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Multiple pathways for elevating extracellular adenosine in the rat hippocampal CA1 region characterized by adenosine sensor cells

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Abstract

Extracellular adenosine in the brain, which modulates various physiological and pathological processes, fluctuates in a complicated manner that reflects the circadian cycle, neuronal activity, metabolism and disease states. The dynamics of extracellular adenosine in the brain are not fully understood, largely because of the lack of simple and reliable methods of measuring time-dependent changes in tissue adenosine distribution. This study describes the development of a biosensor, designated an adenosine sensor cell, expressing adenosine A1 receptor and a genetically-modified G protein. This biosensor was used to characterize extracellular adenosine elevation in brain tissue by measuring intracellular calcium elevation in response to adenosine. Placement of adenosine sensor cells below hippocampal slices successfully detected adenosine releases from these slices in response to neuronal activity and astrocyte swelling by conventional calcium imaging. Pharmacological analyses indicated that high-frequency electrical stimulation induced postsynaptic adenosine release in a manner dependent on L-type calcium channels and calcium-induced calcium release. Adenosine release following treatments that cause astrocyte swelling is independent of calcium channels, but dependent on aquaporin 4, an astrocyte-specific water channel subtype. The ability of ectonucleotidase inhibitors to inhibit adenosine release following astrocyte swelling, but not electrical stimulation, suggests that the former reflects astrocytic ATP release and subsequent enzymatic breakdown, whereas the latter reflects direct adenosine release from neurons. These results suggest that distinct mechanisms are responsible for extracellular adenosine elevations by neurons and astrocytes, allowing exquisite regulation of extracellular adenosine in the brain.

Graphical abstract

Time-dependent changes in the distribution of adenosine, a neuromodulator with various physiological and pathological functions, were analyzed in hippocampal slices using a novel biosensor that images extracellular adenosine. Increases in tissue adenosine following electrical stimulation were attributed to L-type calcium channel dependent adenosine release from

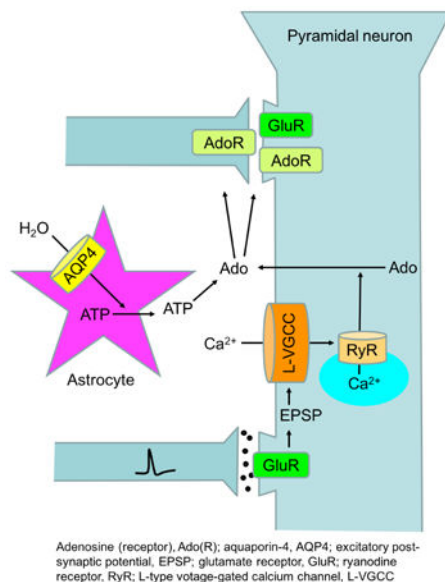
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postsynaptic neurons, whereas astrocyte swelling increased adenosine by releasing ATP and subsequent enzymatic catabolism.



Keywords

adenosine; astrocyte; calcium channel; dendrite; water channel

Introduction

Extracellular adenosine modulates diverse physiological and pathological processes, including neuronal activity, blood flow, metabolism and inflammation, both in peripheral organs (Belardinelli *et al.* 1998, Hansen & Schnermann 2003, Ernst *et al.* 2010) and in the central nervous system (CNS). In the CNS, adenosine modulates, but does not trigger, synaptic responses by A₁ and A_{2A} receptors, which mainly localize at excitatory synapses (Rebola *et al.* 2003, Rebola *et al.* 2005a, Rebola *et al.* 2005b), suggesting that adenosine acts as a prototypic neuromodulator. A₁ receptors, one of the most abundant G protein-coupled receptors in the brain, control basal network activities by suppressing synaptic transmission (Dunwiddie & Masino 2001), whereas A_{2A} receptors play essential roles in some forms of synaptic plasticity (Rebola *et al.* 2008, Costenla *et al.* 2011). In addition, adenosine receptors crosstalk with other neurotransmitter and neuromodulator receptors for fine tuning of synapses (Sebastiao & Ribeiro 2009). Transient elevations of extracellular adenosine following high frequency stimulation have been found to play crucial roles in plastic changes in synaptic transmission, including in heterosynaptic depression (Manzoni *et al.* 1994) and long-term potentiation (Rebola *et al.* 2008). Furthermore, ambient adenosine has been found to fluctuate during physiological and pathological processes, including sleep (Porkka-Heiskanen & Kalinchuk 2011), ischemia (Winn *et al.* 1979) and epilepsy (Boison 2005), thus modulating brain waves (Pietersen *et al.* 2009), cerebral blood flow (Gordon *et al.* 2008) and neuronal survival (Rudolphi *et al.* 1992).

Although adenosine plays crucial roles in brain physiology and pathology, the mechanisms underlying the dynamics of extracellular adenosine in the brain are poorly understood. Results using peripheral and central tissue preparations have suggested several pathways responsible for the elevation of extracellular adenosine, including exocytosis (Klyuch *et al.* 2012), transport (Wall & Dale 2013, Lovatt *et al.* 2012) and enzymatic breakdown of extracellular nucleotides (Heinrich *et al.* 2012, Mi & Jackson 1998). Several pathways are also responsible for reductions in extracellular adenosine, including equilibrative nucleoside transporter-mediated adenosine uptake (Baldwin *et al.* 2004), which is potentiated by the clearance of intracellular adenosine by adenosine kinase (Boison 2005) and/or adenosine deaminase (Lloyd & Fredholm 1995). In peripheral organs, distinct mechanisms regulate extracellular adenosine. For example, elevated extracellular adenosine in cardiac ischemia is due to an imbalance between ATP production and consumption (Headrick *et al.* 2003), whereas elevated renal extracellular adenosine is due to cAMP release and subsequent breakdown by phosphodiesterases and nucleotidases (Mi & Jackson 1998). Thus, the brain, which utilizes adenosine for diverse functions, is thought to be equipped with multiple pathways for elevating extracellular adenosine, allowing exquisite spatiotemporal regulation.

In addition to activity-dependent synaptic releases of ATP (White 1977, Wieraszko *et al.* 1989, Cunha *et al.* 1996), accumulating evidence suggests that astrocytes play central roles in ATP signaling in the CNS. Astrocytes have been shown to release ATP by exocytosis (Lalo *et al.* 2014), gap junction hemichannels (Orellana *et al.* 2011), P_{2X7} receptors (Suadicani *et al.* 2006), and anion channels (Liu *et al.* 2008), and astrocyte-derived ATP is presumably involved in a number of intercellular communications in the CNS (Chen *et al.* 2013a, Davalos *et al.* 2005, Cui *et al.* 2014). Furthermore, astrocyte ATP is converted to adenosine by ectonucleotidases (Yang *et al.* 2015). However, it is still unclear whether astrocytes are the source of ambient extracellular adenosine in physiological and pathological conditions, as well as transient increases in ATP and/or adenosine in response to neuronal activities.

Extracellular adenosine has been measured by HPLC (Andresen *et al.* 1999) and enzymatic electrodes (Wall & Dale 2013). These methods allow accurate quantification of adenosine concentration, but require specialized equipment and techniques and lack spatial resolution. Thus, we hypothesized that the imaging of tissue adenosine will reveal novel aspects of adenosine release mechanisms. This study describes a novel and efficient method for measuring time-dependent changes in tissue adenosine distribution and use of this method to characterize adenosine release in the hippocampus. Adenosine was detected as elevated intracellular calcium in HEK293 cells expressing mouse adenosine A1 receptor and Gqi5, a chimeric Gq alpha subunit in which the five carboxy-terminal amino acids are replaced by the corresponding part of Gi (Conklin *et al.* 1993). These cells, designated adenosine sensor cells, were placed below hippocampal slices, with calcium imaging used to measure adenosine release from slices following electrical or pharmacological stimulation. Our pharmacological analysis of hippocampal elevation of extracellular adenosine, as measured by adenosine sensor cells, revealed multiple pathways, involving neuronal calcium channels and astrocytic water channels. These results illustrate the exquisite regulation of extracellular adenosine in the brain.

Materials and methods

Cell culture

Mouse A1 receptor cDNA was amplified by PCR and subcloned into pcDNA3.1 (Life Technologies, Carlsbad, CA). A DNA fragment containing Gqi5 was generated by PCR (Conklin et al. 1993) and subcloned into pME18S. Adenosine sensor cells were generated by transfecting HEK293 cells (ATCC, Manassa, VA) with these two plasmids using GenePORTER (Genlantis, San Diego CA) and by selecting stable transfectants showing calcium response to adenosine with G418 (Life Technologies) and calcium imaging. The adenosine sensor cells were maintained in DMEM/F12 (Life Technologies) containing 10% fetal calf serum and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin, Life Technologies).

Hippocampal slices

All animal experiments were approved by the Institutional Animal Care and Use Committee (Permission number: 25-09-04) and performed according to the Animal Experimentation Regulations of Kobe University. Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) were housed in cages that were maintained in a room with a temperature (24°C) and humidity-controlled environment on a 12-h light/dark cycle. Water and food were provided *ad libitum*. Rats of both sexes aged 3–4 weeks were decapitated under isoflurane anesthesia; their brains were removed into ice-cold cutting solution (2 mM KCl, 1.5 mM NaH₂PO₄, 26 mM NaHCO₃, 6 mM MgSO₄, 200 µM CaCl₂, 10 mM D-glucose, 220 mM sucrose) saturated with 95% O₂ / 5% CO₂ gas; and 400 µm horizontal slices of brains were obtained using a ZERO-1 microslicer (Dosaka EM, Kyoto, Japan). The slices were maintained in artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 2 mM KCl, 1.5 mM NaH₂PO₄, 26 mM NaHCO₃, 1 mM MgSO₄, 10 mM D-glucose, 2 mM CaCl₂) saturated with 95% O₂ / 5% CO₂ at room temperature for more than one hour before experiments.

Calcium imaging

The adenosine sensor cells were seeded onto coverslips coated with 1 µg/ml poly-D-lysine, and incubated at 30°C for 45 min with 7.5 µM Fura2AM (Dojin, Kumamoto, Japan) or Rhod-4AM (AAT Bioquest, Inc. CA) in basal salt saline (BSS, 129 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 10 mM D-glucose, 2 mM CaCl₂, 10 mM Hepes, pH 7.4). After being loaded with a calcium indicator, the cells were maintained in BSS containing 100 µM sulfinpyrazone, which blocks the sequestration of calcium indicator (Di Virgilio *et al.* 1990). Slices were placed directly onto the cells and held by the threads of a hand-made platinum slice anchor. The distances between the slices and the cells were adjusted so as to make both visible in the same focal plane (e.g. Fig 2B; Slice and Fura2). Fluorescence images were obtained every 3 seconds using an inverted microscope (IX-70, Olympus, Tokyo, Japan), equipped with an objective (UApo/340 20×/0.75, Olympus), a filter exchanger (OSP-EXA, Olympus) and a cooled-CCD camera (Orca-R2, Hamamatsu Photonics, Hamamatsu, Japan). The R340/380 ratio of Fura2AM fluorescence and the F/F₀ ratio of Rhod4AM fluorescence were analyzed using Image J software. Slices and adenosine sensor cells were superfused at 30°C with BSS or aCSF at a perfusion rate of 5 ml/min. Electrical stimulation was provided by glass electrodes filled with aCSF. For KCl stimulation, which was used as an alternative

to electrical stimulation to reduce the volume of expensive chemicals, slices and the adenosine sensor cells were incubated in a chamber of approximately 2 mL volume and equipped with a heater and aerator, followed by the sequential dropwise addition of KCl and adenosine, to achieve the final concentrations indicated. For hypoosmotic treatment, slices and adenosine sensor cells were pretreated for 20 min with modified aCSF, in which two-thirds of the NaCl concentration was replaced by sucrose of the same osmolality, followed by a reduction in osmolality from 354 to 195 mOsm/L by reducing sucrose concentration.

Statistical analysis

Florescence data were obtained for individual cells. Cells were randomly selected for statistical analysis in Figures 1 and 4B. In measuring adenosine release from slices (Figs. 2–7, except for Fig. 4B), adenosine sensor cells subjected to quantitative analysis were randomly selected from those responding to control electrical stimulation (3 mA, 30 Hz for 5 sec), which was applied in aCSF prior to test stimulation. Each slice was subjected to one set of control and test stimulations. Values are expressed as mean \pm SD and compared by Student's t tests (Microsoft Excel) or Dunnett's multiple comparison test (JSTAT), as indicated, with differences having a probability (p) < 0.05 considered statistically significant.

Chemicals

Unless otherwise indicated, all chemicals were purchased from Nacalai Tesque (Kyoto, Japan). Hepes, sulfinpyrazone, 8-cyclopentyltheophylline (8CPT), thapsigargin, *S*-(4-nitrobenzyl)-6-thioinosine (NBTI), dipyridamole, 4-aminopyridine, TGN-020 and CdCl₂ were purchased from Sigma Aldrich (St. Louis, MO). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), ARL67156, (2R)-amino 5-phosphonovaleric acid (D,L-APV) and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) were from TOCRIS (Avonmouth, UK), and ryanodine was from Wako Chemical (Tokyo, Japan).

Results

Characterization of adenosine sensor cells

The intracellular calcium response of adenosine sensor cells, established as described in the Materials and Methods section, following exposure to adenosine or inosine, which had been reported to bind A1 receptor with an affinity similar to adenosine (Nascimento *et al.* 2015), was examined (Fig. 1A). At concentrations of 0.1–100 μ M, adenosine induced a concentration dependent elevation in intracellular calcium, with an EC₅₀ value of 8.0 μ M. In comparison, inosine, at concentrations above 100 μ M, induced detectable calcium elevation, with the calcium elevation in response to a saturating concentration of inosine (1000 μ M) being 67% that induced by 100 μ M adenosine. An A1 receptor antagonist, 1 μ M 8CPT, blocked 90% and 88% of calcium elevations induced by 10 μ M adenosine and 1000 μ M inosine, respectively (Fig. 1B), indicating that calcium elevation in response to adenosine and inosine was mediated by A1 receptors. These results indicate that calcium imaging of adenosine sensor cells can detect adenosine at concentrations over 0.1 μ M and inosine at concentrations over 100 μ M. Since adenosine release accompanying adenosine pool depletion has been reported to increase adenosine concentration at the surface of

hippocampal slices to approximately 50 μM , as measured by calibrated enzymatic electrodes, and the elevation in response to inosine does not exceed that in response to adenosine (Dale *et al.* 2000, Pearson *et al.* 2001), adenosine sensor cells likely detect adenosine, but not inosine, even during maximum adenosine release from hippocampal slices.

Electrically-induced extracellular adenosine elevation in hippocampal slices

Rat hippocampal slices were placed onto Fura2A-loaded adenosine sensor cells, and elevations in extracellular adenosine in the CA1 region in response to high frequency electrical stimulation of the Schaffer collateral (synaptic stimulation) or Striatum oriens (antidromic stimulation) were measured by calcium imaging with an inverted microscope (Fig. 2A). High-frequency synaptic stimulation (30 Hz, 5 sec) with a glass electrode increased calcium in the adenosine sensor cells, with both the area and magnitude of calcium response increased by increasing the stimulation intensity from 0.3 mA to 3 mA (Fig. 2B). In subsequent experiments, electrical stimulation was applied at 3 mA, which reliably induced adenosine sensor cell responses. To determine whether the source of adenosine was presynaptic terminals or postsynaptic neurons, adenosine release from slices in response to synaptic and antidromic stimulation was compared. Calcium elevations in response to both synaptic and antidromic stimulations lasted several minutes and were completely blocked by 8CPT (Fig. 2C). The response of adenosine sensor cells was 30% lower to antidromic than to synaptic stimulation, with both responses blocked more than 90% by 8CPT (Fig. 2D). Thus, both synaptic and antidromic stimulation induce substantial calcium responses of adenosine sensor cells, which were pharmacologically confirmed as reflecting adenosine release from hippocampal slices. The ability of the adenosine sensor cells to detect adenosine releases from these slices was further confirmed by the lack of increased calcium in HEK293 cells negative for expression of A1 receptor and Gqi5 (data not shown). Blocking glutamatergic synapses with 10 μM CNQX and 100 μM D,L-APV significantly reduced adenosine release in response to synaptic stimulation by 62%, while not affecting adenosine release in response to antidromic stimulation (Fig. 3). Because synaptic stimulation excites presynaptic axons and terminals, followed by postsynaptic CA1 pyramidal neurons via glutamatergic synapses, whereas antidromic stimulation directly excites pyramidal neurons, these results indicate that electrically-induced adenosine release is due primarily to the excitation of pyramidal neurons, and does not require glutamate receptor activation. The findings, that response to synaptic stimulation was greater than the response to antidromic stimulation and that CNQX and D,L-APV did not completely inhibit adenosine release in response to synaptic stimulation, suggest that synaptic stimulation induces release from presynaptic and glial components, as well as from pyramidal neurons. However, this was unlikely, because synaptically- and antidromically-induced adenosine releases were found to be mediated by a mechanism in pyramidal neurons, in that these releases were similarly blocked by L-type calcium channel blockers as described later. Thus, it is more likely that synaptic stimulation induced greater adenosine release than antidromic stimulation because synaptic stimulation excites adenosine release sites of pyramidal neurons, presumably dendrites more effectively than antidromic stimulation when applied at the same intensity. In addition, high-frequency synaptic stimulation may have activated muscarinic and/or APV-insensitive extrasynaptic NMDA receptors and caused subsequent

depolarization (Suzuki *et al.* 2008, Fraser & MacVicar 1996) during CNQX- and D,L-APV-insensitive adenosine release.

L-type calcium channels and calcium-induced calcium release are involved in electrically-induced extracellular adenosine elevation

In characterizing extracellular adenosine elevation by adenosine sensor cells, the ability of each pharmacological agent to inhibit adenosine-induced calcium increases was assessed by exposing the cells to adenosine in the presence of each agent (e.g., Fig. 4B(b)), thus excluding agents that significantly alter calcium elevations in these cells. The calcium dependence of electrically-induced adenosine release was assessed by using cadmium, a non-selective calcium channel blocker, and nimodipine, a blocker of L-type calcium channels, the major subtype of voltage-gated calcium channels on the dendrites of CA1 pyramidal neurons (Hell *et al.* 1993). Synaptically- and antidromically-induced adenosine releases were blocked 84% and 82%, respectively, by 100 μ M cadmium and 87% and 79%, respectively, by 50 μ M nimodipine (Fig. 4A). These results indicated that L-type calcium channels of pyramidal neurons are essential for electrically-induced adenosine release. Subsequent pharmacological analysis focused on adenosine release by antidromic stimulation, which directly excites pyramidal neurons. As activation of L-type calcium channels and subsequent calcium-induced calcium release (CICR) have been reported to induce dendritic exocytosis (Kolarow *et al.* 2007), the involvement of CICR in electrically-induced adenosine release was assessed using thapsigargin, an irreversible sarco-endoplasmic calcium ATPase inhibitor, and ryanodine, an intracellular calcium channel blocker. Treatment of brain slices with thapsigargin prior to placement onto adenosine sensor cells blocked antidromically-induced adenosine release by 90% (Fig. 4B(a)). To assess the effect of ryanodine, adenosine release was induced by KCl as described in the Materials and Methods section, rather than by electrical stimulation. KCl-induced oscillatory adenosine release was inhibited 57% by 20 μ M ryanodine and 100% by thapsigargin (Fig. 4B (b), (c)). In addition, treatment with adenosine after KCl stimulation elevated calcium in adenosine sensor cells, even in the presence of ryanodine, or when placed below slices pre-treated with thapsigargin, indicating that the ability of adenosine sensor cells to respond to adenosine was preserved under these conditions (Fig. 4B(b)). As both KCl- and antidromically-induced adenosine releases were similarly blocked by thapsigargin, these adenosine releases are equivalent in involving calcium release. In addition, the inhibition by ryanodine suggests that CICR is the calcium release required for adenosine release following neuronal excitation.

As similar electrically-induced adenosine release in hippocampal slices has been reported to result from ATP release and its subsequent extracellular breakdown by ectonucleotidases (Heinrich *et al.* 2012, Cunha *et al.* 1996), the effects of the ectonucleotidase inhibitors, ARL67156 and AOPCP on KCl-induced adenosine release were evaluated. Slices were treated with a mixture of 100 μ M ARL67156 and 100 μ M AOPCP in the presence of 30 μ M PPADS, a non-selective purinergic antagonist, because inhibition of ectonucleotidases may result in the accumulation of extracellular ATP and subsequent ATP-induced calcium elevations in adenosine sensor cells. The finding, that ARL67156 and AOPCP did not inhibit

KCl-induced adenosine release (Fig. 5(a)), indicated that adenosine, rather than ATP, was released in response to antidromic stimulation.

Activity dependent adenosine release may be a consequence of ATP consumption, occurring after excretion of metabolically accumulated adenosine through equilibrate nucleoside transporter (ENT) (Wall & Dale 2013, Lovatt et al. 2012). Therefore, the ability of the ENT blockers, NBTI and dipyridamole (DIPY), to inhibit adenosine release was tested. A mixture of 5 μ M NBTI and 10 μ M DIPY, however, did not block adenosine release induced by antidromic stimulation (Fig. 5(b)), indicating that electrically-induced adenosine release does not involve ENT.

Extracellular adenosine elevation following astrocyte swelling

Inasmuch as astrocytes release nucleic acids and amino acids following swelling *in vitro* (Haskew-Layton *et al.* 2008, Liu et al. 2008), adenosine release was assessed in slices exposed to a hypoosmotic condition, as described in the Materials and Methods section, or to potassium channel blockers, which induce astrocyte swelling. Prior to slice experiments, the inability of hypoosmotic conditions and potassium channel blockers to induce calcium increases in adenosine sensor cells were confirmed. Exposure to hypoosmotic conditions induced oscillatory adenosine release, which was not detected in the presence of 8CPT and was not affected by cadmium (Fig. 6A), indicating that hypoosmotic treatment induces adenosine release by a pathway distinct from that induced by electrical stimulation. Exposure to the potassium channel blockers, tetraethylammonium (TEA), 4-aminopyridine (4-AP) and barium, which depolarize and swell astrocytes (O'Connor *et al.* 1993), induced oscillatory adenosine release, which was not detected in the presence of 8CPT (Fig. 6B). Cadmium significantly increased TEA-induced adenosine release by 60%, with similar results observed in response to other potassium channel blockers. Thus, adenosine release in response to treatments known to swell astrocytes is distinct from release in response to electrical activities of neurons.

Adenosine release in response to hypoosmotic conditions was further characterized by using an AQP-4 blocker, TGN-020. At a concentration of 100 μ M, TGN-020 significantly reduced hypoosmotically-induced adenosine release by 59%, but did not affect antidromically-induced adenosine release (Fig. 7). As AQP-4 expression is specific to astrocytes in the brain, these results confirmed that astrocyte swelling is involved in adenosine release in response to hypoosmotic treatment, but not to electrical stimulation. Because extracellular breakdown of ATP derived from astrocytes may increase extracellular adenosine in the hippocampus (Pascual *et al.* 2005, Wall & Dale 2013), the effect of ARL67156 and AOPCP on adenosine release in response to hypoosmotic treatment was analyzed. ARL67156 and AOPCP significantly reduced hypoosmotically-induced adenosine release by 55%, indicating that adenosine release in response to hypoosmotic treatment involves ATP breakdown by ectonucleotidases. Collectively, these results suggest that astrocyte swelling increases extracellular adenosine through a mechanism involving ATP release and subsequent breakdown. The partial inhibition by TGN-020 and by ARL67156 and AOPCP suggests that the adenosine release induced by hypoosmotic conditions was not exclusively

derived from ATP released by swelling astrocytes, but from other pathways involving neurons and vasculature.

Discussion

The adenosine sensor cell, in which intracellular calcium was increased in response to extracellular adenosine concentrations above 0.1 μM , allowed the detection of adenosine release from hippocampal slices in response to various conditions. The present pharmacological characterization suggests two distinct pathways for elevating extracellular adenosine; adenosine release from postsynaptic neurons following electrical activities in a manner depending on L-type calcium channels and CICR, and ATP release from swollen astrocytes and subsequent enzymatic breakdown to adenosine.

Measurement of extracellular adenosine elevation by adenosine sensor cells

The EC_{50} of adenosine for inducing calcium increase by adenosine sensor cells, which were HEK293 cells stably expressing mouse A1 receptor and Gqi5, was 8.0 μM . In comparison, the EC_{50} of adenosine for inducing calcium increase by HEK293 cells transiently expressing human A1 receptors and mouse Gqi5 was 1.4 μM (Rittiner *et al.* 2012), whereas the EC_{50} of adenosine for activating potassium current of arterial myocytes via A1 receptors was 3.1 μM (Ragazzi *et al.* 1991). Thus, the potency of adenosine to elicit responses of adenosine sensor cells was slightly lower, presumably reflecting the different levels of expression of receptor and G protein. The minimum adenosine concentration that increased calcium in adenosine sensor cells was 0.1 μM . Extracellular adenosine concentrations have been reported to be 0.2–2 μM in electrically-stimulated thalamic slices (Bekar *et al.*, 2008), 1–2 μM in hippocampal slices prepared from sleeping mice (Schmitt *et al.* 2012) and 10 μM in hippocampal slices stimulated with 25 mM KCl (Heinrich *et al.*, 2012). Thus, adenosine sensor cells are able to detect adenosine release from brain tissue.

Inosine had much lower potency than adenosine in inducing calcium increase in adenosine sensor cells, consistent with findings using HEK293 cells transiently expressing human A1 receptor and mouse Gqi5 (Rittiner *et al.* 2012). This is the advantage of adenosine sensor cells over enzymatic electrodes, which cannot distinguish adenosine from inosine. Extracellular elevation of inosine is reported to be smaller than hypoxia-induced adenosine release, which was 9.1 μM (Dale *et al.* 2000) or negligible during adenosine release following electrical stimulation (Wall & Dale 2013). Hypoxic treatment combined with the removal of extracellular calcium induced robust adenosine release (approximately 50 μM) and depleted the adenosine pool (Dale *et al.* 2000, Pearson *et al.* 2001). Thus, adenosine sensor cells, in which calcium was elevated in response to inosine concentrations above 100 μM , allows the selective detection of adenosine without interference by inosine.

To analyze the dynamics of extracellular adenosine in brain tissue preparations, calcium increases in adenosine sensor cells placed beneath the hippocampal slices were imaged. Slices in direct contact with the adenosine sensor cells did not likely impede the flow of extracellular solution around the cells or cause artifactual changes, including hypoxic adaptation, because bath-applied adenosine induced rapid responses in adenosine sensor cells. Moreover, a similar biosensor cell approach successfully measured ischemia-induced

glutamate release from brain slice preparations (Uchino *et al.* 2001). The spatial resolution of this method, which is limited by the size of the adenosine sensor cells, was sufficient to assess the influence of stimulus intensity on the distribution of adenosine increases. The temporal resolution of the present study was set to obtain 340 nm/380 nm ratio images of Fura2-loaded adenosine sensor cells every 3 sec (0.33 Hz). The time dependent changes in extracellular adenosine reflected in the intracellular calcium of individual cells were equivalent to those in previous studies using enzyme-based biosensor electrodes (Wall & Dale 2013, Klyuch *et al.* 2011). Other studies estimating extracellular adenosine by electrophysiological analysis of synaptic responses reported that even a single electrical stimulation changes the modulation of synaptic responses to adenosine (Mitchell *et al.* 1993), and that extracellular ATP is converted to adenosine within about 100 msec (Dunwiddie *et al.* 1997). Single electrical stimulations did not induce responses of adenosine sensor cells (data not shown), a situation similar to that in which enzyme-based biosensor electrodes detected adenosine release following a single electrical stimulation only in the presence of the potassium channel blocker, 4-AP (Klyuch *et al.* 2011), which induced adenosine release by itself in our study. Thus, neuronal activities are thought to be affected by more localized and faster changes in extracellular than tissue adenosine, as measured by adenosine sensor cells and enzyme-based biosensor electrodes. The adenosine sensor cell is a novel and powerful tool to better understand the role of tissue adenosine, which likely underlies sleep, cerebral circulation and inflammation.

Electrically-induced extracellular adenosine elevation

Electrical stimulation of the hippocampal CA1 region increased extracellular adenosine for several minutes. Glutamate receptor antagonists reduced adenosine release by synaptic stimulation, indicating that excitation of postsynaptic pyramidal neurons is involved in electrically-induced adenosine release. Antidromic stimulation, which directly excites pyramidal neurons, induced the release of adenosine insensitive to glutamate receptor antagonists, indicating that excitation of pyramidal neurons induces adenosine release without glutamate receptor activation. A previous study using enzymatic electrodes showed that hippocampal adenosine release in response to synaptic stimulation was completely blocked by the same glutamate receptor antagonists (Wall & Dale 2013). The results of this representative study using enzymatic electrodes suggest that synaptic stimulation induces neuronal adenosine release by ENT, which was pharmacologically excluded in the present study, and astrocyte ATP release, and both neuronal and astrocytic releases were in a glutamate receptor dependent manner. The discrepancy between this earlier report and our results may be due to the difference in experimental conditions, as well as in methodology. Especially, temperature was higher in experiments using enzymatic electrodes (34–36 °C), than in the present study (30 °C). Higher temperatures were avoided, because significant spontaneous calcium increases in adenosine sensor cells interfered with measurements above 30 °C. These spontaneous calcium increases were presumably due to temperature-dependent increases in ambient adenosine, which also interferes the measurement of postsynaptic potential via A1 receptors under similar high temperature conditions (Masino & Dunwiddie 1999, Masino *et al.* 2001). The fluctuation of ambient adenosine may be too slow or too localized to be detected by enzymatic electrodes; thus experiments using enzymatic electrodes prefer higher temperatures to increase adenosine release, presumably from ENT.

Astrocytes may release ATP in response to synaptic stimulation (Wall and Dale 2013), as shown using a transgenic mouse line expressing dominant negative SNARE, in an astrocyte selective manner (Pascual et al. 2005). However, this astrocytic component of adenosine release may reflect neuronal exocytosis, which was recently shown to be affected in this mouse cell line (Fujita *et al.* 2014).

The present study showed that electrically-induced adenosine release was inhibited by blocking L-type calcium channels or CICR. The exocytosis of brain-derived neurotrophic factor from dendrites of cultured neurons is also mediated by the same L-type calcium channels and CICR (Kolarow et al. 2007), suggesting that similar dendritic exocytosis underlies electrically-induced adenosine release. Exocytosis of adenosine is supported by findings showing synaptophysin-positive vesicles containing concentrative nucleoside transporter 2, which is responsible for adenosine accumulation in vesicles (Melani *et al.* 2012) and the presence of adenosine in the synaptic vesicle fraction (Corti *et al.* 2013).

As antidromically-induced adenosine release is not affected by the AQP-4 blocker, it does not involve ATP release following astrocyte swelling. However, astrocytes may contribute to electrically-induced adenosine release by an as yet unknown pathway. We examined this possibility by using a gliotoxin, fluoroacetate, which had been used to exclude the contribution of astrocyte ATP release to electrically-induced adenosine release measured with an enzymatic electrode (Wall & Dale 2013). However, as previously reported this agent alone increased ambient adenosine concentration (Canals *et al.* 2008) and significantly interfered with the measurement of evoked extracellular adenosine elevation by adenosine sensor cells (data not shown). Thus, the contribution of astrocytes to electrically-induced extracellular adenosine elevation has yet to be determined.

A previous study reported that the accumulation of extracellular adenosine and inosine in hippocampal slices during 5 min electrical stimulation at 10 Hz was reduced by blocking N- or P-type calcium channels (Latini *et al.* 1997). In this study, L-type calcium channels were involved only in the presence of an L-type calcium channel activator, BayK-8644. Thus, the higher frequency (30 Hz) used in the present study is likely required to activate L-type calcium channel dependent postsynaptic adenosine release. In contrast, the presynaptic pathway for elevating extracellular adenosine, which was suggested to be the N- and P-type calcium channel dependent component (Latini *et al.* 1997), in response to brief (5 sec) stimulation, was negligible in the present study. Thus, a single electrical stimulation and brief high-frequency stimulation, presumably resulting in rapid rundown of presynaptic activities, have little effect on extracellular adenosine, whereas continuous activation of presynaptic terminals results in the substantial accumulation of adenosine. The lack of a presynaptic component in the present study is consistent with studies using enzymatic electrodes. In those studies, electrically-induced elevation of extracellular adenosine was largely attributed to postsynaptic neurons and astrocytes in the hippocampus (Wall & Dale 2013), and to presynaptic termini in the cerebellum (Klyuch et al. 2012).

Extracellular adenosine elevation following astrocyte swelling

Hypoosmotic treatment and potassium channel blockers, which have been shown to cause astrocyte swelling, induced adenosine release in a manner independent of calcium channels.

The ability of an AQP-4 blocker to inhibit adenosine release induced by hypoosmotic conditions suggests that water influx and subsequent swelling of astrocytes are most likely involved in calcium channel independent adenosine release. The cocktail of ectonucleotidase inhibitors reduced the elevation of extracellular adenosine in response to hypoosmotic conditions but not to electrical stimulation, suggesting that the elevation in extracellular adenosine in response to hypoosmotic conditions involved ATP release. Although several studies have shown that swelling of cultured astrocytes in response to hypoosmotic conditions or high (>50 mM) KCl results in the release of various nucleic acids and amino acids (O'Connor & Kimelberg 1993, Liu et al. 2008, Rutledge & Kimelberg 1996, Thrane *et al.* 2011), the physiological implications of this release have not yet been fully addressed. Evidence showing that deficient exocytosis of astrocytes leads to low ambient adenosine, especially during sleep (Schmitt et al. 2012), is not necessarily incompatible with the present results, because exocytosis-related cellular machinery for membrane trafficking is presumably required to maintain plasma membranes during swelling.

Implications of multiple pathways for elevating extracellular adenosine

Adenosine signaling is involved in diverse physiological functions and pathological processes, including circulation, angiogenesis, metabolism, immune function and inflammatory diseases, neuronal activities, and neurological and psychiatric disorders (Fredholm *et al.* 2005, Gomes *et al.* 2011). Thus, adenosine and caffeine, a non-selective adenosine receptor antagonist, are used clinically (Mosqueda-Garcia 1992, Sawynok 1995), and a number of drugs are in development targeting this signaling pathway (Chen *et al.* 2013b). However, widespread adenosine signaling hampers specific clinical applications of adenosine receptor ligands. Thus, modulating mechanisms regulating extracellular adenosine concentration are alternative therapeutic options. Indeed, the effects of several clinically-applied drugs, including methotrexate and dipyridamole, may be due in part to alterations in extracellular adenosine concentration, and deep brain stimulation has been shown to reduce tremor by stimulating ATP/adenosine release (Bekar *et al.* 2008). Thus, further understanding of the dynamics and underlying regulatory mechanisms of tissue adenosine is expected to provide future therapeutic targets in adenosine signaling. Basic research and drug discovery in this field will be accelerated by detection of tissue adenosine by adenosine sensor cells.

The present study, involving the pharmacological analysis of extracellular adenosine elevations in response to various conditions, showed that distinct pathways regulate extracellular adenosine. It is noteworthy that extracellular adenosine elevation in response to electrical stimulation depends on dendritic calcium, rather than synaptic transmission. These findings implies that extracellular adenosine reflects activities in the neuronal network and regulates information processing of neuronal circuits.

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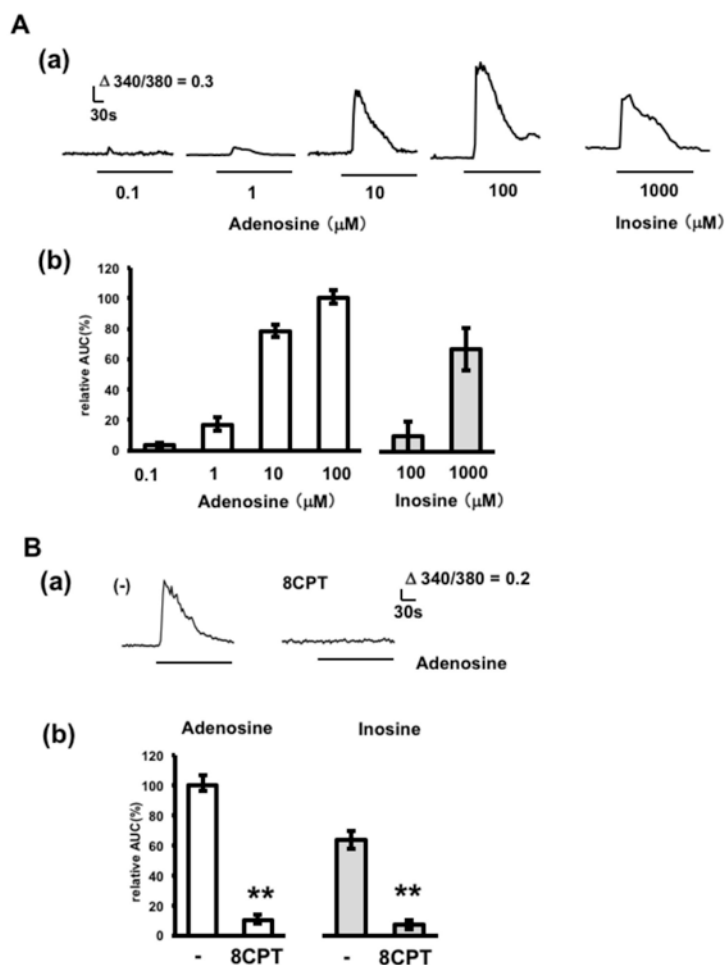
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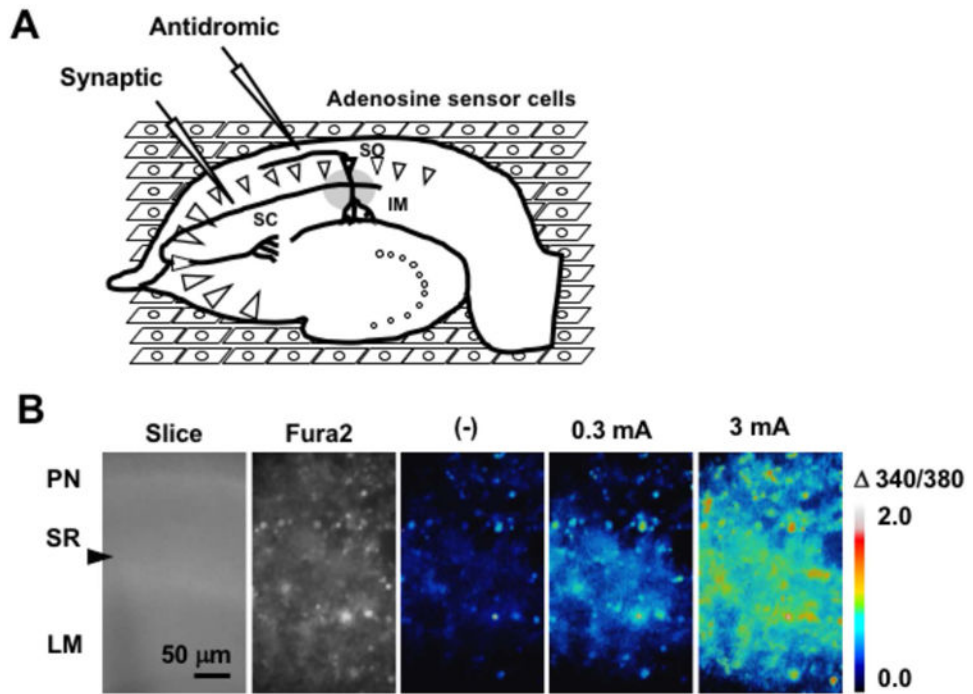
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Abbreviations

AOCPC	$\alpha\beta$ -methylene-ADP
aCSF	artificial cerebrospinal fluid
4-AP	4-aminopyridine
APV	(2R)-amino 5-phosphonovaleric acid
AQP-4	aquaporin 4
CICR	calcium-induced calcium release
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
DIPY	dipyridamole
ENT	equilibrate nucleoside transporter
NBTI	S-(4-nitrobenzyl)-6-thioinosine
PPADS	pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
TEA	tetraethylammonium

**Figure 1.**

Adenosine- and inosine-induced calcium elevations in adenosine sensor cells. A. Concentration dependent effects of adenosine and inosine. (a) Representative calcium increases in response to 0.1-100 μM adenosine and 1000 μM inosine. (c) Average calcium increases (n=29–40 cells from 3–4 experiments). Cells were stimulated with adenosine or inosine at the concentrations indicated. The relative area under the curve during stimulation (relative AUC) was calculated by normalizing to the calcium increase achieved by 100 μM adenosine. B. Effect of an A1 receptor antagonist. Cells were stimulated with 10 μM adenosine or 1000 μM inosine in the presence or absence of 1 μM 8CPT. 8CPT treatment was started 5 min prior to stimulation with adenosine and continued during stimulation (pretreatment; 5 min). (a) Representative adenosine-induced calcium increases in the presence or absence of 8CPT. (b) Average calcium increases (n= 30–40 cells from 3–4 experiments). Relative AUC was calculated by normalizing to the calcium increase to adenosine in the absence of blocker. **p < 0.01, t-test.



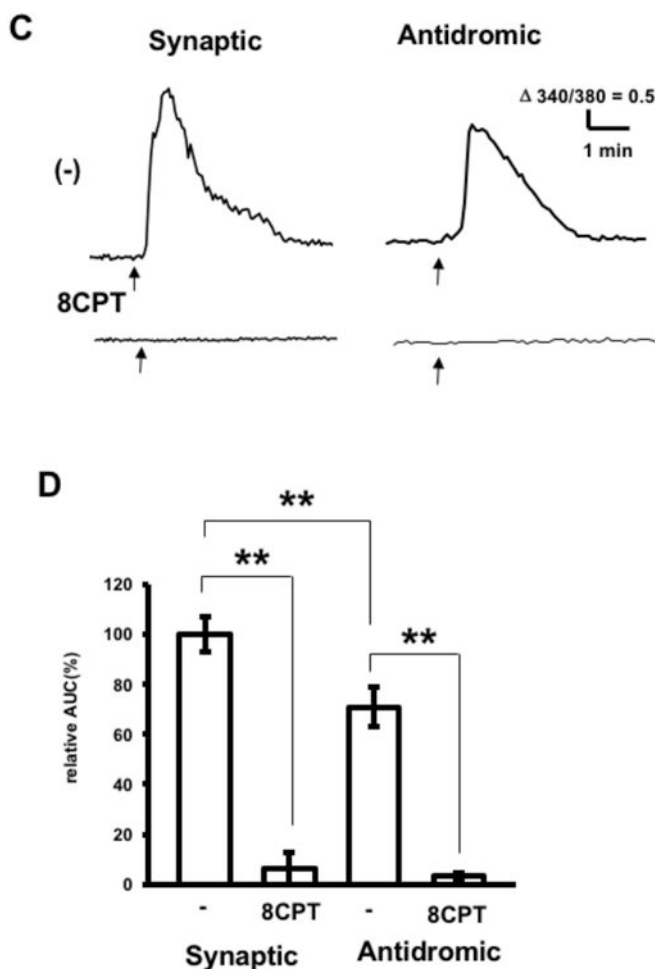


Figure 2.

Electrically-induced adenosine release by hippocampal slices. A. Experimental diagram. SC, Schaffer collateral; SO, Striatum oriens; IM, Imaging area of the inverted microscope (gray circular area). The stimulating electrode was placed on the SC for synaptic stimulation (Synaptic) or on the SO for antidromic stimulation (Antidromic). B. Calcium responses of adenosine sensor cells to synaptic stimulation. Bright field image of a hippocampal slice above the adenosine sensor cells (Slice). PN; Pyramidal neurons in the CA1 region; SR, Stratum radiatum; LM, Stratum lacunosum moleculare. The stimulating glass electrode was placed as indicated by the arrowhead. Fura2 fluorescence image of the adenosine sensor cells (Fura2). Ratio of the image (340 nm/380 nm) before stimulation (-), and 15 sec after high-frequency electrical stimulation (30 Hz for 5 sec) at 0.3 mA or 3 mA. C. Representative calcium increases in adenosine sensor cells in response to synaptic or antidromic stimulation (3 mA, 30 Hz for 5 sec, arrow) in the absence or presence of 8CPT (pretreatment 20 min). D. Average calcium increases in adenosine sensor cells in response to synaptic or antidromic stimulation, with results normalized to response to synaptic stimulation in the absence of 8CPT. Each n= 29–47 cells from 3–5 experiments, **p < 0.01, t-test.

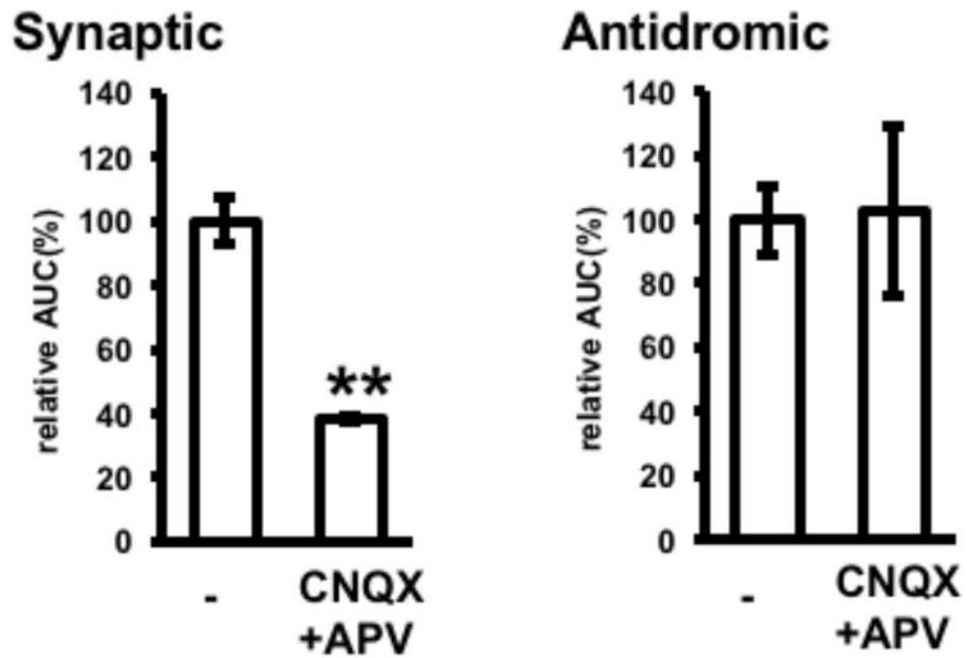


Figure 3. Effects of glutamate receptor antagonists on electrically-induced adenosine release. Adenosine release in response to synaptic or antidromic stimulation was assessed in the absence or presence of 10 μ M CNQX and 100 μ M D,L-APV. $n = 30$ – 49 cells from 3–5 experiments, $**p < 0.01$, t-test

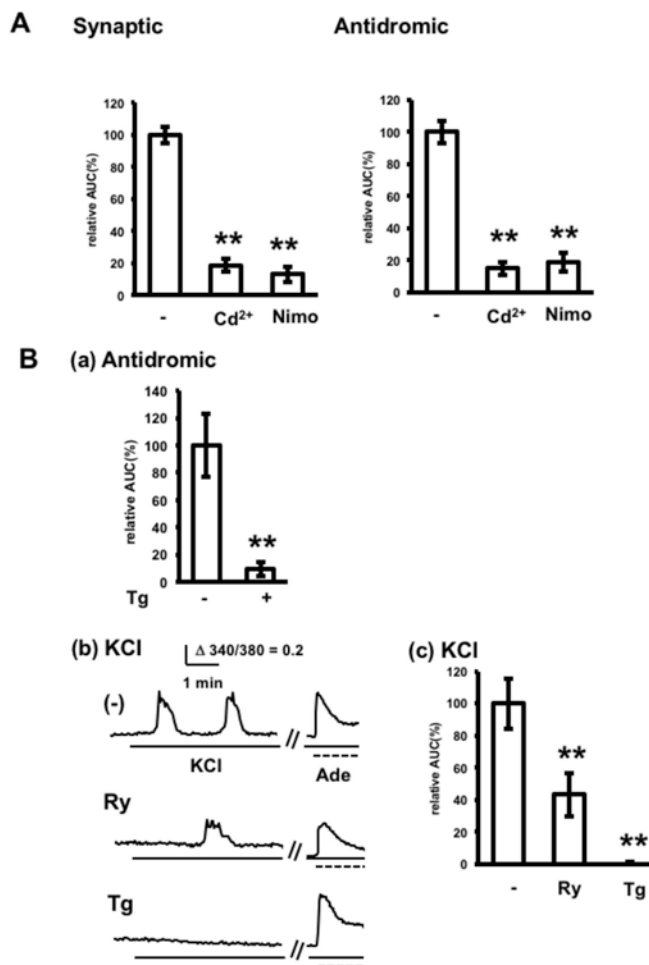


Figure 4. Involvement of L-type calcium channels and CICR in electrically-induced adenosine release. A. Pharmacologic inhibition of calcium channels. Adenosine release in response to synaptic or antidromic stimulation in the absence or presence of 100 μM Cd^{2+} (pretreatment; 10 min) or 50 μM nimodipine (Nimo, pretreatment; 30 min). $n = 23\text{--}30$ cells from 3–4 experiments, $**p < 0.01$, Dunnett's multiple comparison test with control. B. Pharmacologic inhibition of CICR. (a) Adenosine release by normal and thapsigargin-treated (Tg, 1 μM) slices in response to antidromic stimulation. Slices were treated with 1 μM thapsigargin for 15 min, washed three times with normal aCSF, and placed onto adenosine sensor cells in normal aCSF for electrical stimulation. $n = 30$ cells from 3 experiments, $**p < 0.01$, t-test. (b) Effects of ryanodine and thapsigargin on adenosine release in response to KCl stimulation. Representative calcium increases in adenosine sensor cells. Adenosine release was induced by bath application of KCl (final concentration, 25 mM) as indicated by solid lines, and the lack of effect of ryanodine and thapsigargin on the adenosine response of adenosine sensor cells was confirmed by bath applications of adenosine (final concentration, 10 μM) at the end of the experiment (dashed line). Ryanodine treatment (Ry, 20 μM) was started 30 min prior to KCl stimulation. Thapsigargin was used as in Fig 4B(a). (c) Average KCl-induced calcium increases in adenosine sensor cells in the presence or absence of ryanodine or

thapsigargin. Each n= 30 cells from 3 experiments, **p < 0.01, Dunnett's multiple comparison test with control.

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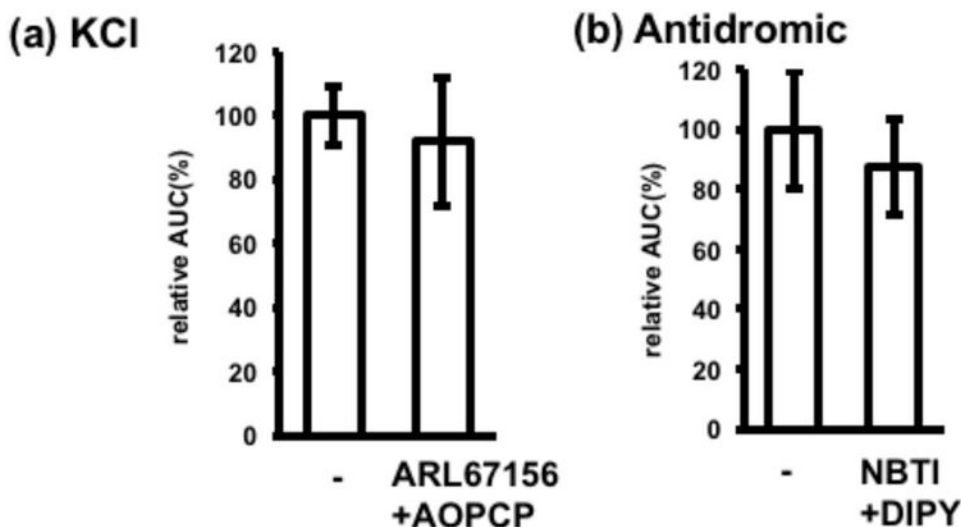


Figure 5.

Lack of involvement of ectonucleotidases and equilibrate nucleoside transporter (ENT) in electrically-induced adenosine release. (a) KCl-induced adenosine release in the absence or presence of 100 μ M ARL67156 and 100 μ M AOPCP (pretreatment; 15 min). As the inhibition of ectonucleotidases may cause extracellular ATP build-up and ATP-induced calcium increase in adenosine sensor cells, a P2 receptor antagonist, 30 μ M PPADS, was added to the extracellular solution. Each $n=30$ cells from 3 experiments, $**p < 0.01$, t-test. (b) Adenosine release in response to antidromic stimulation in the absence or presence of 5 μ M NBTI and 10 μ M DIPY (pretreatment; 30 min). As DIPY has autofluorescence blocking ultraviolet excitation of Fura2AM, Rhod4AM was used. Each $n=28-34$ cells from 3-4 experiments. $**p < 0.01$, t-test.

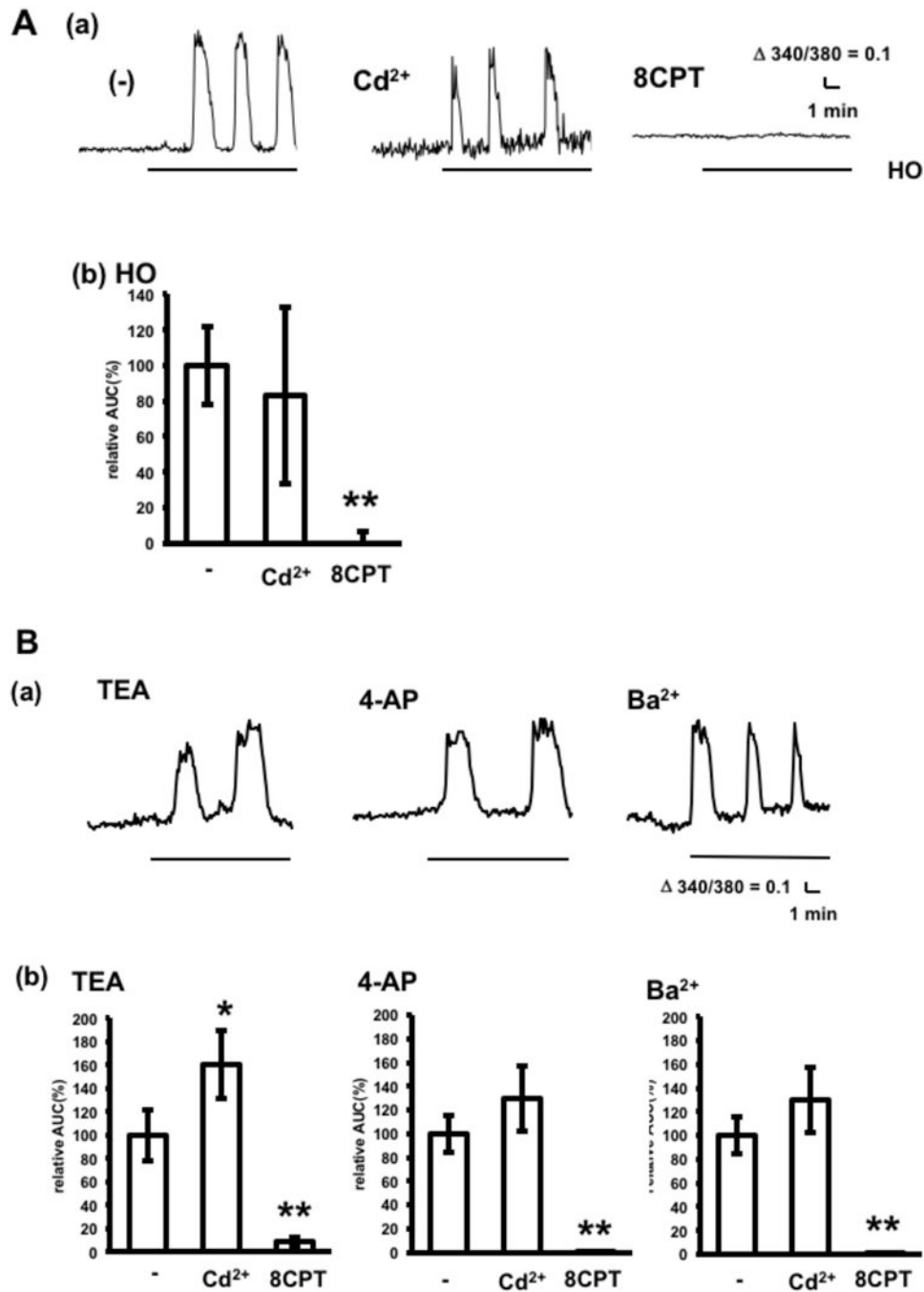


Figure 6. Adenosine release by treatments known to swell astrocytes. A. Calcium increases in adenosine sensor cells below hippocampal slices following hypoosmotic treatment (HO) in the absence or presence of 100 μM Cd^{2+} or 1 μM 8CPT. (a) Representative calcium increases in adenosine sensor cells. (b) Average calcium increases. $n = 18\text{--}30$ cells from three experiments. B. Adenosine release in response to potassium channel blockers. Slices were treated with 25 mM TEA, 100 μM 4-AP or 6 mM Ba^{2+} for 10 min in the presence or absence of 100 μM Cd^{2+} or 1 μM 8CPT. (a) Representative calcium increases in adenosine

sensor cells following treatment with potassium channel blockers. (b) Average calcium increases. n= 29–39 cells from 3–4 experiments. *p < 0.05, **p < 0.01, Dunnett's multiple comparison test with control.

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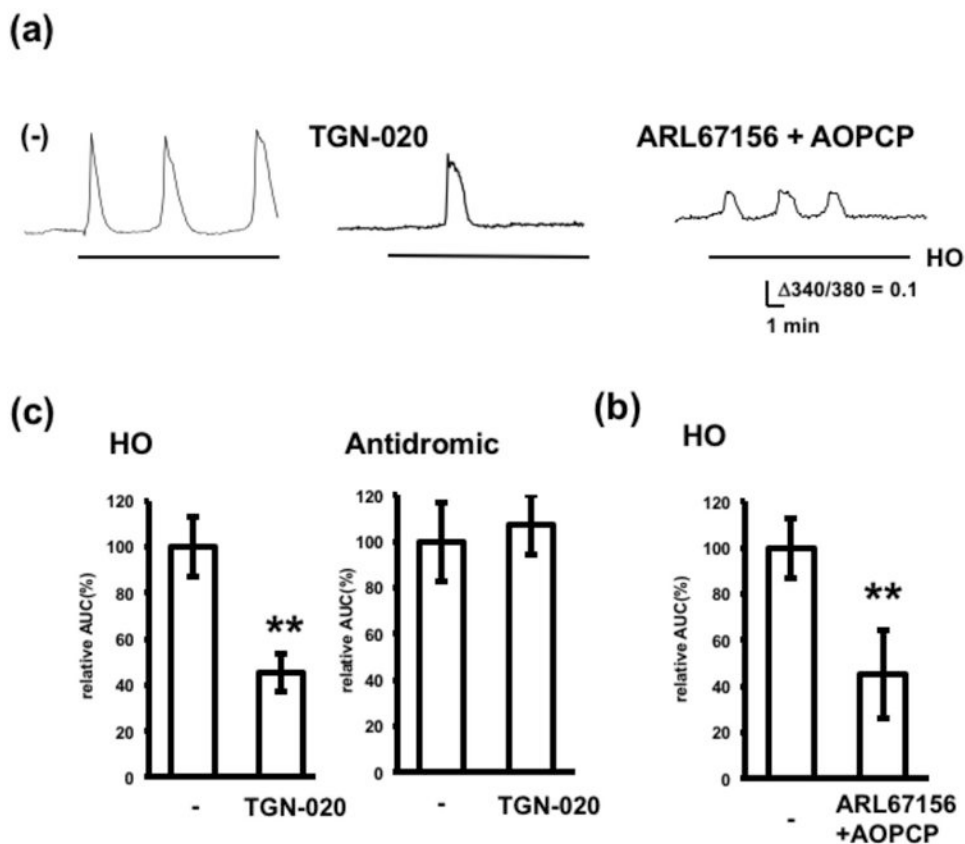


Figure 7. Involvement of AQP-4 and ectonucleotidases in hypoosmotically-induced adenosine release. Adenosine release induced by hypoosmotic conditions or antidromic stimulation was measured in the presence or absence of 100 μ M TGN-020 (pretreatment; 20 min) or the mixture of ARL67156, AOPCP and PPADS (pretreatment; 15 min). (a) Representative calcium increases in the adenosine sensor cells. (b) Effects of TGN-020 on hypoosmotically- and antidromically-induced adenosine release. Average calcium increases. $n = 20\text{--}30$ cells from 3 experiments. $**p < 0.01$, t-test. (c) Effect of ARL67156 and AOPCP on hypoosmotically-induced adenosine release. Average calcium increases. $n = 20\text{--}30$ cells from 3 experiments. $**p < 0.01$, t-test.