Purinergic activation of anion conductance and osmolyte efflux in cultured rat hippocampal neurons

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Volume regulation in many cells occurs in response to changes in the extracellular or intracellular environment triggered by physiological or pathological cell swelling or shrinkage (67). The increased intracellular water content that occurs during cell swelling is restored to normal via loss of ions and organic osmolytes in a process called regulatory volume decrease (RVD). Chloride channels play a very important role in regulating the intracellular content of Cl− and, loss of intracellular amino acids via anion channels has been shown to contribute to RVD of many cell types (19, 37, 64).

Numerous studies have implicated extracellular ATP as an initiating signal for cellular volume regulation (12, 19, 23, 27, 31, 49, 80). In several cell types, extracellular ATP activates a current with electrophysiological characteristics similar to anion currents stimulated by cell swelling (IClswell) (19, 23). In other cells, extracellular ATP potentiates volume-regulated anion channels (VRAC) and amino acid efflux but cannot initiate these responses (29, 57, 90). Our previous studies demonstrated that cultured hippocampal neurons express an anion current that can be activated by extracellular ATP in the absence of osmotic swelling (47). Since ATP can be released by many cell types during insults that cause cell swelling, such as physical injury, tissue pH changes, trauma, or ischemia (10, 40, 54, 76, 81), increases in extracellular ATP may mediate volume regulation in cells of the pathologically swollen brain via anion channel activation. The ATP-activated current in hippocampal neurons also can be carried by the anionic form of the intracellular osmolyte, taurine (47). Therefore, this mechanism may directly participate in net loss of intracellular osmolytes responsible for volume regulation. In these studies, we have characterized the time course of current activation and the pharmacological kinetics of the response to ATP and established the subtype of nucleotide receptor that leads to activation of the anion current. We also have demonstrated that increased extracellular ATP acting via anion channels alters the

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net neuronal contents of taurine and other amino acid osmoles.

**MATERIALS AND METHODS**

**Materials.** Media components and balanced salt solutions for cell cultures were obtained from Invitrogen (Grand Island, NY). Niflumic acid was purchased from ICN Biomedicals (Aurora, OH). 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-5-yl)oxy]butanoic acid (DCPIB) is a product of Tocris Bioscience (Ellisville, MO). All other chemicals, including ATP, ADP, UTP, suramin, reactive blue, brilliant blue G, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), 2-(methylthio)adenosine 5′-diphosphate (2-MeS-ADP), pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS), 2′,3′-O-(4-benzoylbenzoyl) ATP (Bz-ATP), α,β-methylene ATP, and β,γ-methylene ATP were of the highest grade available and were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hanover Park, IL).

**Neuron cultures.** The Institutional Animal Care and Use Committee of Wright State University approved all procedures involving animals. Hippocampal neurons were obtained from rat fetuses on the 18th day of gestation using a method modified from that originally described by Banker and Cowan (5) as previously described (47). Cells were plated at a density of 30,000 cells/cm² in 35-mm plastic petri dishes. Some dishes contained 12-mm glass coverslips to permit transfer of the cells to the electrophysiological recording station. All growth surfaces were coated with polyornithine before the cells were plated. At the initial plating, the medium consisted of Eagle’s minimum essential medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% horse serum. After 4–6 h, this was replaced with growth medium consisting of Neurobasal A plus B27 additives and the same antibiotics (11). Cultures were maintained at 37°C in an atmosphere of 5% CO₂. Every 3–4 days, one-half of the medium was removed and replaced with fresh growth medium. Cells were used for experiments after 10–20 days in culture.

**Electrophysiology.** Whole cell recordings were made from neurons maintained at 35 ± 0.5°C and perfused at a rate that exchanges the 0.5-ml reservoir volume in 45 s. Solutions used for electrodes and perfusion solutions are described in Table 1. Electrodes pulled from borosilicate glass had resistances of 3–6 MΩ when filled with CsCl electrode solution and measured in phosphate-buffered saline (PBS).

Recordings were initiated with a patch electrode containing CsCl or N-methyl-o-glucamine-Cl (NMDG-Cl) while cells were perfused with PBS. Neurons were held at −70 mV in voltage-clamp mode. After a stable recording was established, the perfusion solution was changed to CsCl, NMDG-Cl, or Cs-glucanate solution, and the holding potential was raised to 0 mV. Hippocampal neuron currents then were measured every 30 s by delivering a train of 90-ms voltage steps to values ranging from −120 to +120 mV in 20-mV increments. Current-voltage (I-V) relationships for each recorded cell were determined from the average of the cell current during the last 20 ms of each voltage step. Membrane conductance for each cell was calculated by linear regression of these data between −80 and −20 mV. In some experiments, extracellular CsCl, NMDG-Cl, or Cs-glucanate was replaced with equiosmotic sucrose, and resulting shifts in membrane potential were determined.

**Amino acid content and taurine uptake.** The cellular contents of amino acids and the unidirectional rate of taurine accumulation were determined using cells grown in 35-mm culture dishes as previously described (68, 71). Growth medium was rinsed from the cultures, and the cells were incubated for 30 min in PBS (Table 1). The PBS was then changed to an identical solution, except it also contained 0–100 μM ATP. For uptake studies, this solution also contained 1 μM tritiated taurine with a specific activity of 0.5 μCi/ml.

To measure amino acid contents, cultures were incubated for 30 min. The extracellular fluid was then removed from the culture dish and replaced with 1 ml of 0.6 M HClO₄. Cells were scraped from the dish in this solution and centrifuged at 10,000 g for 1 min. Amino acids were measured in the supernatant by HPLC after derivatization with o-phthalaldehyide. Cellular material adherent on the culture dish was removed with 1 ml of 1.0 M NaOH, and this solution was used to dissolve the pellet from the centrifugation. Protein was determined in the resulting solution by using the method of Lowry et al. (50).

To measure taurine accumulation, a sample of PBS was removed for liquid scintillation counting after 20 min of incubation, and the cells were fixed in 1 ml of 0.6 M HClO₄. The cells were scraped from the dish, and the resulting suspension was sonicated and then centrifuged. Radioactivity was determined in the supernatant. The pellet plus residual cellular material remaining on the dish were dissolved in NaOH for protein determination as described above.

**Data analysis.** Results are means (SD). Curve fits were performed by linear or nonlinear regression algorithms, and SE are provided for the calculated parameters. The time course of conductance changes was

Table 1. Composition of perfusion and patch electrode solutions

<table>
<thead>
<tr>
<th></th>
<th>Perfusion Solutions</th>
<th>Patch Electrode Solutions</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>CsCl</td>
</tr>
<tr>
<td>NaCl</td>
<td>137</td>
<td>100</td>
</tr>
<tr>
<td>CsCl</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>NMDG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gluconic acid</td>
<td>0.5-1.0</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.5-1.0</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.5-1.0</td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.5-1.0</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>5.5-5.5</td>
<td></td>
</tr>
<tr>
<td>EGTA</td>
<td>5.5-5.5</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>90-90</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>7.3</td>
<td>7.3</td>
</tr>
<tr>
<td>pH</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Adjusted with</td>
<td>NaOH</td>
<td>CsOH</td>
</tr>
<tr>
<td>Osmolality, mOsmol/kg H₂O</td>
<td>290</td>
<td>290</td>
</tr>
<tr>
<td>Adjusted with</td>
<td>NaCl</td>
<td>Sucro</td>
</tr>
</tbody>
</table>

All concentrations are given in mM. NMDG, N-methyl-o-glucamine.
analyzed by repeated-measures ANOVA with post hoc Dunnett’s test. Responses to drug exposures or ion substitutions were evaluated using ANOVA followed by Dunnett’s test or Student’s t-test for paired or independent samples, as appropriate. Significant differences were indicated by \( P < 0.05 \).

RESULTS

Isolation of anion currents. Following successful establishment of a whole cell patch recording using the CsCl patch electrode solution, depolarizing voltage steps to \(-50\) mV or greater resulted in transient inward currents characteristic of action currents (Fig. 1A). Cells also typically demonstrated spontaneous transient currents resembling synaptic currents. To ensure that our recordings were from viable neurons, we abandoned recordings from cells that did not demonstrate the action currents in response to depolarizing command voltages. After the perfusing PBS was replaced with 100 mM CsCl perfusion solution, the resting potential was \(-4.9\) (SD 3.4) mV. Once this solution change was complete, the holding potential was moved to 0 mV. Action currents were eliminated during all voltage steps in this configuration, but the current magnitude in response to both depolarizing and hyperpolarizing voltage commands was increased compared with cells perfused with PBS (Fig. 1B). Therefore, ion substitution experiments were performed to determine which ions were responsible for the basal membrane currents. In this analysis, junction potentials created at the AgCl reference electrode were numerically compensated using the algorithm provided with pClamp 8.2 (Axon Instruments, Sunnyvale, CA). Reducing extracellular CsCl to 50 mM by equiosmotic replacement with sucrose produced little change in membrane potential (Fig. 2), suggesting that the membrane was equally permeable to Cs\(^+\) and Cl\(^-\) under these conditions. However, further reductions of the extracellular CsCl concentration to 25 or 10 mM resulted in positive membrane potential shifts of +18.7 (SD 4.9) or +39.7 (SD 6.9) mV, respectively. These values are still below those predicted for a membrane solely permeable to Cl\(^-\) and over this wide range of CsCl concentrations predict a relative permeability \((P_{Cl}/P_{Cs})\) of 4.3 (SE 1.0) using the Goldman-Katz model for two permeable ions. To test whether Cs\(^+\) permeability is responsible for the variance from the predicted membrane potential shift for a single permeant anion, we performed experiments with 100 mM NMDG rather than Cs\(^+\) in extracellular and patch electrode solutions. Under these conditions, reducing extracellular NMDG-Cl from 100 to 50 or 25 mM (with equiosmotic sucrose replacement) resulted in membrane potential shifts of +14.0 (SD 6.2) or +30.6 (SD

Fig. 1. Patch-clamp recordings of hippocampal neurons. Recordings were obtained from a single cell using an electrode solution containing 100 mM CsCl and depict representative results from all cells used in these studies. A: voltage-clamp recordings made within several minutes of establishing a whole cell recording while the cell is perfused with PBS. The capacitive current was electronically eliminated in this recording. The membrane potential was held at \(-70\) mV and stepped between \(-90\) and \(+90\) mV in 20-mV steps. Rapidly inactivating inward currents appeared with voltage steps to \(-50\) mV and higher (arrows). B: membrane currents recorded from the same cell after the perfusate was replaced with 100 mM CsCl solution and the holding potential changed to 0 mV. Capacitive current compensation was not used for this and subsequent recordings. The membrane voltage was stepped between \(-120\) and \(+120\) mV in 20-mV steps. C: membrane currents recorded using the same holding potential and voltage steps following the introduction of 1 mM tetraethylammonium (TEA) plus 100 \(\mu\)M NiCl\(_2\) to the CsCl perfusing solution. D: current-voltage relationships measured from data shown in B (●) and C (○) as indicated. The current for each voltage step was calculated as the average of the last 20 ms of the appropriate tracing.
7.5) mV, respectively (Fig. 2). Over this range of extracellular NMDG-Cl concentrations, we calculated a relative permeability PCl/PNMDG of 12.7 (SE 4.9). In other studies, the membrane potential hyperpolarized when the perfusing solution was changed from 100 mM Cs-gluc onate to 50 mM Cs-gluc onate (with equiosmotic sucrose replacement). Thus we conclude that cultured hippocampal neurons have a significant permeability to Cs⁺ under our basal recording conditions.

We evaluated various ion channel inhibitors for their ability to reduce the cation permeability of the neurons during perfusion with 100 mM CsCl. With 1 mM tetraethylammonium (TEA) added to the perfusion solution, cells showed a depolarizing membrane potential change when extracellular CsCl was reduced to 50 mM (Fig. 2). Little additive effect was observed when 100 μM BaCl and 1 mM TEA were combined (data not shown). However, with 1 mM TEA plus 100 μM NiCl₂ (TN) added to the extracellular solution, a reduction in extracellular CsCl concentration to 50 mM resulted in a membrane potential shift of +16.2 (SD 5.4) mV. This compares favorably with the value of +18.6 mV calculated using the Nernst equation at 35°C and yields a PCl/PNa relative permeability of 8.8 (SE 2.9). The addition of TN also reduced the membrane conductance of cells perfused with 100 mM CsCl by 45% (SD 32%) (Fig. 1D). Thus, in the presence of these cation channel inhibitors, ~90% of the resting membrane conductance is due to current carried by chloride ions. The remaining electrophysiological studies described were performed with TN in all CsCl perfusing solutions.

**ATP-activated anion currents.** Hippocampal neurons with 100 mM CsCl in the patch electrode and 100 mM CsCl plus TN in the perfusion solution had an input resistance of 274 (SD 90) MΩ and a membrane capacitance of 50.4 (SD 19) pF. The I-V curve was slightly outward rectifying with a conductance of 88.8 (SD 37.0) pS/pF measured between −80 and −20 mV (Fig. 3, A and C). Adding 10–100 μM ATP to the perfusion solution increased the membrane conductance within several minutes, reaching a peak value between 5 and 20 min after the start of ATP exposure (Fig. 3D). With 100 μM ATP, the average maximum membrane conductance relative to that measured before ATP exposure (Relative Conductance) was 2.86 (SD 3.3) and appeared 9.00 (SD 4.17) min after the start of exposure (Fig. 3D). This change in membrane conductance was completely inhibited with 100 μM of either of the anion channel inhibitors NPPB or niflumic acid (Fig. 3E). The dose-response curve for the ATP-activated chloride conductance can be modeled as a Michaelis-Menten relationship with a maximal relative conductance of 3.8 and an ATP concentration at half-maximal activation of ~70 μM (Fig. 3F). The calculated intercept of this curve at 0 μM ATP is not statistically different from unity.

**ATP activates anion currents via P2Y₁ receptors.** The nucleotides ATP, ADP, and UTP activated hippocampal neuron chloride conductance (Fig. 4). At equal concentrations of 100 μM, the rank order of the magnitude of the conductance response was ATP > ADP > UTP. For each agonist, the general P2 receptor antagonist suramin (100 μM) completely blocked the conductance increase. The P2Y receptor antagonists reactive blue and PPADS also completely blocked the activation of neuronal chloride conductance when added with 100 μM ATP (Table 2). Indeed, reactive blue significantly decreased neuronal conductance to <75% of control values even in the presence of 100 μM ATP, whereas in the absence of exogenous ATP, 100 μM suramin and reactive blue inhibited the baseline conductance after 6 min of exposure by 25.4% (SD 12.6%) and 16.9% (SD 15.3%), respectively. In contrast, the P2X antagonist brilliant blue G had no effect on the conductance increase caused by exposure to 30 μM ATP. In addition, neither the P1 agonist adenosine nor any of the P2X agonists (α,β-methylene ATP, β,γ-methylene ATP, or Bz-ATP) caused a change in neuronal chloride conductance.

To further define the contri bution of P2Y receptor subtypes to the neuronal anion conduc tance, we utilized the P2Y₁ selective receptor agonist 2-MeS-ADP and the P2Y₁ receptor antagonist MRS2179 (Fig. 5). 2-MeS-ADP increased chloride conductance with a time course similar to that observed with ATP, although the maximal conductance increase was nominally less than that obtained with 100 μM ATP. MRS2179 blocked the conductance increase normally observed with 100 μM ATP. The small decrease in conductance obtained with MRS2179 treatment in the presence of ATP was not statistically significant.

**Amino acid loss during ATP exposure.** Because of the role of anion channels play in amino acid release during cell swelling, the importance of taurine as an intracellular osmol yte involved in cell volume regulation, and the mechanism of extracellular ATP-induced volume regulation described for other cell types (19, 23, 59, 75), we examined whether treatment of the cultured neurons with 100 μM ATP would decrease neuronal contents of taurine and other amino acids. The standard defined growth medium used for these neuronal cultures does not contain taurine. Therefore, for these experiments, cells were grown in medium supplemented with 100 μM taurine for 3 days before experimentation. This treatment increased neuronal taurine contents from 19 (SD 4) nmol/mg protein measured in the absence of taurine supplementation to 144 (SD 31) nmol/mg protein with taurine added to the growth medium, a value approximately twice that of the hippocampus in situ (72).

During a 30-min exposure to PBS containing 0–10 μM ATP, no change in neuronal taurine contents was observed (Fig. 6A).
However, neuronal taurine contents decreased significantly following a 30-min exposure to either 30 or 100 μM ATP. Alanine contents also decreased in a dose-dependent manner from 26.6 (SD 6.2) nmol/mg protein in 0 μM ATP to 16.6 (SD 3.2) nmol/mg protein in 100 μM ATP (Fig. 6B). Neuronal contents of aspartate, glutamate, glutamine, glycine, and γ-aminobutyric acid were not affected by ATP treatment.

To examine whether the ATP-induced losses of taurine and alanine were due to movement through VRAC, these experiments were repeated with cells exposed to 100 μM ATP in the presence of the VRAC inhibitors DCPiB or NPPB (Fig. 7). Similar to results shown in Fig. 6, 30-min exposure to 100 μM ATP caused an ~10 and 40% decrease in neuronal taurine and alanine contents, respectively. These decreases were com-

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by extracellular ATP, the nucleotide receptor mediating this response is completely inhibited with either 20 μM DCPIB or 100 μM NPPB added during ATP exposure. Net loss of intracellular taurine observed during ATP exposure might result from inhibition of active taurine reaccumulation following passive loss through membrane channels. Therefore, we measured unidirectional taurine accumulation to test whether ATP exposure downregulated or inhibited the taurine transporter. We found the rate of taurine accumulation was not affected by 100 μM ATP (Fig. 8). However, taurine accumulation was significantly increased in the presence of the nonselective P2 receptor antagonist suramin (100 μM).

**DISCUSSION**

The present study characterizes a neuronal current activated by extracellular ATP, the nucleotide receptor mediating this electrophysiological response, the effect of nucleotide receptor antagonists on hippocampal neuron chloride conductance.

**Table 2. Effects of purinergic receptor agonists and antagonists on hippocampal neuron chloride conductance**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Relative Conductance after 6-Min Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.97 (0.70)</td>
</tr>
<tr>
<td>30 μM ATP</td>
<td>2.49 (1.8)</td>
</tr>
<tr>
<td>100 μM ATP</td>
<td>4.67 (1.3)</td>
</tr>
<tr>
<td>P2Y antagonists (plus 100 μM ATP)</td>
<td>0.73 (0.07)*</td>
</tr>
<tr>
<td>Reactive blue</td>
<td>1.18 (0.42)*</td>
</tr>
<tr>
<td>PPADS</td>
<td>2.67 (1.37)</td>
</tr>
<tr>
<td>P2X antagonist (plus 30 μM ATP)</td>
<td>0.92 (0.12)*</td>
</tr>
<tr>
<td>Brilliant blue G</td>
<td>1.06 (0.26)*</td>
</tr>
<tr>
<td>P2X agonists</td>
<td>0.98 (0.12)*</td>
</tr>
<tr>
<td>α,β-Methylene ATP</td>
<td>1.00 (0.30)*</td>
</tr>
</tbody>
</table>

Values are means (SD) for 5–10 independent measurements. All drugs were used at a concentration of 100 μM. PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate. *P < 0.05, significantly different from the value obtained during control drug exposure.

Fig. 5. Sensitivity of the neuronal anion conductance to P2Y1 receptor subtype activation and inhibition. Values are the relative conductance measured following introduction of 100 μM 2-(methylthio)adenosine 5′-diphosphate (2-MeS-ADP; ●) or 100 μM ATP plus 1 μM MRS2179 (●) to the perfusing solution at time 0. Each data point is the mean ± SE of 5–7 independent measurements. *P < 0.05, significantly different from unity.

activation on amino acid contents, and the mechanism of ATP-induced taurine loss. We previously demonstrated the presence of an ATP-activated current in cultured hippocampal neurons (47). The pharmacological profile of the ATP response described presently indicates this current is activated by P2Y1 and not P2Y2 receptors. The ATP-induced increase in neuronal membrane conductance is observed with cesium and chloride as the primary conducting ions in the perfusing and electrode solutions and is sensitive to the anion channel blockers nitramide and DCPIB. Thus, we conclude, the ATP-activated current under these conditions is carried by chloride ions moving through the VRAC. Our previous data suggested the channels activated by ATP exposure can conduct taurine in its anionic form. The present results indicate ATP-induced activation of the VRAC can lead to net reduction of cellular taurine contents.

In contrast to previous studies of primary cultured rat astrocytes, hippocampal neurons demonstrate a cesium permeability comparable to that of chloride when depolarized in the presence of 100 mM CsCl in perfusion and electrode solutions. The cesium permeability is only partially inhibited by several potassium channel blockers but is completely blocked by the presence of TEA plus NiCl2 in the perfusing medium. In hippocampal neurons, NiCl2 has been shown to inhibit calcium-sensitive cation channels permeable to potassium and the Na+/Ca2+ exchanger (86, 88). However, because the increased conductance we measured in hippocampal neurons is due to cesium ions and is inhibited with a Ni2+ concentration less than 1 mM, we suggest the current is mediated by a nonspecific cation channel activated by intracellular calcium ions (16, 73). A calcium-dependent cation current activated by metabotropic glutamate receptors has previously been described in CA1 hippocampal neurons held at −60 mV (16); however, we did not test the glutamate-dependence of the nonspecific current identified in our neuron cultures. Because our objective was to examine ATP-activated anion currents, once we determined the neurons were predominantly perme-
able to chloride in the presence of 1 mM TEA plus 100 μM Ni2⁺, we did not further characterize this cation current.

Extracellular ATP activated a neuronal anion current with a time course of several minutes, similar to that observed in rat astrocytes and other cell types (19, 23). This current was inhibited by niflumic acid and demonstrated inactivation at highly depolarizing membrane potentials, characteristics of the anion conductance of anion conductance. Thus a role for P2X or P1 receptors in the ATP-induced activation of the anion current is unlikely. Indeed, the P2Y antagonist reactive blue reduced baseline anion conductance in both the absence and presence of ATP in the perfusing solution, suggesting that P2Y receptors stimulated by endogenously released ATP may partially activate the anion conductance under basal conditions.

The hippocampus expresses a variety of P2Y receptor subtypes including P2Y1, P2Y2, P2Y4, and P2Y6 (see Ref. 13 for review). In situ hybridization also has revealed other nucleotide receptors in the principal cell layers of the mouse hippocampus (45). Freshly isolated rat hippocampal astrocytes express mRNA and immunoreactivity for P2Y1, P2Y2, and P2Y4 receptors (92), and subcellular localization of P2Y1, P2Y2 and P2Y12 receptors has been observed in cultured rat forebrain astrocytes and C6 glioma cells (42). P2Y1 receptors have been observed immunohistochemically in hippocampal pyramidal cells and interneurons of the rat and human (30, 60,

Fig. 6. Amino acid contents of hippocampal neurons after exposure to extracellular ATP. Taurine (A) and alanine contents (B) were measured after 30 min of exposure to various ATP concentrations in PBS. Bars represent means ± SE of data from 6-15 cultures. *P < 0.05, significantly different from the content measured in 0 μM ATP.

In contrast, each of the P2Y antagonists, suramin, reactive blue, and PPADS (77), completely blocked the ATP-activated anion conductance. Thus a role for P2X or P1 receptors in the ATP-induced activation of the anion current is unlikely. Indeed, the P2Y antagonist reactive blue reduced baseline anion conductance in both the absence and presence of ATP in the perfusing solution, suggesting that P2Y receptors stimulated by endogenously released ATP may partially activate the anion conductance under basal conditions.

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Fig. 7. Anion channel inhibitors block net loss of neuronal taurine and alanine contents during exposure to ATP. Taurine (A) and alanine contents (B) are expressed as a percentage of the value measured in control cells before ATP and drug exposure. ATP alone resulted in a significant decrease in the net content of each amino acid. Both 20 μM DCPiB and 100 μM NPPB completely blocked this net amino acid loss. Values are means ± SE of results from triplicate experiments from different sets of cultures. *P < 0.05, significantly different from the content measured before ATP and drug treatment.
61), and functional evidence points to the presence of P2Y₁, P2Y₃, and P2Y₄ receptors that can alter neurotransmitter release or voltage-activated potassium currents in neurons from the rat hippocampus (55, 63, 79). Since we observed only a small conductance change when UTP was applied to the cells, we conclude P2Y₂, P2Y₄ and P2Y₆ receptors are not highly coupled to the increased anion current (2). Furthermore, our results showing activation of anion conductance by the P2Y₁-specific agonist 2-MeS-ADP and inhibition of ATP-induced current activation by the P2Y₁ antagonist MRS2179 suggest the majority of the ATP-activated anion current is mediated by the P2Y₁ receptor subtype, a result similar to that reported for cultured rat astrocytes (19). However, since there are no specific agonists or antagonists for all of the various P2Y receptor subtypes (2), we also used the profile of agonist activities to examine which other receptor subtypes are likely to mediate the increased anion conductance in cultured hippocampal neurons. We found a somewhat greater increase in anion conductance with the application of ATP compared with that of ADP. Although this result argues against the involvement of the ADP-prefering P2Y₁ receptors (2), the presence of extracellular ATPases and nucleotidases may confound this interpretation. The rate of ATP hydrolysis in hippocampal synaptosomes is twice that of ADP hydrolysis and over 10 times that of AMP hydrolysis (20). As a result, even at the high concentrations used in this study, exogenously applied ATP is rapidly converted to ADP, whereas AMP appears at a much slower rate (17). Thus, during the time course of our experiments, ectonucleotidases acting on exogenous ATP would first produce ADP, which still might activate ADP-prefering nucleotide receptors. Exogenous ADP applied to the cells would be converted to AMP, a compound that is a poor substrate for nucleotide receptors.

Because our cultures were prepared from whole hippocampi, a variety of cell types were likely to be present. With our methods, these primary cultures of rat hippocampal neurons contained ~20% astroglial cells as defined by counts of glial fibrillary acidic protein (GFAP)-positive cells and the specific activity of the astrocyte-specific enzyme glutamine synthetase (71). All cells used in these studies were neurons as defined by their general morphological appearance and the presence of inward currents observed upon membrane depolarization; however, the specific neuronal cell type was not characterized. Although each cell responded to application of ATP with an increase in anion conductance, significant variability was observed in the magnitude and the time course of the current activation. This variability in the ATP response may be due to the variety of neuronal subtypes present, including GABAergic and glycinergic interneurons as well as pyramidal neurons (3, 8, 18). At least some hippocampal interneurons express P2Y₁ receptors (61); however, the expression and cell surface density of nucleotide receptor may be variable within each neuronal subtype (25).

Nucleotide receptors have been shown to play an important role in hyposmotic volume regulation of rat hepatoma cells, human hepatocytes, Necturus erythrocytes, and rat astrocytes (19, 23, 38, 48, 49, 80). Additional studies have demonstrated ATP-induced activation of anion channels in both osmotically swollen and normal cells (26, 51, 53, 56, 83, 89). In other cells, extracellular ATP does not initiate an anion current but can potentiate such currents activated by hyposmotic swelling (29, 90). We previously demonstrated a similar potentiation of the ATP-activated anion current by hyposmotic exposure in these cultured hippocampal neurons (47). Since we observed that inhibitors of nucleotide receptors reduce anion currents in isosmotic conditions, we suggest current can be activated by basal levels of extracellular ATP present in these cultures incubated in isosmotic conditions.

The present studies indicate P2Y₁ receptors are coupled to the activation of anion currents and amino acid loss in hippocampal neurons. In rat astrocytes, P2Y₁ receptors mediate the swelling-induced increase in anion conductance (19), whereas P2Y₂ receptors activate anion conductances in cell lines of kidney distal tubule and tracheal epithelial cells (9, 89). In several of these cellular systems, one functional effect of nucleotide receptor-activated anion conductance is net efflux of intracellular osmolytes and reduction in cellular volume. For rat astrocyte cultures, ATP acts through P2Y receptors to increase the efflux of amino acids via anion channels during hyposmotic swelling (57). ATP-induced amino acid efflux from astrocyte cultures also has been reported to occur by activation of P2X receptors (22).

We have previously established that osmotic swelling and activation of nucleotide receptors in hippocampal neurons increase the membrane conductance to taurine (47), a major intracellular osmolyte of the brain (62, 74, 91). The present study demonstrates that exposure of hippocampal neurons to ATP also leads to a dose-dependent reduction in net amino acid contents. Reduction of the ATP-stimulated amino acid efflux by pharmacological inhibitors of the VRAC supports the concept that neuronal volume regulation in isosmotic conditions is mediated via osmolyte-conducting channels that are activated by extracellular nucleotide receptors. Efflux of amino acids such as glutamate, glutamine, and aspartate as well as potentially other organic osmolytes may be increased when anion channels are activated by cell swelling or exposure to ATP (37, 59, 82). However, in these cultured neurons, taurine and alanine were the only amino acids that showed net loss during ATP exposure. This selectivity in amino acid loss may be due to one of several factors. First, permeability through volume-activated anion channels relative to that of chloride varies for
different amino acid species (82). Thus the difference in the percent loss of taurine compared with alanine may represent differences in the permeability of these amino acids through the VRAC. For taurine, the relative permeability is ~75% in MDCK cells (82) and 20% in C6 glioma cells (37). Second, the lack of change in the net content of amino acids other than taurine and alanine may be a result of rapid reuptake into adjacent glial cells or into the neurons from which they were released. Although taurine also is known to be accumulated by neurons and glial cells (6, 32, 33, 71), we found that ATP did not alter unidirectional uptake of taurine into the neuron cultures. Conversely, the broadly acting P2 receptor antagonist suramin increased the rate of taurine accumulation. Although this drug has been shown to directly inhibit volume-activated anion currents in cultured endothelial cells (21), unidirectional accumulation of the extracellular radioactive tracer measured in these studies would not be altered by changes in efflux pathways. Indeed, inhibition of passive taurine-conducting channels would be expected to decrease rather than increase the unidirectional movement of [3H]taurine into the cells. These data also imply that the net taurine loss observed in the presence of ATP is not a result of inhibition of the taurine accumulation pathway. Finally, amino acids such as glutamate, glutamine, and aspartate are metabolically coupled to the Krebs cycle intermediates and thus may be replaced via various transaminase reactions if intracellular contents are lost via increased efflux. Since taurine is synthesized relatively slowly in brain cells compared with other amino acids (7), replacement of lost taurine by anabolic reactions is not possible during the period of our studies.

ATP is released into the extracellular space during conditions associated with cell swelling such as hyposmotic exposure and ischemia (54, 76). In addition, taurine is mobilized into the extracellular space during osmotic, ischemic, and traumatic brain edema (28, 44, 84, 85). Thus our data suggest activation of a purinergic signaling pathway may initiate volume regulation in neurons via the loss of taurine and other amino acid osmolytes during these diverse pathological conditions. Differential sensitivity of neurons and astroglial cells to osmolyte loss initiated by swelling and by extracellular ATP (46, 70) may influence relative volume changes in these cell types and lead to regulation of neuronal volume in the presence of pronounced glial swelling, a characteristic of cytotoxic brain edema. Thus regulation of neuronal volume via ATP released by glial cells would represent an important neuronal-glial interaction involved in the adaptation of brain tissue to traumatic or metabolic insults that can precipitate brain edema.

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ATP-activated neuronal anion conductance


