAP-25, syntaxin-1, and synaptobrevin-2 in lysates from the rat intact whole brain (Brain) and cultured rat hippocampal astrocytes (Astrocyte). Note that a similar result was obtained with 3 independent experiments.

Astrocytes express SNARE proteins

We next examined expression of SNARE proteins relevant to synaptic vesicle exocytosis in cultured rat hippocampal astrocytes. In the Western blot analysis, the immunoreactive signals for SNAP-25, syntaxin-1, and synaptobrevin-2 were detected at 25, 35, and 15 kDa, respectively (Fig. 2), indicating that neurons actually express these SNARE proteins. For cultured astrocytes, expression of syntaxin-1 and synaprobrevin-2 were confirmed at the same molecular weight as for the brain, but an anti-SNAP-25 antibody produced immunoreactive signal at 23 kDa, which is consistent with SNAP-23 (Fig. 2). This, in the light of the facts that astrocytes express SNAP-23 instead of SNAP-25, still interacting with syntaxin-1 and synaprobrevin-2 [11, 35], suggests that SNARE proteins engineer transmitter release from synaptic-like vesicles for astrocytes in a fashion similar to for neurons.

A_{2a} adenosine receptor mediates glutamate release from synaptic-like vesicles in astrocytes

We monitored real-time glutamate release from cultured rat hippocampal astrocytes by detecting NADH fluorescent signals. CGS21680 (1 μM), an agonist of A_{2a} adenosine receptor, increased NADH fluorescent signals (Fig. 3B), while the A_1 adenosine receptor agonist CHA (10 μM)(Fig. 3A), the A_{2b} adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA)(10 μM)(Fig. 3C), or the A_3 adenosine receptor agonist 2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-*N*-methyl-uronamide (2-Cl-IB-MECA)(50 μM)(Fig. 3D) had no effect on the signals. This indicates that A_{2a} adenosine receptor mediates glutamate release from astrocytes.

CGS21680 (1 μM)-induced increase in NADH fluorescent signals was abolished by DMPX (1 μM), an inhibitor of A_{2a} adenosine receptor (P<0.0001 as compared with that in the absence of DMPX, ANOVA) (Fig. 4A), suggesting glutamate release from astrocytes as mediated via A_{2a} adenosine receptors. The CGS21680 effect, alternatively, was inhibited by H-89 (1 μM), an inhibitor of PKA (P<0.0001 as compared with that in the absence of H-89, ANOVA)(Fig. 4B). This, in the light of the fact that A_{2a} adenosine receptors are linked to G_s protein involving PKA activation, accounts for the implication of A_{2a} adenosine receptor/PKA in glutamate release from astrocytes.

CGS21680-induced increase in NADH fluorescent signals was significantly prevented by the vesicular transport inhibitors, brefeldin A (60 nM)(P<0.0001 as compared with that in the absence of brefeldin A, ANOVA)(Fig. 4C), bafilomycin A1 (4 μM)(P<0.0001 as compared with that in the absence of bafilomycin A1, ANOVA)(Fig. 4D), latrunculin B (10 μM)(P<0.0001 as compared with that in the absence of latrunculin B, ANOVA)(Fig. 4E), and jasplakinolide (300 nM) (P<0.0001 as compared with that in the absence of jasplakinolide, ANOVA)(Fig. 4F). For cells pretreated with botulinum toxin-A (BoTX-A)(0.1 U/ml) for 24 h, CGS21680 (1 μM) caused less increase in NADH signals (Fig. 4G). BoTX-A degrades SNAP-25, thereby preventing vesicular exocytosis of transmitters. Moreover, BoTX-A is shown to degrade SNAP-23 too in non-neuronal tissues [36]. Collectively, these results indicate that CGS21680 stimulates glutamate release from astrocytes by vesicular exocytosis.

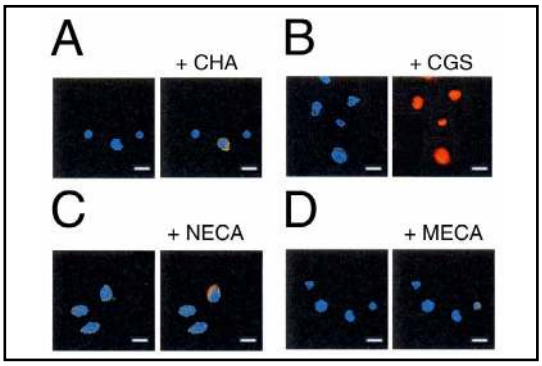

Fig. 3. NADH imaging analysis in cultured rat hippocampal astrocytes. Glutamate release from cells was visualized by detecting NADH fluorescent signals before and after bath-application with CHA (10 μM)(A), CGS21680 (CGS)(1 μM)(B), NECA (10 μM)(C), or 2-Cl-IB-MECA (MECA)(50 μM)(D). Images before and 90 s after bath-application with agonists are shown. Bars in images, 10 μm. Note that similar results were obtained from 8 independent cells.

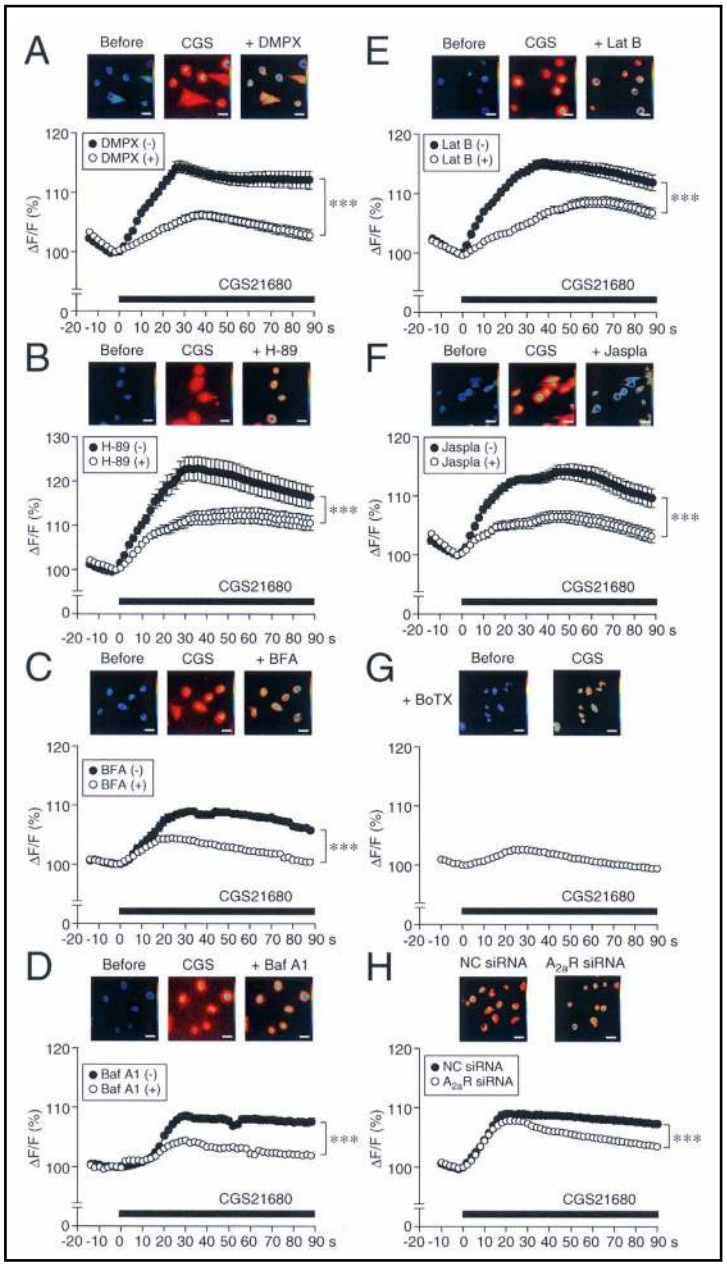

Fig. 4. CGS21680-induced glutamate release from cultured rat hippocampal astrocytes. Glutamate release from cells was visualized by detecting NADH fluorescent signals before and after bath-application with CGS21680 (1 μM) in presence and absence of DMPX (1 μM) (n=31 independent cells) (A), H-89 (1 μM) (n=31 independent cells) (B), brefeldin A (BFA)(60 nM)(n=26 independent cells) (C), bafilomycin A1 (Baf A1)(4 μM)(n=26 independent cells) (D), latrunculin B (Lat B)(10 μM)(n=16 independent cells) (E), or jasplakinolide (Jaspla) (300 nM)(n=16 independent cells) (F). Cells were treated with BoTX-A (0.1 U/ml) 24 h prior to NADH imaging assay (n=31 independent cells) (G). NADH imaging assay was carried out in cells transfected with the NC siRNA (n=50 independent cells) or the A_{2a}R siRNA (n=50 independent cells) (H). Bars in images, 10 μm. In the graphs, each point represents the mean (± SEM) percentage of basal NADH fluorescent signals (0 s). ***P<0.0001, ANOVA.

To obtain further evidence for A_{2a} adenosine receptor/PKA-dependent glutamate release from astrocytes, we constructed the A_{2a}R siRNA. In the Western blot analysis, signal intensities

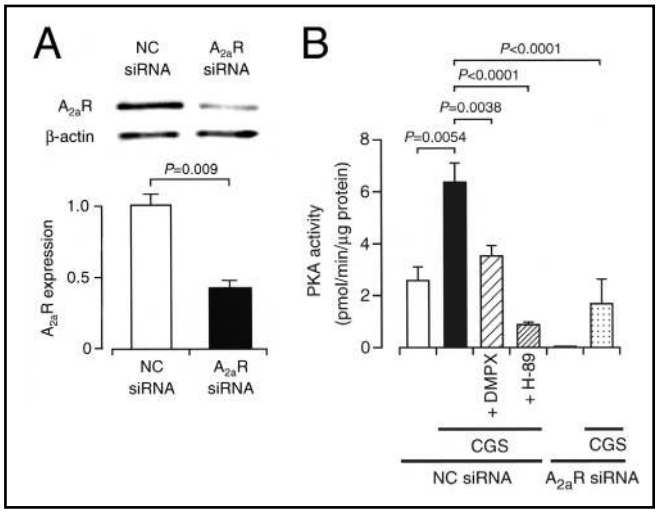

Fig. 5. Knocking-down of A_{2a} adenosine receptor. Cultured rat hippocampal astrocytes were transfected with the NC siRNA or the A_{2a}R siRNA. (A) Western blotting using an anti-A_{2a} adenosine receptor antibody was carried out 48 h after transfection. Signal intensities for A_{2a} adenosine receptor were normalized by those for β-actin, and the ratio of normalized signal intensities for A_{2a} adenosine receptor in cells transfected with the A_{2a}R siRNA against those in cells transfected with the NC siRNA was calculated. In the graph, each column represents the mean (± SEM) A_{2a} adenosine receptor expression ratio (n=4 independent experiments). *P* value, unpaired *t*-test. (B) *In situ* PKA assay was carried out in cells transfected with the NC siRNA or the A_{2a}R siRNA before and after treatment with CGS21680 (1 μM) (CGS) in the presence and absence of DMPX (1 μM) or H-89 (1 μM). In the graph, each column represents the mean (± SEM) PKA activity (pmol/min/μg protein)(n=6 independent experiments). *P* values, Dunnett's test.

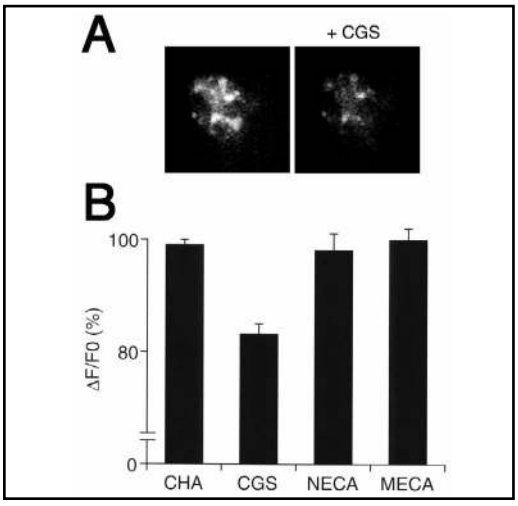

Fig. 6. FM1-43 dye release from cultured rat hippocampal astrocytes. (A) FM1-43 was taken up into the vesicle membrane in cells and FM1-43 fluorescent signals were monitored in individual vesicles in each astrocyte. FM1-43 fluorescent images before and 5 min after bath-application with CGS21680 (1 μM) (+ CGS). Note that FM1-43 fluorescent intensities in individual vesicles are decreased after bath-application with CGS21680. (B) In the graph, each column represents the mean (± SEM) percentage of basal FM1-43 fluorescent signals 5 min after application with CHA (10 μM), CGS21680 (CGS) (1 μM), NECA (10 μM), or 2-Cl-IB-MECA (MECA) (50 μM) (n=8 independent cells).

for A_{2a} adenosine receptor were normalized by those for β-actin, and the ratio of normalized signal intensities for A_{2a} adenosine receptor in cells transfected with the A_{2a}R siRNA against those in cells transfected with the NC siRNA was calculated. For cultured rat hippocampal astrocytes transfected with the A_{2a}R siRNA, expression of A_{2a} adenosine receptor protein was clearly suppressed as compared with the expression for cells transfected with the NC siRNA (Fig. 5A), confirming A_{2a} adenosine receptor knock-down. A_{2a} adenosine receptor is linked to G_s protein involving activation of adenylate cyclase followed by cAMP production and PKA activation. In the *in situ* PKA assay, CGS21680 (1 μM) activated PKA in cultured rat hippocampal astrocytes transfected with the NC siRNA, and the CGS21680-induced PKA activation was prevented by DMPX (1 μM) or H-89 (1 μM)(Fig. 5B). For cells transfected with A_{2a}R siRNA, PKA was little activated under the basal conditions and CGS21680 (1 μM)-induced PKA activation was significantly suppressed as compared with the activation for cells transfected with the NC siRNA (Fig. 5B). This provides evidence that A_{2a} adenosine receptor engages PKA activation in astrocytes.

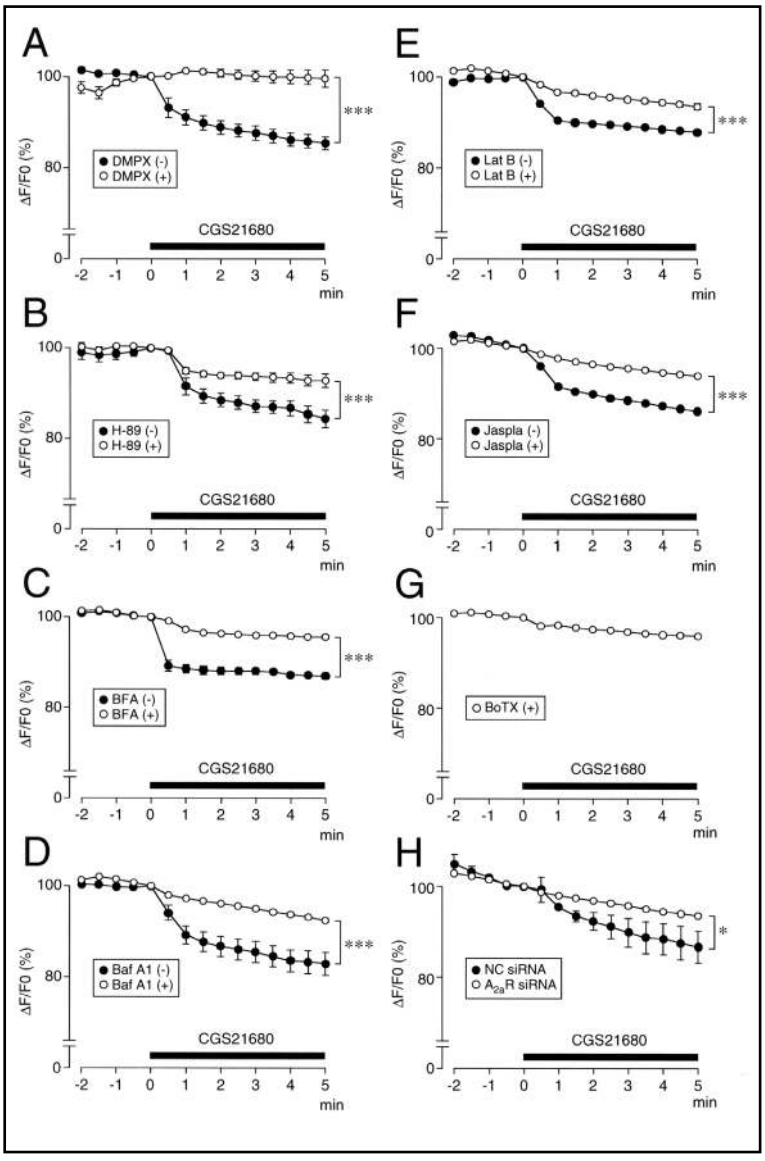

Fig. 7. FM1-43 imaging analysis in cultured rat hippocampal astrocytes. FM1-43 was taken up into the vesicle membrane in cells and FM1-43 fluorescent signals were detected before and after bath-application with CGS21680 (1 μM) in presence and absence of DMPX (1 μM)(n=12 independent cells) (A), H-89 (1 μM)(n=9 independent cells) (B), brefeldin A (BFA)(60 nM) (n=25 independent cells) (C), bafilomycin A1 (Baf A1)(4 μM)(n=19 independent cells) (D), latrunculin B (Lat B)(10 μM)(n=28 independent cells) (E), or jasplakinolide (Jaspla) (300 nM)(n=40 independent cells) (F). Cells were treated with BoTX-A (0.1 U/ml) 24 h prior to FM1-43 imaging assay (n=38 independent cells) (G). FM1-43 imaging assay was carried out in cells transfected with the NC siRNA (n=32 independent cells) or the A_{2a}R siRNA (n=32 independent cells) (H). In the graphs, each point represents the mean (± SEM) percentage of basal FM1-43 fluorescent signals (0 s). ***P<0.0001; *P=0.040, ANOVA.

For astrocytes transfected with the A_{2a}R siRNA, no significant increase in NADH signals was obtained with CGS21680 (1 μM)(P<0.0001 as compared with the CGS21680 effect for cells transfected with the NC siRNA, ANOVA)(Fig. 4H). This further supports the note for glutamate release from synaptic-like vesicles in astrocytes through an A_{2a} adenosine receptor/PKA signaling pathway.

A_{2a} adenosine receptor stimulates FM1-43 dye release from astrocytes

To obtain further evidence for A_{2a} adenosine receptor-mediated glutamate release from synaptic-like vesicles in astrocytes, we carried out FM1-43 imaging assay. In this assay, destaining of fluorescent signals for FM1-43, taken up into astrocytes, reflects transmitter release from astrocytes. We monitored FM1-43 dye release from individual puncta in each astrocyte. CGS21680 (1 μM) decreased FM1-43 fluorescent signals, while no effect was obtained with CHA (10 μM), NECA (10 μM), or 2-Cl-IB-MECA (50 μM) (Fig. 6A,B).

CGS21680 (1 μM)-induced decrease in FM1-43 fluorescent signals was inhibited by DMPX (1 μM)(P<0.0001 as compared with that in the absence of DMPX, ANOVA)(Fig. 7A) or H-89 (1 μM)(P<0.0001 as compared with that in the absence of H-89, ANOVA)(Fig. 7B). CGS21680-induced decrease in FM1-43 fluorescent signals was significantly prevented

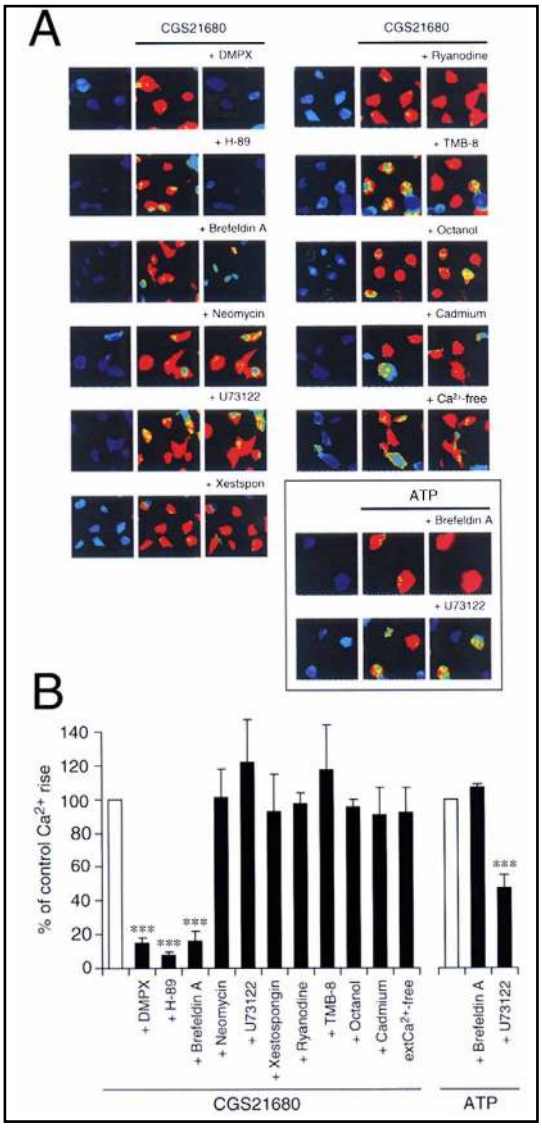

Fig. 8. CGS21680-regulated intracellular Ca^{2+} mobilizations in cultured rat hippocampal astrocytes. (A) Intracellular Ca^{2+} mobilizations were monitored before and after bath-application with CGS21680 (1 μM) in the absence and presence of DMPX (1 μM), H-89 (1 μM), brefeldin A (60 nM), neomycin (500 μM), U73122 (10 μM), xestospongin (50 μM), ryanodine (40 μM), TMB-8 (1 mM), octanol (1 mM), or cadmium (200 μM) in a Ca^{2+}-containing and -free extracellular solution. In a different set of experiments, intracellular Ca^{2+} mobilizations were assayed before and after bath-application with ATP (10 μM) in the absence and presence of brefeldin A (60 nM) or U73122 (10 μM). Illustrated Ca^{2+} imagings were monitored before and after bath-application with CGS21680 or ATP for 10 s in the absence and presence of inhibitors. Note that a series of assays was carried out using same cells. (B) In the graph, each column represents the mean (± SEM) percentage of control Ca^{2+} rise after bath-application with CGS21680 or ATP in the absence of inhibitors for 10 s (n=6 independent experiments). ***P<0.0001, Dunnett's test.

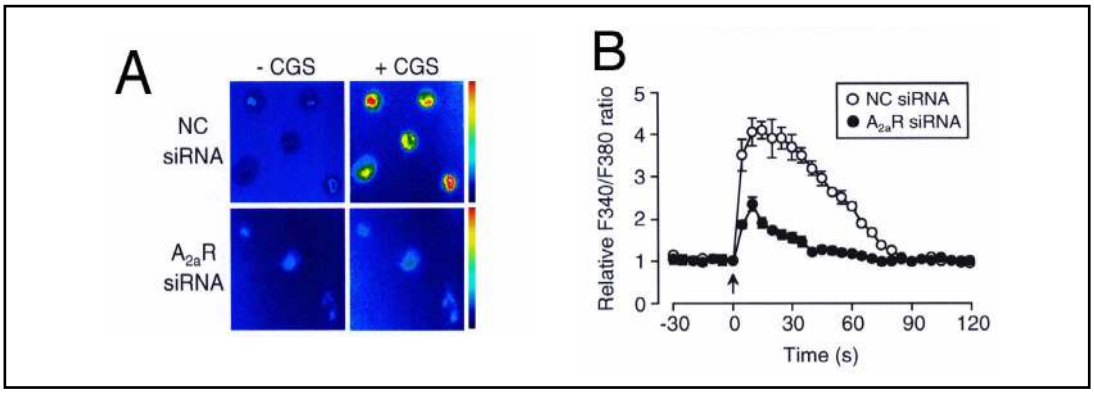

Fig. 9. A_{2a} adenosine receptor-dependent Ca^{2+} rise in cultured rat hippocampal astrocytes. (A) Intracellular Ca^{2+} mobilizations were monitored before and after bath-application with CGS21680 (1 μM) in cells transfected with the NC siRNA or the A_{2a}R siRNA. Illustrated Ca^{2+} imagings were monitored before and after bath-application with CGS21680 for 10 s. (B) In the graph, each point represents the mean (± SEM) relative F340/F380 ratio against basal untreated ratios (n=4 independent experiments). An arrow indicates the start of CGS21680 application.

by brefeldin A (60 nM)(P<0.0001 as compared with that in the absence of brefeldin A, ANOVA)(Fig. 7C), bafilomycin A1 (4 μM)(P<0.0001 as compared with that in the absence of bafilomycin A1, ANOVA)(Fig. 7D), latrunculin B (10 μM)(P<0.0001 as compared with that in the absence of latrunculin B, ANOVA)(Fig. 7E), and jasplakinolide (300 nM)(P<0.0001 as compared with that in the absence of jasplakinolide, ANOVA)(Fig. 7F). The CGS21680 effect was not found for cells pretreated with BoTX-A (0.1 U/ml) for 24 h (Fig. 7G) or transfected with the A_{2a}R siRNA (Fig. 7H). These results, taken together with the results of NADH monitoring, indicate that A_{2a} adenosine receptor mediates glutamate release from synaptic-like vesicles in astrocytes in a PKA-dependent manner.

A_{2a} adenosine receptor increases intracellular Ca^{2+} concentrations by calcium efflux from an IP_3- and ryanodine-insensitive intracellular calcium stores

We finally examined whether A_{2a} adenosine receptor regulates intracellular Ca^{2+} mobilizations in cultured rat hippocampal astrocytes. Ca^{2+} levels were quantified by fura-2 imaging. CGS21680 (1 μM) increased intracellular Ca^{2+} concentrations in Ca^{2+}-containing extracellular solution, and the effect was still found in Ca^{2+}-free extracellular solution (Fig. 8A,B). This interprets that CGS21680 stimulates Ca^{2+} efflux through an intracellular calcium store. CGS21680-induced intracellular Ca^{2+} rise was abolished by DMPX (1 μM) or H-89 (1 μM)(Fig. 8A,B), indicating that A_{2a} adenosine receptor mediates Ca^{2+} efflux from intracellular stores in a PKA-dependent manner. The Ca^{2+} rise for cells transfected with the A_{2a}R siRNA was significantly suppressed as compared with that for cells transfected with the NC siRNA (Fig. 9A,B). This indicates that PKA-dependent increase in intracellular Ca^{2+} concentrations is mediated via A_{2a} adenosine receptor.

Intriguingly, the Ca^{2+} rise was not inhibited by the phospholipase C inhibitors, neomycin (500 μM) and U73122 (10 μM), the IP_3 receptor inhibitor, xestospongin (50 μM) (Fig. 8A,B), excluding Ca^{2+} efflux through IP_3 receptors expressed on the ER. Astrocytes express P2Y receptor linked to G_q protein involving phospholipase C activation to hydrolyze phosphatidylinositol into diacylglycerol and IP_3 [37]. ATP (10 μM) increased intracellular Ca^{2+} concentrations, and the effect was inhibited by U73122 (10 μM)(Fig. 8A,B). This implies that unlike CGS21680 ATP increases intracellular calcium concentrations by Ca^{2+} efflux through IP_3 receptors following P2Y receptor activation. In addition, CGS21680-induced intracellular Ca^{2+} rise was not inhibited by the ER calcium release blocker, TMB-8 (1 mM) (Fig. 8A,B). Collectively, these results indicate that IP_3 receptor on the ER is not a target for A_{2a} adenosine receptor-regulated intracellular Ca^{2+} mobilizations in astrocytes.

The effect of CGS21680 on Ca^{2+} rise was not affected by the ryanodine receptor inhibitor, ryanodine (40 μM)(Fig. 8A,B), indicating that A_{2a} adenosine receptor does not regulate Ca^{2+} efflux through ryanodine receptors. Moreover, the CGS21680 effect was not inhibited by the gap-junction inhibitor, octanol (1 mM), or the non-selective calcium channel blocker, cadmium (200 μM)(Fig. 8A,B), ruling out the implication of gap-junctions or plasma membrane calcium channels in the intracellular Ca^{2+} rise.

Surprisingly, CGS21680-induced intracellular Ca^{2+} rise was significantly prevented by the vesicular transport inhibitor, brefeldin A (60 nM), although brefeldin A had no effect on ATP-induced intracellular Ca^{2+} rise (Fig. 8A,B). This might account for Ca^{2+} efflux from vesicular calcium stores under the control of A_{2a} adenosine receptor/PKA.

A_{2a} adenosine receptor thus appears to increase intracellular Ca^{2+} concentrations by calcium efflux from an IP_3- and ryanodine-insensitive intracellular calcium stores in astrocytes.

Discussion

Several avenues of evidence have pointed to glutamate release from astrocytes through three distinct pathways; Ca^{2+}-dependent vesicular exocytosis, reverse transport by glutamate transporters, and anion transporter-mediated release [6-8]. In support of

vesicular glutamate release from astrocytes, astrocytes express synaptic vesicular proteins such as synaptophysin, synaptobrevin-2, cellubrevin, syntaxin-1A, SNAP-23, munc-18-1, and complexin 2 or vesicular glutamate transporters such as VGLUT1 and VGLUT2 [11, 13-16]. In support of this note, the present study confirmed expression of SNARE proteins such as SNAP-23, syntaxin-1, and synaptobrevin-2, which are required for vesicular exocytosis, in cultured rat hippocampal astrocytes. Glutamate release from astrocytes is shown to be inhibited by clostridial toxins, that cleave synaptic proteins [10, 12] or botulinum toxin-B, that cleaves synaptobrevin [9]. In the present study, glutamate was condensed in fractions reactive to antibodies against synaptophysin, a synaptic vesicle marker, and vesicular glutamate transporters, separated from cultured rat hippocampal astrocytes, indicating that astrocytes express synaptic-like vesicles containing glutamate. Moreover, aspartate, glycine, GABA, and serine were also condensed in fractions consistent with those for glutamate. Taken together, these results suggest that astrocytes have the machinery for release of glutamate, perhaps aspartate, glycine, GABA, and serine as well, from synaptic-like vesicles for astrocytes, similar to that for neurons.

In the glutamate assay using cultured rat hippocampal astrocytes, we have found that adenosine stimulates glutamate release from astrocytes, which is inhibited by DMPX, an inhibitor of A_{2a} adenosine receptors, or H-89, an inhibitor of PKA [30], and that CGS21680, an agonist of A_{2a} adenosine receptor, stimulates glutamate release from astrocytes, while CHA, an agonist of A_1 adenosine receptor, has no effect [31]. In the NADH imaging assay using cultured rat hippocampal astrocytes, adenosine increased NADH fluorescent signals, that reflects glutamate release from astrocytes, and the adenosine effect was inhibited by DMPX, but not the A_1 adenosine receptor antagonist 8-cyclopentyltheophylline, the A_{2b} adenosine receptor antagonist MRS1706, or the A_3 adenosine receptor antagonist MRS1191 (data not shown). In the present study, CGS21680 increased NADH fluorescent signals, but such effect was not found with CHA, the A_{2b} adenosine receptor agonist NECA, or the A_3 adenosine receptor agonist 2-Cl-IB-MECA. These data indicate that of adenosine receptors A_{2a} adenosine receptor mediates glutamate release from astrocytes.

CGS21680-induced increase in the NADH fluorescent signals was still obtained with extracellular Ca^{2+}-free solution (data not shown), suggesting no requirement for extracellular Ca^{2+} influx in the glutamate release. In addition, the CGS21680 effect was not affected by the glutamate transporter inhibitors L-*trans*-pyrrolidine-2,4-dicarboxylic acid and dihydrokainic acid or deleting extracellular Na^+ (data not shown), which rules out reverse transport through glutamate transporters in CGS21680-induced glutamate release from astrocytes. CGS21680-induced increase in the NADH fluorescent signals was significantly inhibited by DMPX, H-89, vesicular transport inhibitors such as brefeldin A, bafilomycin A1, latrunculin B, and jasplakinolide, or BoTX-A, that cleaves SNAP-23, or by knocking-down A_{2a} adenosine receptor. This accounts for A_{2a} adenosine receptor/PKA-mediated glutamate release from synaptic-like vesicles in astrocytes.

In the FM1-43 imaging analysis, CGS21680 decreased FM1-43 fluorescent signals in cultured rat hippocampal astrocytes, but CHA, NECA, or 2-Cl-IB-MECA had no effect on the signals. CGS21680-induced decrease in FM1-43 fluorescent signals was inhibited by DMPX, H-89, brefeldin A, bafilomycin A1, latrunculin B, jasplakinolide, or BoTX-A, or by knocking-down A_{2a} adenosine receptor. FM1-43 allows us to monitor activity-dependent synaptic vesicular uptake and release for neurons; staining of FM1-43 fluorescent signals corresponds to vesicular uptake, but conversely destaining of the signals corresponds to vesicular release [38]. Intriguingly, FM1-43 is also taken up into lysosomes in astrocytes and extracellular ATP or glutamate induces lysosomal exocytosis [39]. The possibility that CGS21680-induced decrease in FM1-43 fluorescent signals reflects lysosomal exocytosis might not be presently ruled out; however, the fact that FM1-43 was taken up into astrocytes under the conditions of high extracellular K^+ (70 mM), i.e., in an activity-dependent manner, supports vesicular uptake of FM1-43. The results of FM1-43 imaging experiments, taken together with the results of NADH imaging experiments, indicate that A_{2a} adenosine receptor mediates PKA-dependent glutamate release from synaptic-like vesicles in astrocytes.

Accumulating evidence has pointed to adenosine receptors in astrocytes for the control of glucose metabolism, astrogliosis, cell proliferation, cell volume changes, cell death, and the release of neurotrophic factors and interleukins [40, 41]. Of adenosine receptors A_{2a} adenosine receptor in the brain is shown to inhibit glutamate uptake as a short-term action and reduce expression of mRNAs for GLT-1 and GLAST glutamate transporters as a long-term action, presumably leading to a sustained decrease of glutamate uptake [42]. These A_{2a} adenosine receptor actions might cause neuronal death responsible for a variety of brain disease including neurodegenerative disease [43, 44]. In the present study, A_{2a} adenosine receptor promotes glutamate release from synaptic-like vesicles in astrocytes, to increase extracellular glutamate concentrations in the brain. This action might also contribute to progression of neuronal death under some pathological conditions. A_{2a} adenosine receptor blockage, accordingly, could protect from neuronal death relevant to brain diseases such as Alzheimer disease and Parkinson disease. To address this point, further investigations need to be carried out.

For cultured rat hippocampal astrocytes CGS21680 increased intracellular Ca^{2+} concentrations both in Ca^{2+}-containing and -free extracellular solution, and the effect was inhibited by DMPX, H-89, or knocking-down A_{2a} adenosine receptor. This indicates that A_{2a} adenosine receptor stimulates Ca^{2+} efflux from an intracellular calcium store under the control of PKA, thereby increasing intracellular Ca^{2+} concentrations.

PKA phosphorylation of IP_3 receptor is shown to stimulate intracellular Ca^{2+} mobilizations [45]. CGS21680-induced Ca^{2+} rise was not affected by the phospholipase C inhibitors, neomycin and U73122, the IP_3 receptor inhibitor, xestospongin, or the ER calcium release blocker, TMB-8. This denies IP_3 receptor on the ER as a target of A_{2a} adenosine receptor/PKA responsible for CGS21680-induced intracellular Ca^{2+} rise in astrocytes. The Ca^{2+} rise, alternatively, was not inhibited by the ryanodine receptor inhibitor, ryanodine, excluding the participation of ryanodine receptors in A_{2a} adenosine receptor/PKA-mediated Ca^{2+} rise. The Ca^{2+} rise was not still affected by the gap-junction inhibitor, octanol or the non-selective calcium channel blocker, cadmium, indicating that A_{2a} adenosine receptor/PKA-mediated Ca^{2+} rise is not due to Ca^{2+} influx through gap-junctions or calcium channels on the plasma membrane.

One of the most striking findings in the present study is that the vesicular transport inhibitor, brefeldin A, prevented intracellular Ca^{2+} rise induced by CGS21680, but not ATP. This raises the possibility that CGS21680 increases intracellular calcium concentrations due to vesicular Ca^{2+} efflux through an A_{2a} adenosine receptor/PKA signaling pathway. Then, the question addressing is from what intracellular calcium store Ca^{2+} released in response to CGS21680 stimulation. Accumulating evidence has pointed to intracellular Ca^{2+} stores except for the ER that include the Golgi, mitochondria, nucleus, and lysosomes [46]. The Golgi apparatus is an IP_3-sensitive Ca^{2+} store [47]. In the present study, ATP-induced increase in intracellular Ca^{2+} concentrations was attenuated by U73122, suggesting that P2Y receptor might stimulate Ca^{2+} efflux through IP_3 receptors on not only the ER but the Golgi apparatus; however, this is not the case with A_{2a} adenosine receptor/PKA, since CGS21680-induced Ca^{2+} rise was insensitive to IP_3 receptor. Secretory pathway Ca^{2+}-ATPase pump (SPCA), that is specifically localized in the Golgi apparatus, plays a critical role in the Golgi homeostasis of Ca^{2+} [48]. In pancreatic acinar cells, intracellular Ca^{2+} mobilizations are regulated by capacitative Ca^{2+} entry channel (CCE), also known as store-operated Ca^{2+} channel, Ca^{2+} pump in the ER (SERCA), and the Ca^{2+} pumps in the plasma membrane (PMCA), in addition to IP_3 receptor [49]. Furthermore, evidence suggests that vesicle-associated Ca^{2+} channel regulates fusion pore expansion and vesicle content release [50]. It is presently unknown how A_{2a} adenosine receptor/PKA regulates intracellular Ca^{2+} mobilizations in astrocytes. To address this question, we are currently conducting further experiments.

In summary, the results of the present study show that A_{2a} adenosine receptor mediates PKA-dependent glutamate release from synaptic-like vesicles and Ca^{2+} efflux from an IP_3- and ryanodine-insensitive calcium store in astrocytes. This may represent a novel A_{2a} adenosine receptor/PKA signaling pathway relevant to glial transmitter release

in association with intracellular Ca^{2+} mobilizations.

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