ATP potently modulates anion channel-mediated excitatory amino acid release from cultured astrocytes

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Mongin, Alexander A., and Harold K. Kimelberg. ATP potently modulates anion channel-mediated excitatory amino acid release from cultured astrocytes. Am J Physiol Cell Physiol 283: C569–C578, 2002. First published April 18, 2002; 10.1152/ajpcell.00438.2001.—Volume-dependent ATP release and subsequent activation of purinergic P2Y receptors have been implicated as an autocrine mechanism triggering activation of volume-regulated anion channels (VRACs) in hepatoma cells. In the brain ATP is released by both neurons and astrocytes and participates in intercellular communication. We explored whether ATP triggers or modulates the release of excitatory amino acid (EAAs) via VRACs in astrocytes in primary culture. Under basal conditions exogenous ATP (10 μM) activated a small EAA release in 70–80% of the cultures tested. In both moderately (5% reduction of medium osmolality) and substantially (35% reduction of medium osmolality) swollen astrocytes, exogenous ATP greatly potentiated EAA release. The effects of ATP were mimicked by P2Y agonists and eliminated by P2Y antagonists or the ATP scavenger apyrase. In contrast, the same pharmacological maneuvers did not inhibit volume-dependent EAA release in the absence of exogenous ATP, ruling out a requirement of autocrine ATP release for VRAC activation. The ATP effect in nonswollen and moderately swollen cells was eliminated by a 5–10% increase in medium osmolality or by anion channel blockers but was insensitive to tetanus toxin pretreatment, further supporting VRAC involvement. Our data suggest that in astrocytes ATP does not trigger EAA release itself but acts synergistically with cell swelling. Moderate cell swelling and ATP may serve as two cooperative signals in bidirectional neuron-astrocyte communication in vivo.

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Adenosine, in regulation of EAA release under conditions of mild “physiological” swelling, resembling changes on physiological neuronal excitation. The results of this study have been presented and published in a preliminary form (44).

MATERIALS AND METHODS

Materials. d-[3H]aspartate (specific activity 18 Ci/mM) was obtained from Du Pont-NEN Research Products (Boston, MA). Dispase (neutral protease dispase grade II) was purchased from Boehringer Mannheim (Indianapolis, IN). All cell culture reagents were from GIBCO (Grand Island, NY). 1’-(N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl)-4-phenoxyphosphorazone (KN-62) and tetanus toxin were from Calbiochem (La Jolla, CA). Alloxazine, β,γ-methylene-δ-adenosine 5’-triphosphate disodium salt (β,γ-MeATP), and pyridoxal phosphate-6-azophenyl-2‘,4′-disulfonic acid (PPADS) were from RBI (Natick, MA). (S)-dihydroxyphenylglycine (DHPG) and reactive blue 2 were from Tocris Cookson (Ballwin, MO). Suramin, ATP, two-sodium salt, 2-methylthioadenosine 5’-triphosphate (2-MeSATP), and other chemicals, unless otherwise specified, were from Sigma (St. Louis, MO).

Cell cultures. Confluent primary astrocyte cultures were prepared from the cerebral cortex of newborn Sprague-Dawley rats as described by Frangakis and Kimelberg (19), with minor modifications as described below. All animal procedures were performed according to the NIH guide for animal care and approved by the institutional animal care committee. The cerebral cortices were separated from meninges and basal ganglia, and tissue was dissociated with the neutral protease dispase. Dissociated cells were seeded on poly-lysine-coated 18 × 18-mm glass coverslips (Carolina Biological Supply, Burlington, NC) and grown for 3–4 wk in minimal essential medium (MEM) supplemented with 10% heat-inactivated horse serum (HIHS), 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified 5% CO2–95% air atmosphere. Culture medium was replaced twice a week. After 2 wk of cultivation, penicillin and streptomycin were removed from the culture medium. Immunocytochemistry showed that ≥98% of the cells stained positively for the astrocytic marker glial fibrillary acid protein.

Excitatory amino acid efflux measurements. Excitatory amino acid efflux measurements were performed as previously described (43). Astrocytes grown on glass coverslips were loaded overnight with d-[3H]aspartate (4 μCi/ml, final concentration 220 nM) in 2.5 ml of MEM containing 10% HIHS in an incubator set for 5% CO2–95% air at 37°C. Before the start of the efflux measurements, the cells were washed of extracellular isotope and serum-containing medium in HEPES-buffered solution. The basal HEPES-buffered medium contained (in mM) 122 NaCl, 3.5 KCl, 0.4 MgSO4, 1.3 CaCl2, 1.2 KH2PO4, 10 n-glucose, and 25 HEPES. pH was adjusted to 7.4 with NaOH (~15 mM). The coverslips were inserted into a Lucite perfusion chamber that had a depression precisely cut in the bottom to accommodate the coverslip and a Teflon screw top leaving a space above the cells of ~100 μm in height. The cells were superfused at a flow rate of 1.0 ml/min in an incubator set at 37°C with HEPES-buffered medium. In hyposmotic media NaCl concentration was reduced to 115 mM (~7 mM NaCl, a 5% decrease in medium osmolality) or to 72 mM (~50 mM NaCl, a 35% decrease in medium osmolality). Hypersmotic media were made by adding sucrose. The osmolalities of all buffers were checked with a freezing point osmometer (Advanced Instruments, Needham Heights, MA). Superfusate fractions were collected at 1-min intervals. At the end of each experiment, the isotope remaining in the cells was extracted with a solution containing 1% sodium dodecyl sulfate plus 4 mM EDTA. Four milliliters of Ecoscint scintillation cocktail (National Diagnostics, Atlanta, GA) was added, and each fraction was counted for 3H in a Packard Tri-Carb 1900TR liquid scintillation analyzer (Packard Instrument, Meriden, CT). Percent fractional isotope release for each time point was calculated by dividing radioactivity released in each 1-min interval by the radioactivity left in the cells (the sum of all the radioactive counts in the remaining fractions up to the beginning of the fraction being measured plus the radioactivity left in the cell digest) with a custom computer program.

ATP assays. ATP assays were done with luciferin-luciferase reaction as described elsewhere (68). Cells in 12-well culture plates were washed several times with isosmotic medium and then exposed for 1–10 min to isosmotic or hypoosmotic medium (for compositions see Excitatory amino acid efflux measurements). Aliquots of extracellular medium (100 μl) were taken for extracellular ATP determination. Extracellular medium was then aspirated, cells were lysed with 0.1% Triton X-100 (1 ml), and 40-μl aliquots were used for intracellular ATP content measurements in the same well.

Statistical analysis. Data are presented as mean ± SE values of 3–10 experiments performed on at least two different astrocyte preparations. Effects of all agonists and antagonists were always compared with the controls performed on the same day and on the same culture preparation. The data were analyzed by one-way ANOVA followed by post hoc Newman-Keuls when multiple comparisons were made.

RESULTS

Synergistic effect of moderate cell swelling and ATP on EAA release in astrocytes. In 14 of 18 astrocyte cultures tested, 10 μM ATP under isosmotic conditions induced transient stimulation of d-[3H]aspartate release with maximal release rates of 50–120% over basal release levels (Fig. 1). Four cultures did not respond to ATP under isosmotic conditions (data not shown). Because nonpathological neuronal excitation can induce moderate swelling of astrocytes (1), likely due to uptake of released K+ and glutamate (31), we tested ATP effects on EAA release in cells subjected to mild hypotonic stress. Five percent reduction of extracellular medium osmolality by itself led to a twenty to forty-five percent increase in EAA release over the basal levels (Fig. 1B). When 10 μM ATP was added to the hypoosmotic medium, it caused a larger transient potentiation of EAA release compared with the ATP effect under basal conditions (Fig. 1). It should be stressed that ATP-induced potentiation was found even in those cultures not responding to ATP under basal conditions. The ATP-induced release increased with a further decrease in medium osmolality (Fig. 1). In contrast, when medium osmolality was increased by 5% and 10% (14 and 28 mM sucrose addition, respectively), this potently suppressed the ATP-induced EAA release to near-basal levels (Fig. 1).

EAA release in cultured astrocytes can occur by several transport mechanisms via VRACs (32), a Ca2+- sensitive mechanism exhibiting several characteristics of neuronal exocytosis (5, 54) and reversal of Na+-
dependent glutamate transporters, which may be seen only with elevated extracellular K\(^+\) concentration and intracellular Na\(^+\) concentration (67). Therefore, we tested for the involvement of VRACs in ATP-induced D-[3H]aspartate efflux by using the broad-spectrum anion channel inhibitors 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and DIDS. These compounds reduced the basal EAA release by 25–40% (Fig. 2A) and largely inhibited the ATP-induced release under conditions of mild hypotonic cell swelling (Fig. 2B). When tested in the same cell cultures, the ATP-induced release under conditions of moderate cell swelling was equally sensitive to 100 μM NPPB, and more sensitive to 200 μM DIDS, compared with the well-characterized VRAC-mediated EAA release induced by substantial hyposmotic cell swelling (Fig. 2B). High extracellular ATP concentration (10 mM), known to inhibit VRAC-mediated Cl\(^-\) currents (52), suppressed EAA release by ~50% in substantially swollen cells and produced a complete inhibition in moderately swollen cells (Fig. 2B). In addition, we tested phloretin, an inhibitor recently found to discriminate between VRACs and several other types of Cl\(^-\) channels (17). Phloretin at 100 μM inhibited the release in both moderately and substantially swollen cells and was more potent in moderately swollen cells, just like DIDS and ATP (Fig. 2B).

To check for a potential contribution of a Ca\(^{2+}\)-dependent exocytosis-like mechanism to the ATP effect, we pretreated cells with tetanus toxin for 24 h (60). Such pretreatment had no effect on ATP-induced EAA release in moderately swollen cells or hyposmotic medium-induced release in substantially swollen cells (Fig. 3).

Effects of P2 receptor agonists and antagonists on astrocytic EAA release. To explore what receptor subtype mediates the ATP effects under conditions of moderate cell swelling, we used agonists and antagonists for P2Y/P2U and P2X receptors. 2-MeSATP, a potent agonist for P2Y\(_1\) and some P2X receptors, and UTP, which is active at P2X\(_1\) and some P2X receptors, and UTP, which is active at P2X receptors (group of UTP-sensi-
tive P2Y receptors including P2Y2, P2Y4, and P2Y6), both increased EAA release in moderately swollen cells (Fig. 4A). Besides several P2Y receptor subtypes, cultured astrocytes also express two P2X receptors, P2X1 and P2X7 (3, 41). These receptors seem not to be involved in the ATP effects, because both the P2X1 agonist β,γ-MeATP (10 μM, Fig. 4A) and the P2X7 antagonist KN-62 (1 μM, n = 3, P > 0.85; data not shown) were ineffective. The P2Y1 antagonist reactive blue 2 (10 μM) completely inhibited the effect of 2-MeSATP and partially suppressed the effects of ATP and UTP (Fig. 4B). Although in the experiments presented in Fig. 4B inhibition of the ATP effect by reactive blue 2 was not statistically significant, this partial inhibition was reproduced in two other cultures. As an additional approach to blocking ATP receptors, we used a 10-min preexposure to ATP to desensitize P2 receptors (64). Preincubation with ATP completely suppressed further ATP-induced potentiation of EAA release in moderately swollen cells (Fig. 4C). Adenosine and the selective group I metabotropic glutamate receptor agonist DHPG, both known to increase intracellular Ca2+ concentration in cultured and acutely isolated astrocytes (6, 62), did not affect EAA release in astrocyte cultures exposed to mild hypotonic stress (Fig. 4A).

Substantial cell swelling induces ATP release from cultured astrocytes. In several cell types, and particularly in hepatoma cells, swelling induces endogenous ATP release, which then activates volume-dependent anion channels (79). We found a large release of endogenous ATP, measured as accumulation of ATP in extracellular medium under nonperfusion conditions, in confluent astrocyte cultures exposed to substantial hypoosmotic stress (35% reduction in medium osmolality; Fig. 5A). During the first 2 min of hypotonic exposure, the extracellular ATP concentration increased ~4.5 times, equivalent to the release of ~5% of the total cell ATP content. After this first phase the rate of ATP release was decreased despite the persistence of an osmotic gradient. The hypoosmotic medium-induced ATP release was not associated with significant changes in intracellular ATP content (Fig. 5B), suggesting a compensatory increase in cellular ATP production. This also rules out cell lysis as a reason for ATP release. In our previous work (43), we also did not
observe any significant cell lysis in hypoosmotic media by monitoring the release of preloaded $^{51}$Cr. In parallel experiments performed on the same culture preparation, a change of medium from isosmotic to hypoosmotic led to a ~70% increase in extracellular ATP content; this rise was 2.7 times smaller compared with hypoosmotic conditions (Fig. 5A). These results indicate that mechanostimulation of astrocytes during changes of medium is not the major cause for the ATP release under hypoosmotic conditions.

Effects of extracellular ATP scavenging and purinergic receptor antagonists on EAA release activated by substantial cell swelling. To check whether the autocrine release of endogenous ATP is the cause for activation of astrocytic volume-dependent anion channels, as reported by Wang et al. (79) for hepatoma cells, we added apyrase to hydrolyze extracellular ATP and also several P2 receptor inhibitors. Scavenging of extracellular ATP with apyrase (5 U/ml) reduced swelling-activated $\text{d-[^{3}H]aspartate}$ release by 20–30% (Fig. 6A), but this effect was not statistically significant. Efficiency of ATP scavenging by apyrase was checked in the experiments with exogenously added $10 \mu M$ ATP, where apyrase completely inhibited the ATP-induced potentiation of EAA release ($n = 3$; data not shown).

The broad-spectrum P2 receptor antagonists suramin (100 $\mu M$) and PPADS (50 $\mu M$) (64) blocked the hypoosmotic medium-induced EAA release by 67% and 60%, respectively (Fig. 6, B and C). In contrast to these agents, reactive blue 2, a more selective inhibitor of the P2Y receptor subfamily (it also inhibits P2X2 receptors; Ref. 27), failed to block the swelling-induced EAA release at concentrations of 10 and 50 $\mu M$ (Fig. 6C).

P2Y receptor-mediated modulation of EAA release in substantially swollen astrocytes. Although endogenous ATP release does not seem to be necessary for the

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**Fig. 5.** ATP release (A) and ATP content (B) in primary astrocyte cultures exposed to substantial hypoosmotic swelling (35% reduction in medium osmolarity). At time 0 basal HEPES-buffered solution (287 mosM) was replaced with the same solution (Iso) or a hypoosmotic (Hypo, $-50 \text{ mM NaCl}$, 190 mosM) solution. Two, five, and ten minutes later, samples of extracellular media were taken for ATP assay. Cells were lysed with 0.1% Triton X-100 for measurements of ATP content in the same well as described in MATERIALS AND METHODS. Data are means ± SE of 9 wells. *$P < 0.05$, **$P < 0.01$ compared with ATP release before a change of medium.

**Fig. 6.** Effects of extracellular ATP scavenging and P2 receptor antagonists on astrocytic EAA release induced by substantial hypoosmotic swelling (35% reduction in medium osmolarity). A: swelling-activated $\text{d-[^{3}H]aspartate}$ release from astrocyte cultures in the presence of the ATP scavenger apyrase (5 U/ml). Data are means ± SE of 3 experiments in each condition performed in the same culture preparation. B: representative effect of the P2 inhibitor suramin (100 $\mu M$) on swelling-activated $\text{d-[^{3}H]aspartate}$ release. C: comparison of the average effects of the P2 receptor antagonists suramin, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), and reactive blue 2 on hypoosmotic medium-induced astrocytic EAA release. Data are means ± SE of 3–4 experiments for each condition using a total of 2 different culture preparations. Inhibition was calculated for the maximal release at 22–23 min. **$P < 0.01$ compared with controls performed in the same cultures.
activation of astrocytic VRACs (see Fig. 6A), this does not exclude the possibility of modulatory effects of ATP in substantially swollen cells. Because we measure the volume-dependent EAA release with a superfusion system, removal of endogenously released ATP will likely diminish the activation of purinergic receptors, masking potential modulatory effects of ATP on VRACs. We therefore tested the effects of exogenous ATP, P2X, and P2Y receptor agonists added to the superfusion medium on EAA release. At concentrations of 10 μM, ATP and the P2Y agonists UTP and 2-MeSATP increased volume-dependent δ[^3H]aspartate release 2.5–3.5 times with a potency in the order of UTP > ATP = 2-MeSATP (Figs. 7, A and B). In contrast, βγ-MeATP (10 μM), an agonist selective toward P2X1 and P2X3 receptors (27), did not significantly affect the swelling-induced EAA release (Fig. 7B). The ATP-induced increment in EAA release was nearly completely suppressed by the P2Y antagonist reactive blue 2 (Fig. 7A; for the lack of reactive blue 2 effect on release induced by substantial cell swelling, see Fig. 6C). Potentiating effects of other P2Y agonists were also suppressed by reactive blue 2. As in the case of moderately swollen cells, the 2-MeSATP-induced increment in EAA release was completely inhibited by reactive blue 2 (~95% inhibition, n = 3; data not shown), but the UTP effect was only partially sensitive to reactive blue 2 (~40% inhibition, n = 5; data not shown). Desensitization of purinergic receptors by a 10-min preexposure to ATP completely inhibited any subsequent ATP-induced increment in EAA release in substantially swollen cells (Fig. 7C). It should be noted, however, that although ATP receptor desensitization eliminated the ATP effect, the volume-dependent component of EAA release remained unchanged (compare with the control volume-dependent release in Fig. 7C). These data further support the view that activation of the P2Y receptors is not a necessary step in the activation of anion channels but is rather involved in channel modulation.

Effect of adenosine on volume-dependent EAA release in substantially swollen cells. In brain tissue extracellular ATP is rapidly hydrolyzed to adenosine by ecto-ATPases and ecto-5′-nucleotidase (80). Astrocytes in situ and in culture express several types of adenosine receptors (62, 63), and adenosine receptors have also been implicated in the activation (8, 9) or modulation (46) of volume-dependent anion channels in tracheal and ciliary epithelial cells. We therefore tested the effects of adenosine and the A2B adenosine receptor antagonist alloxazine, on volume-dependent EAA release in substantially swollen cells. Adenosine (100 μM) potentiated hyposmotic medium-induced δ[^3H]aspartate release by ~100% (Fig. 8). This was different from the lack of adenosine effect in moderately swollen cells (Fig. 4A). In substantially swollen cells, alloxazine (2 μM) suppressed the adenosine effect by 70% but by itself did not affect control volume-dependent EAA release (Fig. 8).

DISCUSSION

In this study we demonstrate that ATP works synergistically with cell swelling in activating EAA release from primary astrocyte cultures. This release is likely mediated by volume-dependent anion channels, which are permeable to Cl⁻ and small organic anions and uncharged molecules (35, 73). The ATP effect strongly depends on cell volume; even a small degree of cell shrinkage completely eliminates the ATP-induced EAA release observed in 80% astrocyte cultures not
subjected to osmotic gradients. This is opposite to the idea of a direct volume-independent activation of anion channels by ATP in nonswollen cells and is important for understanding the mechanisms of ATP actions in vivo. Our pharmacological data suggest that ATP exerts its modulatory effects on EAA release from astrocytes via activation of P2Y receptors.

**Extracellular ATP is a potent modulator of astrocytic volume-dependent anion channels but is not required for their activation.** ATP can be released from cells by a large variety of factors including changes in cell volume, hypoxia, mechanical stress, cAMP, and receptor stimulation (reviewed in Refs. 10 and 18). In the CNS and peripheral nervous system (PNS), ATP is also coreleased synaptically with other neurotransmitters (16, 30, 64). From this perspective, the finding by Wang et al. (79) on the autocrine activation of anion channels by ATP with or without changes in cell volume may have special importance in the brain. It is possible that the ATP-induced glutamate release from astrocytes, which has been proposed to mediate glial-neuronal communication (7, 18), is due to activation of VRACs showing significant permeability toward EAs (26, 32). Similar to hepatoma cells, Darby et al. (13) found both inhibition of volume-sensitive Cl− currents by P2 antagonists or apyrase in swollen cultured astrocytes and activation of Cl− currents by exogenous ATP in nonswollen cells. More recently, Jeremic et al. (28) observed stimulation of NPPB-sensitive endogenous glutamate and aspartate release in astrocyte cultures on application of 100 μM ATP.

Our findings argue against autocrine ATP release as a necessary and sufficient signal for VRAC activation in astrocyte cultures. In our experiments exogenous ATP addition induced only a small EAA release under basal conditions, but this effect was completely suppressed by small increases in medium osmolarity. The simplest explanation for this observation is that ATP modulates a small fraction of VRACs that are active under basal conditions in nonswollen astroglial cells (14, 42; see also effect of anion channel blockers on basal EAA release in Fig. 2A). It is possible that ATP-induced Cl− currents observed by others in nonswollen cells (13, 79) may at least partially reflect activation of (an)other subtype(s) of anion channels, i.e., Ca2+-dependent Cl− channels. Alternatively, even a small degree of swelling in electrophysiological experiments may “sensitize” VRACs to the ATP action.

Although we found the release of exogenous ATP in substantially swollen cells, in striking contrast to hepatoma cells (79), swelling-activated EAA release in our experiments under control hypoosmotic conditions was not significantly affected by scavenging of extracellular ATP with apyrase, by desensitization of purinergic receptors, or by the P2Y antagonist reactive blue 2. However, all these pharmacological treatments, including apyrase, prevented the effects of exogenous ATP. The inhibitory effects of suramin and PPADS are unlikely to be due to the inhibition of P2 receptors because of the following reasons. First, omission of extracellular Ca2+ does not inhibit volume-dependent taurine (43) and EAA (unpublished observations) release, thus ruling out the requirement of P2X receptors. A contribution of endogenously activated P2Y receptors to EAA release under control hypoosmotic conditions also seems unlikely because this release has not been eliminated by apyrase and reactive blue 2. Suramin and PPADS exert a large variety of effects unrelated to purinergic receptors, including the direct voltage-dependent block of the volume-dependent Cl− channels (15). Therefore, volume-dependent ATP release and VRAC activation in astrocytes are two unconnected processes. The same conclusion has been reached for epithelial cells (23, 76). Thus the finding by Wang et al. (79) of an obligatory autocrine role for ATP in cell volume regulation is not a general phenomenon for all cell types, and in fact the role of ATP was recently found to be nonobligatory in the same hepatoma cells (65).

Another question is whether endogenous ATP release may modulate (as opposite to direct activation) astrocytic VRACs. Because a continuous superfusion in our experiments dilutes and removes extracellular ATP, the modulatory role of endogenous ATP could be underestimated. In situ, limited extracellular space and several potential sources for ATP release may result in high local ATP concentrations and the potentiation of astrocytic EAA release. As seen from our data, this ATP-induced EAA release is minimal under isosmotic conditions but is drastically upregulated even by small increases in astrocyte volume. Moderate cell swelling may therefore serve as a physiological cosignal for the ATP-induced modulation of EAA release in astrocytes in situ.

**Multiple purinergic receptors are involved in modulation of EAA release.** Cultured astrocytes express at least two ionotropic P2X receptors, P2X1 and P2X7 (3, 41), as well as four subtypes of metabotropic ATP receptors, P2Y1, P2Y2, P2Y4, and P2Y6 (29, 37). To date, no pharmacological tools allow us to reliably distinguish between each purinergic receptor subtype.
Nonetheless, some conclusions can be drawn based on the pharmacological data obtained in the present study. A contribution of P2X4 and P2X7 can be ruled out on the basis of the insensitivity to the P2X4 agonist β,γ-MeATP and the P2X7 antagonist KN-62, respectively. The most important observation seems to be the ability of UTP to mimic the effects of ATP on EAA release. UTP activates P2Y2 and P2Y4 and P2Y6 (collectively termed P2U) receptors, whereas it is completely inactive at any of the P2X or P2Y1 receptors (27, 64). Thus P2Y2, P2Y4, and/or P2Y6 are good candidates for the effects observed in our study. P2Y2 is insensitive to reactive blue 2, whereas P2Y4 and P2Y6 possess a low sensitivity to this compound (IC50 ≈ 20 μM; Ref. 27). Reactive blue 2 partially inhibited the ATP and UTP effects in moderately swollen cells and was highly potent against ATP effects in substantially swollen cells. 2-MeSATP, which discriminates P2Y1 from all P2U receptors, potentiated the EAA release in both moderately and substantially swollen cells, and these effects were highly sensitive to reactive blue 2, consistent with the involvement of P2Y1 (27, 50). Overall these data suggest that both P2Y1 and P2U-like receptors can equally contribute to potentiation of EAA release. However, depending on the degree of cell swelling the endogenous agonist ATP seems to act preferentially via P2U (moderately swollen cells, low sensitivity to reactive blue 2) or P2Y1 (substantially swollen cells, high sensitivity to reactive blue 2). The potent effects of 2-MeSATP in moderately swollen cells and UTP in substantially swollen cells somewhat contradict this model. Potential explanations for this discrepancy include a UTP/2-MeSATP-induced ATP release followed by a cross-activation of other receptor subtypes, a phenomenon contributing to the propagation of Ca2+ waves (21) and/or a deviation of the pharmacological profiles of astrocytic P2Y receptors from their cloned counterparts (37). In endothelial cells hypoxosmotic medium-induced ATP release accelerates volume regulation via activation of P2Y receptors with pharmacological properties similar to those found in the present study (72).

Anion channels or Ca2+-dependent vesicular release? Recently much attention has been paid to the phenomenon of astrocytic Ca2+-dependent glutamate release, which shows many similarities to the vesicular glutamate release in neuronal cells (2, 5, 22, 54). Cultured astrocytes express many proteins constituting the apparatus of exocytosis (55) and respond to physiological elevations of cytoplasmic Ca2+ levels with endogenous glutamate release (56). Therefore, one should consider whether the effects of ATP on EAA release are due to stimulation of astrocytic Ca2+-dependent vesicular-like glutamate release, especially in nonswollen and moderately swollen cells. Several facts are inconsistent with such a hypothesis. First, the ATP-induced EAA release in moderately swollen cells shows a high sensitivity to anion channel blockers, which is similar to the volume-dependent release induced by substantial hypoxosmotic shock. An extensive line of experimental evidence suggests that at least the latter release is attributed to activation of VRACs (26, 57, 59, 66, 73). Second, ATP effects are strictly dependent on medium osmolarity and are completely inhibited by moderate cell shrinkage. Third, ATP-induced EAA release in moderately swollen cells was completely insensitive to 24-h pretreatment with tetanus toxin, in contrast to the astrocytic vesicular-like glutamate release (60). Together, the data on ATP-induced modulation of astrocytic EAA release can be most parsimoniously explained by the modulation of VRACs. This process may be complementary or alternative to the vesicular-like astrocytic glutamate release described elsewhere (2, 5, 22, 54).

Possible physiological and pathological significance of ATP-induced EAA release. Volume-dependent anion channels contribute to EAA release and likely to neural tissue damage under various pathological conditions associated with astrocyte swelling (4, 33, 61, 71). As seen from our data in substantially swollen cells, ATP release from damaged or swollen neural cells may significantly increase pathological VRAC-mediated EAA release. Because activation of the VRACs is dependent on intracellular ATP, their contribution to EAA release in the infarction core is less likely (26, 52, 68). However, in the penumbra, intracellular ATP content remains as high as 70% of the normal tissue levels (40). Therefore, modulation of swelling-activated EAA release by released extracellular ATP and adenosine may persist in the penumbra over the total duration of ischemic episode and during reperfusion.

In contrast to pathological conditions, very little is known to date regarding the normal physiological functions of VRACs in the brain. In supraoptic and paraventricular nuclei of the hypothalamus, taurine is concentrated in glial cells and tonically released via VRACs in response to small changes in extracellular osmolarity (14). This release modulates electric activity and vasopressin release in magnocellular neurons, thereby contributing to hormonal control of body fluid homeostasis (24, 25). Similarly, VRAC-mediated glutamate release may contribute to regulation of neuronal activity in other brain areas. However, on the basis of in vitro studies we know that volume-dependent glutamate release requires significant changes in cell volume above a threshold level (67), which seems unlikely in the normal CNS. Our data strongly suggest that ATP may be a factor, which provides potent activation of VRACs under physiological conditions. Any degree of astrocytic swelling due to increases in extracellular K+ and/or glutamate concentration should drastically enhance the efficiency of an ATP signal, offering an additional level of regulation of astrocytic EAA release. Severalfold stimulation of basal astrocytic glutamate release is unlikely enough to activate low-affinity non-NMDA glutamate receptors. However, the anion channel-mediated glutamate release in astrocytic processes surrounding glutamatergic synapses may be sufficient to modulate neuronal NMDA receptor activity and contribute to the processes of synaptic plasticity such as long-term potentiation. Interestingly, ATP receptors have a certain specificity in this regard because 100...
μM adenosine or 200 μM DHPG, two agents causing the phospholipase C/inositol 1,4,5-trisphosphate-mediated Ca++ increases in cultured and acutely isolated astrocytes (6, 62), were unable to upregulate EAA release in moderately swollen cells.

In summary, the novel findings of our study are that ATP-induced stimulation of astrocytic EAA release is strictly dependent on cell volume and can be strongly upregulated within physiological ranges of cell swelling. The volume dependence, high sensitivity to anion channel blockers, and insensitivity to long-term pre-treatment with tetanus toxin are most consistent with EAA release via volume-regulated anion channels. Furthermore, pharmacological analysis suggests that ATP exerts its action via activation of multiple P2Y receptor subtypes (both P2Y1 and P2U-like).

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REFERENCES

ATP-STIMULATED EAA RELEASE IN ASTROCYTES