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Title	Different mechanisms of extracellular adenosine accumulation by reduction of the external Ca2+ concentration and inhibition of adenosine metabolism in spinal astrocytes
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Citation	Journal of pharmacological sciences, 128(1), 47-53 https://doi.org/10.1016/j.jphs.2015.04.008
Issue Date	2015-05
Doc URL	https://hdl.handle.net/2115/59983
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	J.Pharmacol.Sci.v128 .pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Journal of Pharmacological Sciences 128 (2015) 47–53

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Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full paper

Different mechanisms of extracellular adenosine accumulation by reduction of the external Ca^{2+} concentration and inhibition of adenosine metabolism in spinal astrocytes

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ARTICLE INFO

Article history:
Received 24 February 2015
Received in revised form
26 March 2015
Accepted 21 April 2015
Available online 30 April 2015

Keywords:
ATP
Gap junction hemichannels
Nucleoside transporters
Adenosine kinase
Adenosine deaminase

ABSTRACT

Extracellular adenosine is a neuromodulator in the central nervous system. Astrocytes mainly participate in adenosine production, and extracellular adenosine accumulates under physiological and pathophysiological conditions. Inhibition of intracellular adenosine metabolism and reduction of the external Ca^{2+} concentration ($[Ca^{2+}]_e$) participate in adenosine accumulation, but the precise mechanisms remain unclear. This study investigated the mechanisms underlying extracellular adenosine accumulation in cultured rat spinal astrocytes. The combination of adenosine kinase and deaminase (ADK/ADA) inhibition and a reduced $[Ca^{2+}]_e$ increased the extracellular adenosine level. ADK/ADA inhibitors increased the level of extracellular adenosine but not of adenine nucleotides, which was suppressed by inhibition of equilibrative nucleoside transporter (ENT) 2. Unlike ADK/ADA inhibition, a reduced $[Ca^{2+}]_e$ increased the extracellular level not only of adenosine but also of ATP. This adenosine increase was enhanced by ENT2 inhibition, and suppressed by sodium polyoxotungstate (ecto-nucleoside triphosphate diphosphohydrolase inhibitor). Gap junction inhibitors suppressed the increases in adenosine and adenine nucleotide levels by reduction of $[Ca^{2+}]_e$. These results indicate that extracellular adenosine accumulation by ADK/ADA inhibition is due to the adenosine release via ENT2, while that by reduction of $[Ca^{2+}]_e$ is due to breakdown of ATP released via gap junction hemichannels, after which ENT2 incorporates adenosine into the cells.

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1. Introduction

In the central nervous system (CNS), astrocytes produce adenosine, which plays an important role as a neuromodulator (1). Adenosine is transported across cellular membrane via adenosine gradient-dependent equilibrative nucleoside transporter (ENT); in particular, ENT1 and ENT2 reportedly play a major role in adenosine transport in the CNS (2,3). ENT1 is sensitive to low concentrations of S-(4-nitrobenzyl)-6-thioinosine (NBTI) (4), while higher concentrations of NBTI and dipyridamole (DIP) are necessary to inhibit ENT2 (2). The intracellular adenosine level is thought to be kept low owning to the conversion of adenosine into AMP and inosine via adenosine kinase (ADK) and adenosine deaminase (ADA), respectively (5). On the other hand, astrocytes reportedly release ATP by exocytosis (6) and gap junction hemichannels (7,8), which is in turn broken down to adenosine via a series of ecto-enzymes including ecto-nucleoside triphosphate diphosphohydrolases (ecto-NTPDases) and ecto-5'-nucleotidase (9). Thereafter, adenosine is incorporated into cells by nucleoside transporters (NTs). Thus, the analysis of not only adenosine but also of adenine nucleotides is often important to reveal the mechanisms underlying adenosine accumulation.

Inhibition of intracellular adenosine metabolism reportedly induces extracellular adenosine accumulation under hypoxic/ischemic conditions owing to an increased intracellular concentration of adenosine in the brain and spinal cord (10–12). In rat hippocampus, ENT1 and/or ENT2 are reportedly responsible for adenosine efflux upon ADK inhibition (13).

On the other hand, reduction of the external Ca^{2+} concentration ($[Ca^{2+}]_e$) also causes adenosine accumulation in rat brain (14–16).

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Peer review under responsibility of Japanese Pharmacological Society.
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http://dx.doi.org/10.1016/j.jphs.2015.04.008
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In the brain, $[Ca^{2+}]_e$ is reduced by hypoxic/ischemic conditions (17). However, experimental Ca^{2+}-free conditions, in which Ca^{2+} is replaced with Mg^{2+}, are suspected to induce the release of adenosine by inhibiting intracellular adenosine metabolism because Mg^{2+} can inhibit ADK activities (18,19). Therefore, the mechanisms underlying adenosine accumulation by reduction of $[Ca^{2+}]_e$ remain unclear.

In the spinal cord, it was reported that adenosine mediates antinociceptive and neuroprotective effects via adenosine receptors (20,21). The astrocytic turnover of adenosine and adenine nucleotides has been well-documented in brain but not spinal cord. Therefore, we investigated the mechanisms underlying adenosine accumulation by inhibition of intracellular adenosine metabolic enzymes and reduction of $[Ca^{2+}]_e$ in cultured astrocytes of rat spinal cord via high-performance liquid chromatography (HPLC) and luciferin/luciferase method.

2. Materials and methods

2.1. Spinal astrocyte cultures

All animal care and experimental protocols were approved by the Committee on Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University (No. 13020/13-0029). Every effort was made to minimize animal suffering and to reduce the number of animals used.

Primary cultures of spinal astrocytes were obtained from Wistar rats (0–3 days old, Clea Japan, Tokyo, Japan). Neonatal rats were killed by decapitation and the isolated spinal cord was minced in divalent cation-free Hanks' balanced salt solution. Following digestion with papain (10 U/ml) and DNase (0.1 mg/ml) at room temperature for 20 min, tissues were mechanically dissociated with a Pasteur pipette in culture medium. The culture medium was Dulbecco's Modified Eagle Medium (DMEM)-F12 containing 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml of streptomycin. Thereafter, the cell suspension was transferred to a poly-L-lysine-coated T75 flask, and the medium was replaced after 1–2 h. Cells were cultured in a humidified environment containing 5% CO_2 at 37 °C and fed every 2 or 3 days with new medium. When cells reached 80–90% confluency, flasks were shaken vigorously at 250 rpm for at least 12 h to remove all cells except for astrocytes. The remaining astrocytes were trypsinized and seeded into poly-L-lysine-coated 6-well plates. Cells were used for experiments after they had reached confluency. Almost all cells were positive for the astrocyte marker glial fibrillary acidic protein (GFAP) (Fig. S1). All cultured astrocytes were used within 3 weeks of isolation.

2.2. Extracellular purine assay

Astrocyte cultures in 6-well plates were incubated with 1 ml of artificial cerebrospinal fluid (ACSF). After the medium was changed, the amount of extracellular adenosine increased, peaked at approximately 20 min, and was maintained at this level for 2 h (Fig. S2). Therefore, to examine the effects of ADK and ADA inhibitors, we pre-incubated cells with ACSF for at least 1 h unless otherwise noted. Thereafter, drugs were directly added from stocks to each well without changing the medium, and the culture plates were gently rotated several times. To examine the effects of a reduced $[Ca^{2+}]_e$, astrocyte cultures were incubated with nominal Ca^{2+}-free ACSF, for 1 h in most experiments. Gap27 and ^{10}Panx1 were pretreated with normal ACSF for 30 min. After collecting a sample (500 μl), cells were suspended in 0.1 N NaOH and sonicated. The protein content of cell lysates was measured using the Quick Start protein assay (Bio-Rad, Hercules, CA, USA). The composition of ACSF was as follows: 138 mM NaCl, 3.5 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM HEPES, and 10 mM glucose (pH 7.3 with NaOH). To reduce $[Ca^{2+}]_e$, $CaCl_2$ in ACSF was replaced with equimolar $MgCl_2$.

2.3. Measurement of purines by HPLC analysis

The purines (adenosine, AMP, ADP, and ATP) in the collected samples were eteno-derivatized according to a previously described method (22). The concentrations of ethenoderivatives of purines were determined by reverse-phase HPLC with an Accucore aQ column (4.6 × 150 mm, ThermoFisher Scientific, Tokyo, Japan) at 45 °C and a fluorescence detector (FP-540D, Nihon Koden, Tokyo, Japan). The mobile phase buffer consisted of 100 mM KH_2PO_4, 5 mM tetrabutylammonium bromide, and 2.0% CH_3CN (pH 3.3 with H_3PO_4). The flow rate was 0.8 ml/min. The detection limits were about 2 nM for adenosine, AMP, and ADP, and about 10 nM for ATP. The amounts of purines were expressed as the extracellular amount per milligram of protein content in cell lysates (pmol/mg or nmol/mg).

2.4. Measurement of ATP by luciferin/luciferase analysis

After boiling, the collected sample was chilled on ice, and the concentration of ATP was determined by luciferin/luciferase assay using the ATP Determination Kit (Invitrogen, Carlsbad, CA, USA) with a microplate reader (SH-9000 Lab, Corona Electric, Hitachinaka, Japan). The detection limit for ATP was about 1 nM.

2.5. RT-PCR

The oligonucleotide primers used to amplify ENT, ecto-NTPDase, connexin 43 (Cx43) and pannexin (Panx) are listed in Table S1. Total RNA was extracted from cultured spinal astrocytes and the brain of rats using TRI Reagent (Sigma–Aldrich, St. Louis, MO, USA). First-strand cDNA was synthesized and subsequently amplified using ReverTra Ace (TOYOBO, Osaka, Japan) with Oligo(dT)20 primer and Taq DNA polymerase (Promega, Tokyo, Japan), respectively. Amplification products were separated and analyzed by electrophoresis of 2.0% agarose gels containing ethidium bromide, and visualized under ultraviolet light.

2.6. Drugs

ABT-702 dihydrochloride, adenosine, AMP sodium salt, ATP disodium salt, ARL67156 trisodium salt, carbenoxolone disodium salt, dipyridamole (DIP), S-(4-nitrobenzyl)-6-thioinosine (NBTI), α,β-methylene ADP sodium salt, mefloquine hydrochloride, brilliant blue G and A438079 were purchased from Sigma–Aldrich. ADP disodium salt was from Wako Pure Chemical (Osaka, Japan). Erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA) and sodium polyoxotungstate (POM-1) were from Tocris (Bristol, UK). Flufenamic acid was from LKT Laboratories Inc. (St. Paul, MN, USA). Gap27 and ^{10}Panx1 were synthesized by Invitrogen (Tokyo, Japan).

2.7. Data analysis

Each experiment was performed in at least three different cultures. Results are expressed as mean ± SEM. Two groups were statistically compared using the unpaired Student's *t*-test. For multiple comparisons, ANOVA with Dunnett's test was used. A p value of less than 0.05 was considered significant.

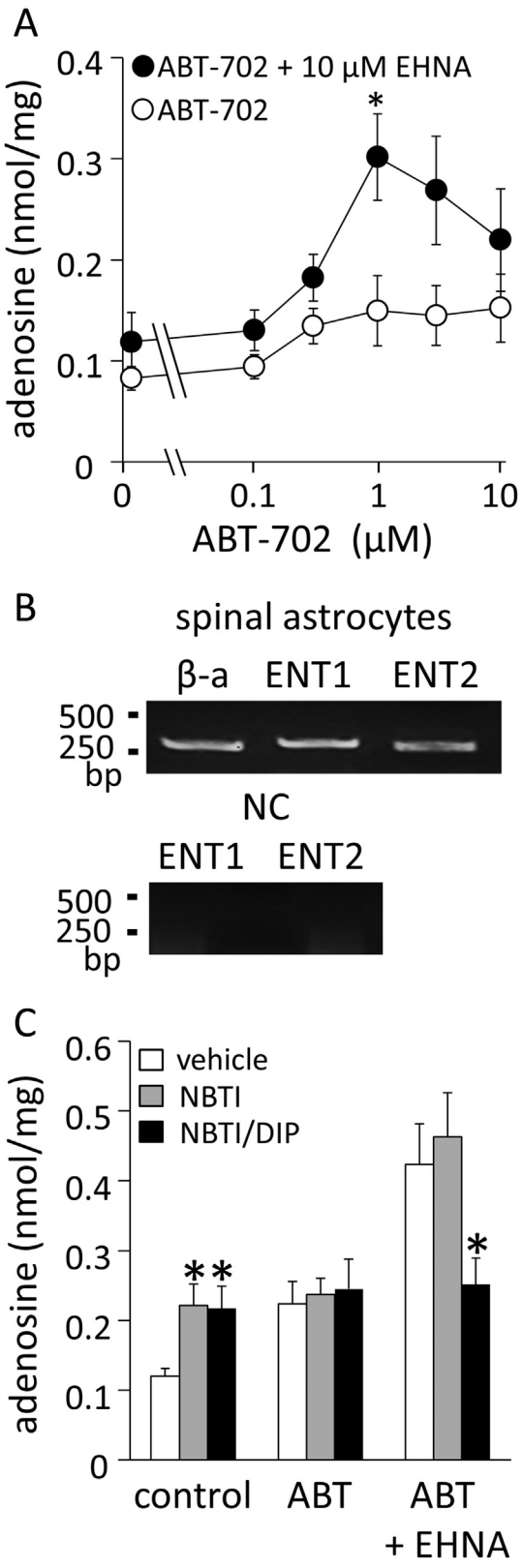


Fig. 1. Inhibition of adenosine metabolic enzymes releases adenosine through ENT2. (A) The increase in the extracellular adenosine level by ABT-702 treatment (0.1–10 μM) in the presence or absence of EHNA (10 μM) for 30 min. *$p < 0.05$ versus ABT-702 (Student's *t*-test), n = 8–10. (B) RT-PCR analysis of mRNA expression of ENT1, ENT2 and β-actin (β-a) in spinal astrocytes. Samples without reverse transcriptase were considered as negative controls (NC). (C) Effects of ENT inhibitors on the adenosine level. Spinal astrocytes were incubated for 30 min with ABT-702 (0.1 μM) or ABT-702 (1 μM)/EHNA (10 μM) in the presence of NBTI (100 nM) or NBTI (5 μM)/DIP (10 μM). *$p < 0.05$ versus vehicle (Dunnett's test), n = 12–14.

3. Results

3.1. Extracellular adenosine accumulation by inhibition of adenosine metabolic enzymes

We first examined the effects of inhibitors of adenosine metabolic enzymes, ADK and ADA, on the adenosine level. ABT-702, an ADK inhibitor, tended to increase the extracellular adenosine level in a concentration-dependent manner (Fig. 1A). EHNA (10 μM), an ADA inhibitor, alone had little effect on the adenosine level. However, in the presence of EHNA, ABT-702 markedly increased the level of adenosine, but not that of AMP (control: 12.4 ± 3.3 pmol/mg, ABT-702/EHNA: 10.1 ± 3.5 pmol/mg, n = 10). In HPLC analysis, levels of ADP and ATP were below the detection limit after 30 min-incubation, regardless of the presence or absence of ABT-702/EHNA. Luciferin/luciferase analysis showed that ABT-702/EHNA had no effect on the ATP level (control: 2.8 ± 0.7 pmol/mg, ABT-702/EHNA: 2.2 ± 0.7 pmol/mg, n = 12).

RT-PCR analysis revealed that rat spinal astrocytes expressed ENT1 and ENT2 (Fig. 1B). After pre-incubation with normal ACSF for 1 h, treatment with a low concentration (100 nM) of NBTI for 30 min significantly increased the adenosine level (Fig. 1C). A mixture of NBTI (5 μM) and DIP (10 μM) (NBTI/DIP) did not further increase the adenosine level. ABT-702 (0.3 μM) increased the adenosine level, which was not affected by NBTI and NBTI/DIP. ABT-702/EHNA greatly enhanced the increase in the adenosine level, which was suppressed by NBTI/DIP but not by NBTI alone.

3.2. Extracellular adenosine accumulation by reduction of the external Ca^{2+} concentration

Mg^{2+} reportedly inhibits ADK activity (18,19). We then incubated cells in ACSF containing various concentrations of Ca^{2+} and/or Mg^{2+} for 1 h (Fig. 2). Regardless of the presence or absence of Mg^{2+}, the extracellular adenosine level markedly increased in the absence of Ca^{2+}.

Unlike the effects of ABT-702/EHNA, a decreased Ca^{2+} concentration in ACSF increased the level not only of adenosine but also of adenine nucleotides, but did not greatly change the ratio of adenosine to adenine nucleotides (Fig. 3A). Purine accumulation in Ca^{2+}-free ACSF was significantly enhanced by NBTI/DIP but not by NBTI alone (Fig. 3B). NBTI/DIP enhanced the increase in the level of adenosine but not that of adenine nucleotides.

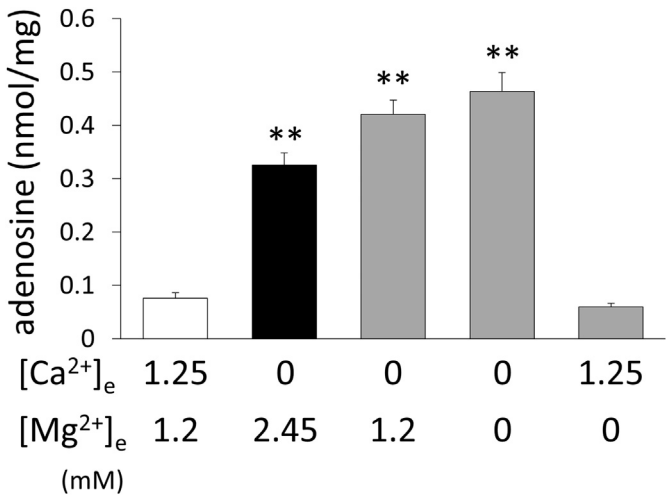
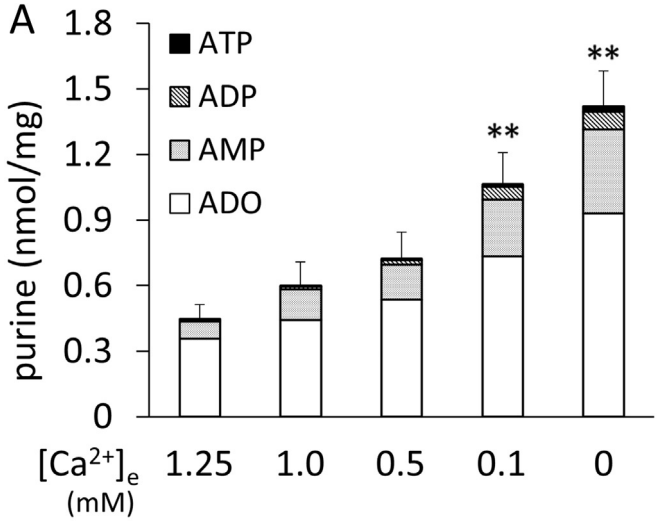


Fig. 2. Removal of extracellular Ca^{2+} increases the adenosine level. The extracellular concentrations (mM) of Ca^{2+} ($[Ca^{2+}]_e$) and Mg^{2+} ($[Mg^{2+}]_e$) were changed. **$p < 0.01$ versus 1.25 mM $[Ca^{2+}]_e$ and 1.2 mM $[Mg^{2+}]_e$ (Dunnett's test), n = 9–18.



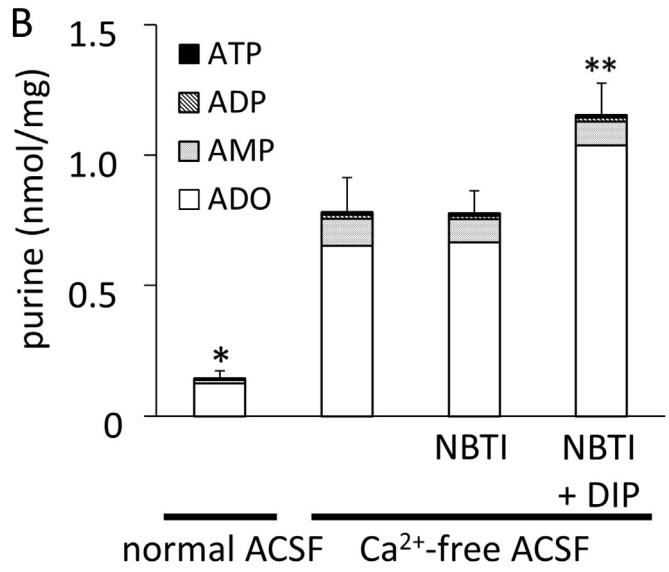
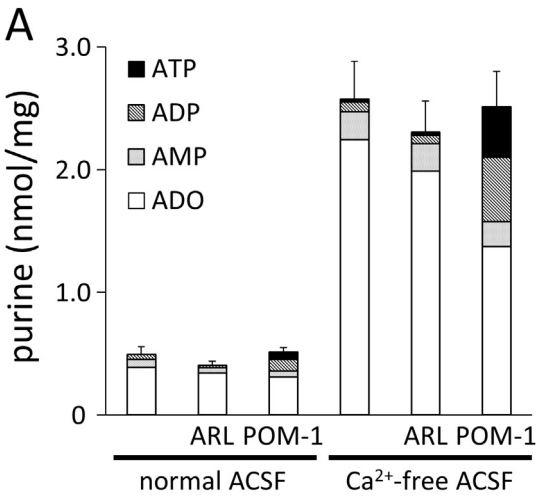


Fig. 3. ENT2 incorporates adenosine that is increased by changing the solution to Ca^{2+}-free ACSF. (A) Ca^{2+} concentration-dependence of purine levels. The extracellular concentration of Ca^{2+} ($[Ca^{2+}]_e$) was changed, and spinal astrocytes were incubated for 1 h **p < 0.01 versus 1.25 mM $[Ca^{2+}]_e$ (Dunnett's test), n = 9. (B) Effect of ENT inhibition on the increase in purine levels evoked by changing the solution to Ca^{2+}-free ACSF. Spinal astrocytes were incubated for 1 h with Ca^{2+}-free ACSF containing NBTI (100 nM) or NBTI (5 μM)/DIP (10 μM). *p < 0.05, **p < 0.01 versus Ca^{2+}-free (Dunnett's test), n = 10. ADO, adenosine.



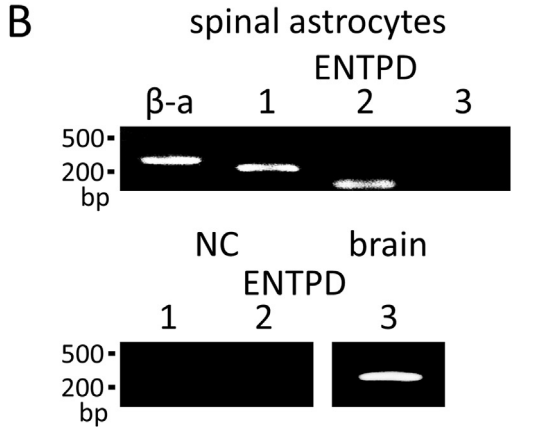


Fig. 4. Ecto-NTPDase contributes to the increase in the adenosine level evoked by changing the solution to normal ACSF and Ca^{2+}-free ACSF. (A) Effect of ecto-NTPDase inhibitors on purine levels in normal ACSF and Ca^{2+}-free ACSF. Spinal astrocytes were incubated for 1 h with normal ACSF or Ca^{2+}-free ACSF containing ARL67156 (ARL, 50 μM) or POM-1 (100 μM), n = 8–14. (B) RT-PCR analysis of mRNA expression of ecto-NTPDase (ENTPD) 1–3 and β-actin (β-a) in spinal astrocytes. Samples without reverse transcriptase were considered as negative controls (NC). Rat brain cDNA was used as a positive control for ENTPD3. ADO, adenosine.

ARL67156 (50 μM), an ecto-NTPDase inhibitor, had no effect on total purine accumulation and the ratio of purine (Fig. 4A and Table 1). Another ecto-NTPDase inhibitor, POM-1 (100 μM), also had no effect on total purine accumulation but significantly increased the levels of ATP and ADP in normal and Ca^{2+}-free ACSF, which was accompanied by a decrease in the adenosine level, indicating the contribution of ATP release and its subsequent breakdown to the increased adenosine level. RT-PCR analysis confirmed that rat spinal astrocytes expressed NTPDase1 and 2 (Fig. 4B).

3.3. Gap junction hemichannels contribute to ATP release

The time course of purine levels in normal and Ca^{2+}-free ACSF was examined. Just after changing the solution to normal ACSF, ATP, ADP, and AMP, but not adenosine, were detectable. The levels of these adenine nucleotides decreased exponentially and were low at 1 h (Fig. 5), indicative of nucleotide release in response to the solution change. Changing the solution to Ca^{2+}-free ACSF greatly increased the amount of ATP, which peaked at around 1 min, and decreased exponentially thereafter. The levels of ADP and AMP just after changing the solution to Ca^{2+}-free ACSF were not different from those in normal ACSF. However, in Ca^{2+}-free ACSF, these levels peaked at around 10 min and gradually decreased thereafter. By contrast, the adenosine level was extremely low just after changing the solution to Ca^{2+}-free ACSF and then gradually increased, peaking at around 1 h.

Cultured rat spinal astrocytes expressed Cx43 and Panx1 mRNA (Fig. 6A). The non-selective gap junction inhibitors carbenoxolone (10 μM), mefloquine (10 μM), and flufenamic acid (100 μM) inhibited the increase in total purines in Ca^{2+}-free ACSF, but not in normal ACSF (Fig. 6B). Next, we measured the levels of adenine nucleotides and adenosine for 10-min incubation with normal or Ca^{2+}-free ACSF in the presence of selective gap junction inhibitors. The respective connexin and pannexin inhibitors, Gap27 and ^{10}Panx1, inhibited the increase in the total purine level in Ca^{2+}-free ACSF (Fig. 6C). However, simultaneous treatment with Gap27 and

Table 1
Effects of ecto-NTPDase inhibitors on purine levels.

	ADO	AMP	ADP	ATP
Normal	77.6 ± 4.2 (385.3 ± 55.8)	13.8 ± 2.1 (66.8 ± 15.3)	7.7 ± 2.3 (38.1 ± 14.6)	0.9 ± 0.1 (3.5 ± 0.4)
Normal + ARL	83.8 ± 2.0 (341.7 ± 35.1)	10.6 ± 1.4 (41.5 ± 5.7)	4.8 ± 0.7 (18.3 ± 2.4)	0.8 ± 0.1 (3.3 ± 0.4)
Normal + POM-1	60.0 ± 2.5$^{\#\#}$ (305.9 ± 25.4)	9.8 ± 1.2 (51.9 ± 9.1)	18.6 ± 1.9$^{\#\#}$ (93.8 ± 11.2$^{\#}$)	11.6 ± 0.4$^{\#\#}$ (58.9 ± 4.5$^{\#\#}$)
Ca^{2+}-free	85.6 ± 1.7 (2240.4 ± 290.6)	10.2 ± 1.5 (229.0 ± 31.6)	3.2 ± 0.3 (78.9 ± 12.0)	1.0 ± 0.2 (24.2 ± 3.4)
Ca^{2+}-free + ARL	84.0 ± 2.0 (1985.4 ± 248.3)	11.4 ± 1.7 (223.4 ± 28.1)	3.3 ± 0.4 (69.2 ± 9.3)	1.2 ± 0.2 (24.8 ± 3.5)
Ca^{2+}-free + POM-1	52.6 ± 2.8** (1369.9 ± 195.1*)	9.1 ± 1.1 (203.4 ± 23.1)	21.3 ± 1.1** (524.7 ± 67.0**)	17.1 ± 1.3** (410.4 ± 49.7**)

Each value is mean ± SEM. The ratio to the total purine level (%) is shown, together with the total amount of purines (pmol/mg) in parentheses. *p < 0.05, **p < 0.01 versus Ca^{2+}-free (Dunnett's test). $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 versus normal (Dunnett's test), n = 8–14. Medium was changed to normal artificial cerebrospinal fluid (ACSF) or Ca^{2+}-free ACSF containing ARL67156 (ARL, 50 μM) or sodium polyoxotungstate (POM-1, 100 μM), and then cells were incubated for 1 h. ADO, adenosine; ecto-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase.

[10]Panx1 did not have an additive effect (data not shown). Gap27 and [10]Panx1 had no effect on the total purine level in normal ACSF. Panx1 reportedly forms a complex with $P2X_7$ receptors (23). The $P2X_7$ receptor antagonists brilliant blue G (10 μM) and A438079 (10 μM) slightly inhibited the increase in the total purine level in normal or Ca^{2+}-free ACSF (Fig. S3).

4. Discussion

The present study shows that inhibition of adenosine metabolic enzymes and reduction of $[Ca^{2+}]_e$ increase the extracellular adenosine level in rat cultured spinal astrocytes. Inhibition of ADK and/or ADA increases extracellular adenosine levels in various regions of the CNS. As the intracellular adenosine level is kept low by ADK and ADA, the inhibition of these enzymes increases the intracellular adenosine, which in turn is transported into extracellular spaces (10–12). However, in cultured rat spinal astrocytes, the extracellular adenosine level was slightly increased by an ADK inhibitor but not an ADA inhibitor, and was synergistically increased by a combination of both inhibitors. These results suggest that, in spinal astrocytes, a main pathway for adenosine turnover is the phosphorylation of adenosine to AMP, that cytosolic adenosine is greatly broken down to inosine by ADA when its level is increased upon ADK inhibition, and that intracellular adenosine is released into the extracellular space when its level is increased upon ADK and/or ADA inhibition. In rat hippocampal slices, at least half of the adenosine efflux by ADK inhibition is reportedly mediated via ENTs (13). Astrocytes are thought to constantly release ATP, which is again incorporated into cells by NTs after breakdown to adenosine. In this study, a low concentration of NBTI increased the extracellular adenosine level in resting conditions, suggesting that adenosine influx is associated with ENT1. However, the increased adenosine efflux by an ADK inhibitor alone was not affected by ENT inhibition. It seems likely that adenosine influx is balanced by its efflux under this condition. ADK and ADA inhibition elicited a great increase in the extracellular adenosine level, which was inhibited by NBTI/DIP. When the intracellular adenosine level is greatly increased, it is suggested to be transported into the extracellular space by ENT2, which has a low affinity and a high capacity for adenosine transport (24).

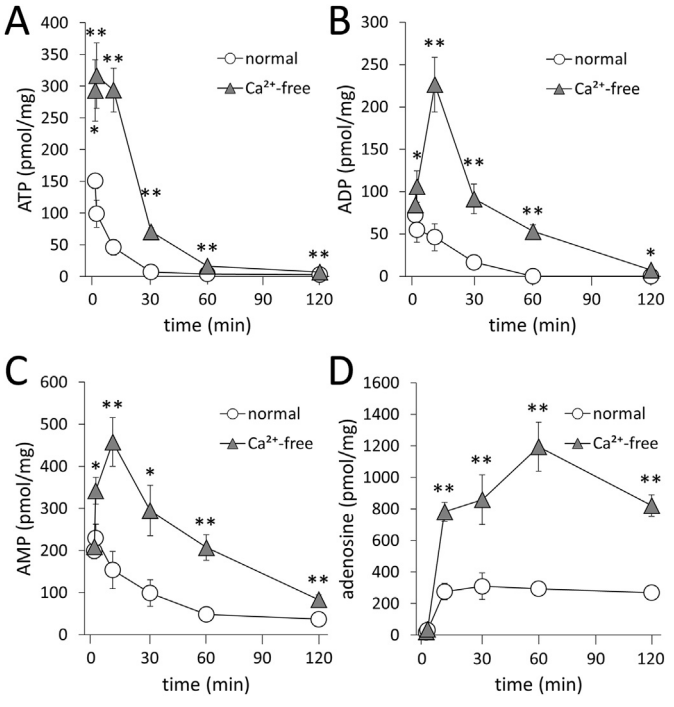


Fig. 5. The time course of ATP (A), ADP (B), AMP (C), and adenosine (D) levels in normal ACSF and Ca^{2+}-free ACSF. Spinal astrocytes were incubated with normal ACSF or Ca^{2+}-free ACSF for 10 s or 1, 10, 30, 60, or 120 min *p < 0.05, **p < 0.01 versus normal ACSF (Student's *t*-test), n = 8–9.

Reduction of $[Ca^{2+}]_e$ increased the extracellular adenosine level. Mg^{2+} is often used to replace Ca^{2+} for experimental Ca^{2+}-free conditions (15,16). Mg^{2+} reportedly inhibits ADK activities (18,19). However, in the present study, adenosine accumulation induced by Ca^{2+}-free ACSF was not affected regardless of the presence or absence of Mg^{2+}, indicating that inhibition of adenosine metabolism by Mg^{2+} is not associated with adenosine accumulation in Ca^{2+}-free ACSF. Ischemia reduces $[Ca^{2+}]_e$ to approximately 0.1 mM in the brain (17), which was sufficient to cause adenosine accumulation in spinal astrocytes in the present study. These results suggest that reduction of $[Ca^{2+}]_e$ is an important factor for extracellular adenosine accumulation during ischemia.

In this study, NBTI/DIP increased the adenosine level in Ca^{2+}-free ACSF, suggesting the involvement of ENT2 to the uptake of adenosine. It is likely that the increased adenosine level in Ca^{2+}-free ACSF is due to adenine nucleotides released from astrocytes, which are broken down to adenosine by a series of ecto-enzymes including ecto-NTPDases. NTPDase 1–3 and 8 are reportedly membrane-bound ecto-enzymes with ATP- and/or ADP-hydrolyzing activity (9). Our study showed that rat spinal astrocytes expressed NTPDase1 and 2, and that POM-1, but not ARL67156, significantly increased the levels of ATP and ADP concomitant with a decrease in that of adenosine. Similarly, in rat cerebellar slices, the inhibitory effect of ARL67156 on ATP breakdown is weaker than that of POM-1 (25). Moreover, ARL67156 reportedly inhibits NTPDase1 and 3, POM-1 inhibits NTPDase1, 2, and 3 (26), and NTPDase2 is a predominant subtype in rat brain astrocytes (27). Taken together, it is suggested that NTPDase2 is mainly responsible for hydrolysis of ATP in rat spinal astrocytes.

The time course of adenine nucleotide and adenosine accumulation in response to solution change to normal or Ca^{2+}-free ACSF

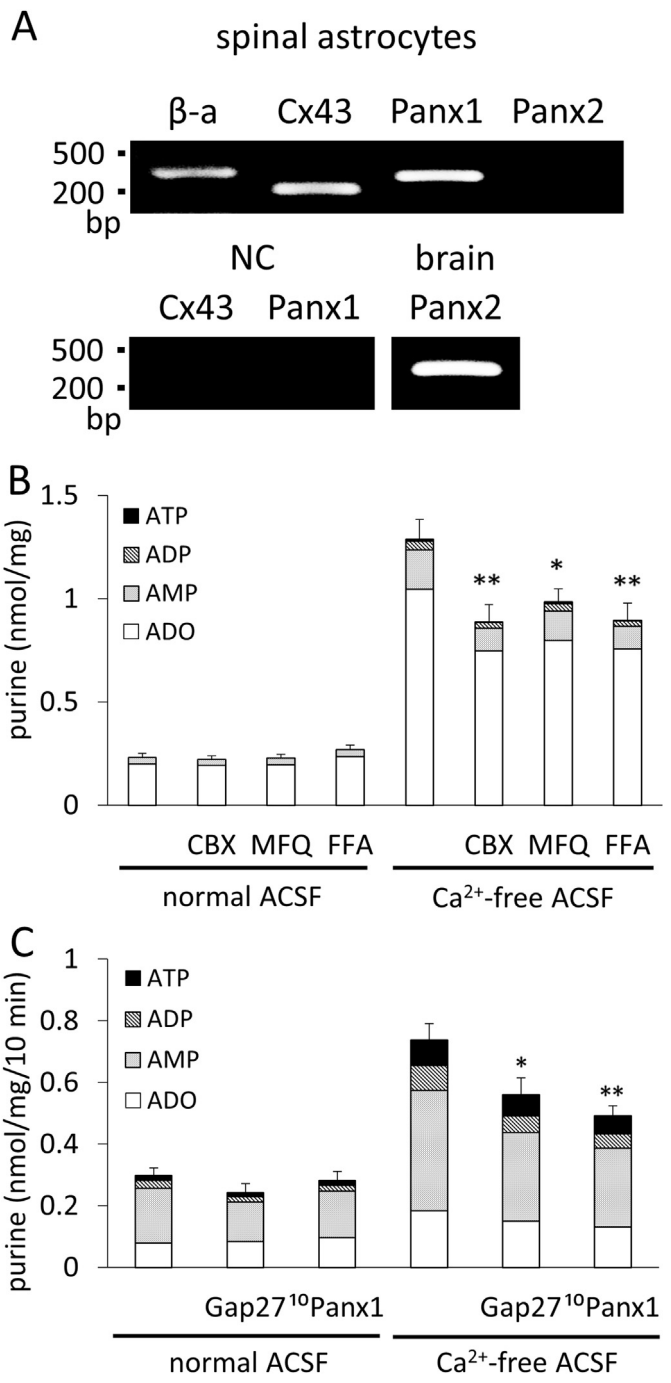


Fig. 6. The removal of extracellular Ca^{2+} releases ATP through gap junction hemichannels. (A) RT-PCR analysis of mRNA expression of Cx43, Panx1, Panx2, and β-actin (β-a) in spinal astrocytes. cDNA without reverse transcriptase was considered as a negative control (NC). Rat brain cDNA was used as positive control of Panx2. (B) Effects of non-selective gap junction inhibitors on purine levels. Spinal astrocytes were incubated for 1 h with normal ACSF or Ca^{2+}-free ACSF containing CBX (10 μM), MFQ (10 μM), or FFA (100 μM). *$p < 0.05$, **$p < 0.01$ versus Ca^{2+}-free ACSF (Dunnett's test), $n = 8–9$. (C) Effects of Gap27 and ^{10}Panx1 on purine levels at 10 min in normal ACSF and Ca^{2+}-free ACSF. Spinal astrocytes were pre-incubated for 30 min with normal ACSF containing Gap27 (50 μM) or ^{10}Panx1 (50 μM), and then incubated for 10 min with normal ACSF or Ca^{2+}-free ACSF containing these inhibitors. *$p < 0.05$, **$p < 0.01$ versus Ca^{2+}-free ACSF (Dunnett's test), $n = 9–11$. CBX, carbenoxolone; MFQ, mefloquine; FFA, flufenamic acid.

showed a great difference among purines. Although, just after changing the solution, the amount of ATP was higher in Ca^{2+}-free ACSF than in normal ACSF, the amounts of ADP and AMP were the same in both types of ACSF. This is indicative of the release of adenine nucleotides upon changing the solution *per se*. On the other hand, adenosine accumulation occurred 10 min after the solution change, suggesting the production of adenosine. The time course of adenine nucleotide elimination seems to be exponential; therefore, it is likely that the released ATP is broken down into ADP or AMP by ecto-NTPDase, after which AMP is broken down to adenosine by ecto-5'-nucleotidase.

In astrocytes, there are reportedly several pathways for ATP release such as exocytosis (6) and gap junction hemichannels (7,8). Exocytotic release of ATP by reduction of $[Ca^{2+}]_e$ is unlikely because this process generally depends on Ca^{2+}. Non-selective gap junction inhibitors inhibited the increase in purine levels in Ca^{2+}-free ACSF, suggesting that gap junction hemichannels contribute to the ATP release. Our data showed that rat spinal astrocytes expressed Cx43 and Panx1, and that selective connexin and pannexin inhibitors inhibited the increase in purine levels in Ca^{2+}-free ACSF. Although Cx43 and Panx1 both seemed to be responsible for ATP release, simultaneous treatment with Gap27 and ^{10}Panx1 did not show any additive effect (data not shown). Cx43 hemichannels reportedly open in response to reduction of $[Ca^{2+}]_e$, whereas Panx1 channels are insensitive to external Ca^{2+} (28), suggesting that reduction of $[Ca^{2+}]_e$ induces ATP release mainly through Cx43 hemichannels. In rat spinal astrocytes, Panx1 is reportedly opened by FGF-1-induced increase in the intracellular Ca^{2+} concentration (29). In present study, however, it is unlikely that the opening of Panx1 is mediated by Ca^{2+} influx in Ca^{2+}-free ACSF. Panx1 reportedly forms a complex with $P2X_7$ receptors, the activation of which by a high concentration of ATP opens Panx1 (23). In the present study, $P2X_7$ receptor antagonists tended to inhibit the increase in purine levels in Ca^{2+}-free ACSF; therefore, Panx1 channels and $P2X_7$ receptors might be slightly involved in ATP release in Ca^{2+}-free ACSF.

As mentioned above, changing the solution caused the release of adenine nucleotides. Mechanical stimulation reportedly opens Cx43 hemichannels and Panx1 channels and releases ATP (30–33). However, gap junction inhibitors had effects on purine levels in Ca^{2+}-free ACSF but not in normal ACSF. Therefore, it is suggested that the great increase in ATP release in Ca^{2+}-free ACSF is not simply owing to mechanical stimulation. Mechanical stimulation reportedly releases ATP through secretory granules (34), maxi-anion channels (35), and mechanosensitive ion channels (36,37). It is possible that these channels participate in ATP release induced by solution change. Further studies are required to elucidate the precise mechanisms underlying ATP release from astrocytes upon changing the solution.

In this study, it is suggested that adenosine accumulation induced by the inhibition of adenosine metabolic enzymes is due to the release of intracellular adenosine through ENT2, whereas adenosine accumulation evoked by reduction of $[Ca^{2+}]_e$ is due to ATP release through gap junction hemichannels. Released ATP is rapidly degraded into adenosine by a series of ecto-enzymes including NTPDases. In isolated tissues and in vivo, this conversion of ATP into adenosine might occur more rapidly. However, under hypoxic/ischemic conditions in the CNS including the spinal cord, it is still unclear which of these is a main pathway. Further studies are required to elucidate the precise mechanisms underlying adenosine accumulation induced by hypoxia/ischemia.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from JSPS (No. 26450440 to K.O.). All experiments were conducted in compliance with the ARRIVE guidelines.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2015.04.008.

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