

Alexander A. Mongin, Harold K. Kimelberg and Brian A. MacVicar
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ATP regulates anion channel-mediated organic osmolyte release from cultured rat astrocytes via multiple Ca²⁺-sensitive mechanisms

A. A. Mongin and H. K. Kimelberg

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Is autocrine ATP release required for activation of volume-sensitive chloride channels?

To the Editor: We read with great interest the paper by Darby et al. in the April 2003 issue of the *Journal of Neurophysiology* (Darby et al. 2003). The authors demonstrated that, in cultured astrocytes, hyposmotic cell swelling causes release of ATP via a pathway with pharmacological profile similar to the multidrug resistance transporter. This ATP then stimulates P2Y1-like receptors and activates, via appropriate intracellular signaling mechanisms, volume-regulated anion channels (VRACs), measured as whole cell Cl^- currents. They also show that in nonswollen astrocytes, exogenous ATP activates Cl^- currents resembling currents carried by VRACs. These data fit the previously proposed model of autocrine ATP release that is obligatory for the activation of VRACs in hepatoma cells (Wang et al. 1996). It is important to note that the significance of such findings in astrocytes extends beyond the “academic” question of how volume-sensitive chloride channels are activated in response to cell volume changes. Astrocytic VRACs, as well as their counterparts in other cells, are permeable toward a variety of small organic anions and uncharged molecules, including the excitatory amino acids glutamate and aspartate (Kimelberg et al. 1990). Therefore in the brain, ATP released during synaptic transmission or astrocyte-to-astrocyte communication may cause VRAC-mediated glutamate release from astrocytes. Such astrocytic glutamate release is now considered a key feedback signal in neuron-astrocyte communication (Haydon 2001), with ATP likely acting as the main neuron-to-astrocyte and astrocyte-to-astrocyte intercellular messenger (Fields and Stevens 2000).

We have also studied the role of ATP in activation of astrocytic VRACs, measured as release of preloaded [^3H]-labeled excitatory amino acid D-aspartate. We found that, in cultured astrocytes, exogenous 10 μM ATP activates a transient excitatory amino acid release in nonswollen cells and strongly potentiates amino acid release in hyposmotically swollen cells (Mongin and Kimelberg 2002; Fig. 1). This ATP-induced D-[^3H]aspartate efflux was potently inhibited by the VRAC blockers 5-nitro-2-(3-phenylpropylamino) benzoic acid, DIDS, and phloretin. It was also suppressed by a 10% increase in medium osmolarity (Mongin and Kimelberg 2002). On the basis of these data, we propose that ATP is incapable of direct VRAC activation, but instead, positively modulates a small fraction of VRACs that are active in nonswollen cells. The ATP-induced D-[^3H]aspartate release in our experiments involving nonswollen cells did not exceed one-tenth of the release induced by a 100 mOsm reduction in medium osmolarity. In contrast, Darby and colleagues report a substantially higher activation of the Cl^- currents by ATP in nonswollen astrocytes, reaching one-half of the value of the hyposmotic Cl^- currents (Darby et al. 2003). If translated to ATP-induced VRAC-mediated glutamate release, such effect should have a large impact on normal brain physiology.

The effect of ATP seen by Darby et al. was concentration-dependent, with the maximum values at 1 and 5 mM. However, millimolar concentrations of extracellular ATP block VRAC-mediated excitatory amino acid release in astrocytes (Haskew et al. 2002; Mongin and Kimelberg 2002), as well as VRAC-

mediated Cl^- currents in several cell lines, with the IC_{50} varying between 0.2 and 5 mM ATP (Okada 1997). As seen in Fig. 1, in cultured astrocytes, 10 μM , 100 μM , and 1 mM ATP activated the transient D-[^3H]aspartate release with equal potency. However, at 5 and 10 mM ATP, the first transient phase of D-[^3H]aspartate release is not present and is presumably inhibited by high concentration of ATP. Instead we observed a delayed and sustained amino acid efflux. This latter release phase is unlikely to be due to VRAC activation. It may be due to extracellular $\text{Mg}^{2+}/\text{Ca}^{2+}$ chelation by ATP, promoting glutamate release through astrocytic connexin hemi-channels or P2X7 receptor-channels, as reported recently (Duan et al. 2003; Ye et al. 2003).

Based on the foregoing, we propose that ATP release is not obligatory or sufficient for VRAC activation in astroglial cells. Instead ATP likely *modulates* active VRACs in both swollen and nonswollen cells. A similar conclusion has been reached in a few recent studies in intestinal epithelial and hepatoma cells employing both an electrophysiological approach and ion flux measurements (Hazama et al. 1999; Junankar et al. 2002). The ATP-induced Cl^- currents in nonswollen astrocytes may result from activation of multiple Cl^- channels. At high ATP concentrations (such as 5–10 mM), the contribution of non-VRAC channels likely predominates. Distinction between permeability pathways is very important since VRAC activation may be

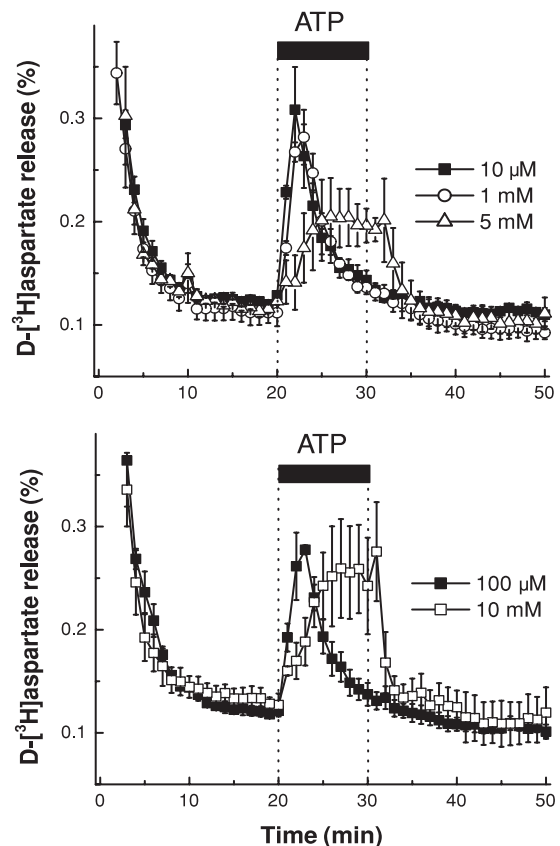


FIG. 1. Effect of exogenous ATP on D-[^3H]aspartate release in primary astrocyte cultures. Astrocytes were preloaded overnight with D-[^3H]aspartate and perfused with iso-osmotic medium in a Lucite chamber. The perfusate was switched to one containing 10 μM –10 mM ATP as indicated. Data are means \pm SE of 3–4 experiments performed on 2 cell culture preparations.

a source of physiological astrocytic glutamate release. On the other hand, it has been shown that cell swelling activates more than one type of Cl^- channel (Zhang and Jacob 1997). Therefore the differences between the ATP effects on Cl^- currents and organic osmolyte efflux may also be explained by contribution of several permeability pathways to what is currently collectively known as a VRAC. We believe that further studies looking at the mechanisms of VRAC activation in nonswollen cells may have important implications for our understanding of astrocyte functions in the brain.

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Alexander A. Mongin

Center for Neuropharmacology and Neuroscience
Albany Medical College

Harold K. Kimelberg

Department of Brain and Heart Cell Rescue
Ordway Research Institute
Albany, New York 12208

REPLY

To the Editor: We thank Mongin and Kimelberg for their interest in our work. We also recognize the contributions from this laboratory in the area of aspartate release from volume-regulated anion channels (Mongin and Kimelberg 2002; Seki et al. 1999). We have evidence that glutamate release from these channels contributes to spreading depression propagation (Basarsky et al. 1999). However, we would like to clarify some

issues that are raised by this letter. Certainly, the final point of the letter by Mongin and Kimelberg should be kept foremost in mind in any discussion on this issue. As they state there are several species of Cl^- channels that are activated by swelling (Zhang and Jacob 1997). In addition, there are several mechanisms by which glutamate and aspartate efflux can occur from astrocytes and other cell types. Mongin and Kimelberg should also be cautious in interpreting their measurements of radiolabeled aspartate efflux as solely due to efflux from anion channels. Their measurements are quite distant from Cl^- channel activation, which is turning out to be a complex phenomenon in astrocytes (Crepel et al. 1998).

The major issue mentioned by Mongin and Kimelberg is our use of high concentrations of ATP (1 to 5 mM). This experiment should be understood in the context of the study. Our argument that ATP release is involved in activating volume-activated Cl^- currents ($I_{\text{Cl,swell}}$) is based on: 1) ATP receptor (P2Y₁) antagonists depressed the activation of $I_{\text{Cl,swell}}$; 2) apyrase, an enzyme that degrades ATP, reduced $I_{\text{Cl,swell}}$; and 3) application of ATP and other P2Y agonists activated a Cl^- current that appeared similar to $I_{\text{Cl,swell}}$. We observed that the degree of block of $I_{\text{Cl,swell}}$ by P2Y₁ antagonists and activation by ATP was only a fraction (~50%) of the total current. We therefore applied quite high concentrations of ATP (1 to 5 mM) to ensure that we were at saturating concentrations. We did not observe ATP-mediated depression under these conditions. Another reason that we used high ATP concentrations was to examine the potential role for P2X₇ receptors. Previously we showed that P2X₇ receptor activation triggered Erk1/2 and p38 MAP kinase activation in astrocytes, thereby leading to increased MCP-1 synthesis (Panenka et al. 2001). P2X₇ receptors are activated by high ATP concentrations and by BzATP. Therefore, we tested the possibility of P2X₇ receptor activation in our paper with high ATP concentrations and with BzATP. Our data did not support a P2X₇ receptor involvement because the P2X₇ agonist BzATP was not more effective than ATP at 100 μM and there was no substantial further activation at the high ATP concentrations. Finally, it should be noted that ATP concentrations of 100 μM were used in our experiments to examine the effectiveness of ATP vs. other P₂ agonists (see Fig. 3 in our article, Darby et al. 2003). ADP was more efficacious than ATP at activating $I_{\text{Cl,swell}}$, which is consistent with a P2Y₁ receptor involvement.

In their previous studies and in this letter, Mongin and Kimelberg have provided interesting data on factors that modify the release of aspartate from astrocytes in cell culture. However, it is unreasonable to dismiss actual recordings of Cl^- channel activation based on measurements of radiolabeled release of aspartate. The cellular response to swelling is complex, and it is conceivable that cellular swelling and the attendant activation of MAP kinases could be an important modifier of numerous cellular processes, some of which could modify aspartate release or Cl^- channel activation. The recent elegant description of glutamate release through hemichannels stresses the multiplicity of glutamate release mechanisms (Ye et al. 2003). In light of these findings, it will be important for future experiments to delineate unequivocally the roles for hemichannels vs. anion channels in the release of glutamate.

Finally, our results indicate that ATP release only contributes to ~50% of the activation of $I_{\text{Cl,swell}}$. This suggests that either another signaling factor is released or that the residual

current represents a different population of Cl^- channels (Zhang and Jacob 1997). The analysis of these currents and the relationship of these currents to the release of aspartate that Mongin and Kimelberg have studied is an important next step.

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Brian A. MacVicar
Brain Research Center
University of British Columbia
Vancouver, British Columbia