ATP Released From Astrocytes During Swelling Activates Chloride Channels

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Darby, Mark, J. Brent Kuzmiski, William Panenka, Denise Feighan, and Brian A. MacVicar. ATP released from astrocytes during swelling activates chloride channels. J Neurophysiol 89: 1870–1877, 2003; 10.1152/jn.00510.2002. ATP release from astrocytes contributes to calcium ([Ca^{2+}]_i) wave propagation and may modulate neuronal excitability. In epithelial cells and hepatocytes, cell swelling causes ATP release, which leads to the activation of a volume-sensitive Cl^- current (I_{Cl,swell}) through an autocrine pathway involving purinergic receptors. Astrocyte swelling is counterbalanced by a regulatory volume decrease, involving efflux of metabolites and in involvement of purinergic receptors. ATP release mediates I_{Cl,swell} in astrocytes through the activation of P2Y1 receptors. Thus ATP release mediates I_{Cl,swell} in astrocytes through the activation of P2Y1 receptors. The multidrug resistance protein (MRP) transport inhibitors probenicid, indomethacin, and MK-571 all potently inhibited I_{Cl,swell}. ATP release from astrocytes in HOS was observed directly using luciferin-luciferase andMK-571 reversibly depressed this HOS-induced ATP efflux. We conclude that ATP release via MRP and subsequent autocrine activation of purinergic receptors contributes to the activation of I_{Cl,swell} in astrocytes by HOS-induced swelling.

INTRODUCTION

The release of ATP from astrocytes is an important intercellular signal. For example, ATP release from astrocytes (Harden and Lazarowski 1999; Wang et al. 2000) possibly through gap junction hemichannels (Stout et al. 2002) has been shown to be important in [Ca^{2+}]_i wave propagation (Cotrina et al. 1998a,b; Guthrie et al. 1999; Hassinger et al. 1996). In other cell types such as hepatoma and epithelial cells (Roman et al. 1999; Schwiebert et al. 1995; Wang et al. 1996) are present in astrocytes. For example, astrocytes exhibit a Cl^- current (I_{Cl,swell}) through an autocrine pathway involving purinergic receptors. ATP release to inhibitors of transport via MRP. Our results show that ATP release from astrocytes contributes to the activation of Cl^- channels during cellular swelling. We tried several approaches. The first was to see if apyrase (an enzyme that degrades ATP) or purinergic receptor antagonists depressed the activation of I_{Cl,swell} during swelling. Second, we determined whether ATP (and other P2 agonists) evoked Cl^- currents and whether ATP-evoked Cl^- currents were sensitive to blockers of I_{Cl,swell}. Third, we examined whether inhibitors of either p-glycoprotein or MRP function could depress I_{Cl,swell}. Fourth, we directly measured ATP release from astrocytes and examined the sensitivity of ATP release to inhibitors of transport via MRP. Our results show that ATP release during hypo-osmotically induced swelling acts on P2 receptors to activate I_{Cl,swell} in astrocytes.

METHODS

Astrocyte primary cell cultures

Astrocyte primary cultures (McCarthy and de Vellis 1980) were obtained from Sprague Dawley rats (1 day postnatal). Cortical tissue that is dependent on MAP kinase activation (Crepel et al. 1998; Lascola and Kraig 1996). Astrocytes are known to swell in response to a number of stimuli including increased external K^+(K^+_o) (MacVicar et al. 2002) and neurotransmitters (reviewed in Kimelberg 1995; Strange 1993). Multidrug resistance protein (MRP) and p-glycoprotein, the protein product of the multidrug resistance gene (MDR), are two ATP-binding cassette proteins that are expressed in astrocytes (Declèves et al. 2000). Finally, purinergic P2Y receptors are expressed on astrocytes both in cell culture (Centeneri et al. 1997; Cotrina et al. 1998a,b; Fam et al. 2000; Scemes et al. 2000) and in vivo (Franke et al. 2001; Zhu and Kimelberg 2001). Therefore all of the components that are involved in the swelling-induced release of ATP and activation of I_{Cl,swell} in hepatoma and epithelial cells (Roman et al. 1999; Schwiebert et al. 1995; Wang et al. 1996) are present in astrocytes.

The activation of I_{Cl,swell} is part of the cellular changes that occur in response to increased cell volume (Strange et al. 1996). Efflux of Cl^- and amino acids through the channel underlying I_{Cl,swell} in conjunction with efflux of K^+ through other channels is part of the active process to decrease volume, termed regulatory volume decrease (RVD) (Pasantes-Morales et al. 1994a,b). I_{Cl,swell} also allows the efflux of larger amino acids and can contribute to the non-[Ca^{2+}]_i-dependent release of glutamate during spreading depression (Basarsky et al. 1999).

The goal of this study was to determine whether ATP release from astrocytes contributes to the activation of Cl^- channels during cellular swelling. We tried several approaches. The first was to see if apyrase (an enzyme that degrades ATP) or purinergic receptor antagonists depressed the activation of I_{Cl,swell} during swelling. Second, we determined whether ATP (and other P2 agonists) evoked Cl^- currents and whether ATP-evoked Cl^- currents were sensitive to blockers of I_{Cl,swell}. Third, we examined whether inhibitors of either p-glycoprotein or MRP function could depress I_{Cl,swell}. Fourth, we directly measured ATP release from astrocytes and examined the sensitivity of ATP release to inhibitors of transport via MRP. Our results show that ATP release during hypo-osmotically induced swelling acts on P2 receptors to activate I_{Cl,swell} in astrocytes.

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and varied only slightly when the intracellular [Cl]/HCl conditions that the liquid junction potential (LJP) was small (1 astrocytes (Crepel et al. 1998). We previously showed under these conditions that the liquid junction potential (LJP) was small (1 astrocytes (Crepel et al. 1998). Thus the membrane potential was not corrected for the LJP.

Materials

Culture reagents were obtained from Canadian Life Technologies (Burlington, Ontario), aspartic acid from Fisher (Edmonton, Alberta), and sucrose, glucose, BaCl2, MgCl2, and CaCl2 were from VWR (Edmonton, Alberta). All other drugs including the grade I and grade VI apyrase were purchased from Sigma (Oakville, Ontario, Canada).

Results

Hypo-osmotic induced current is blocked by enzyme to degrade ATP

Hypo-osmotic solutions (HOS) consistently induced a Cl− current in astrocytes termed IClswell with properties identical to what we previously reported (Crepel et al. 1998). Figure 1 (A−C) shows the typical HOS activation of IClswell and the subtraction of the ramp currents that we used to quantify the magnitude of IClswell. We examined the actions of apyrase, which metabolizes ATP and ADP, on the magnitude of IClswell. A reduction of IClswell by apyrase would support a role for extracellular ATP and/or ADP in the activation of this current (e.g. Roman et al. 1999; Schwiebert et al. 1995; Wang et al. 1996). Two different forms of apyrase with different degrees of ATPase/ADPase ratio (grade I vs. grade VI; 5–20 units/ml) were used and both reversibly decreased the amplitude of the HOS-activated IClswell (Fig. 1, D−F). The preparation with the highest ATPase/ADPase ratio (G-VI) depressed IClswell with similar efficacy as the form with lower ATPase/ADPase ratio. This suggests that ATP and/or ADP and not a degradation product are the active factors released during swelling.

Purinergic receptor antagonists block IClswell

If ATP release is important in the response to swelling, then purinergic receptor activation should be necessary for the activation of IClswell by HOS. Therefore we examined the response of the HOS activated IClswell to antagonists of purinergic receptors (Fig. 2). In all experiments, we first tested the reproducibility and consistency of the activation of IClswell by HOS. To do this, we activated IClswell twice with HOS before receptor antagonists were applied. The second application of HOS consistently induced IClswell with a magnitude similar to the first. In Fig. 2, C and D, the second HOS-activated current was normalized to the first and was plotted as 0 concentration. Suramin, a wide-spectrum purinergic antagonist, reversibly depressed the HOS-activated IClswell (Fig. 2, A−C; maximum depression at 100 μM, 61 ± 2%, n = 4), RB2 (50 μM), a relatively selective P2Y antagonist, also blocked HOS activated IClswell to a similar extent as suramin (64 ± 5%, n = 4; Fig. 2E). Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (100 μM), an antagonist to P2X receptors but not P2U (Charlton et al. 1996; King et al. 1998), also depressed the HOS-activated IClswell (39.5 ± 6.2%, n = 3; Fig. 2E).

ATP activates a Cl− current

The next step was to determine whether ATP itself and purinergic receptor agonists induced a Cl− current with properties similar to the HOS-activated IClswell. We applied ATP to ensure that the putative ligand mimicked the actions of HOS in activating a Cl− current. ATP activated a Cl− current that reversed at potentials (1 mM ATP; reversal at −6.8 ± 5.4 mV; n = 4) not significantly different from the Cl− equilibrium potential (−8.6 mV) and the IClswell reversal potential (−16 ± 8 mV, n = 4, P > 0.3; Fig. 3, A and B). 5-Nitro-2-(3-
phenylpropylamino) benzoic acid (NPPB, 1 mM; n = 3), which blocks \( I_{\text{Cl,swell}} \) (Crepel et al. 1998), also totally blocked the ATP-activated \( I_{\text{Cl,swell}} \) current. NPPB does not alter purinergic receptors directly (Feranchak et al. 2000; Mitchell et al. 1998).

There were differences in the magnitude of the \( I_{\text{Cl,swell}} \) current activated by ATP and the HOS-activated \( I_{\text{Cl,swell}} \). Figure 3C demonstrates that the current was maximally activated by 1 mM ATP; corresponding to current amplitude that was 53 ± 17% (n = 4) of the preceding HOS-activated current. Increasing the concentration of ATP to 5 mM did not activate a larger current. Therefore the maximum ATP-induced current was significantly less than the HOS-activated \( I_{\text{Cl,swell}} \). We then compared the percent depression induced by suramin, the wide spectrum purinergic antagonist. A supra-maximal concentration of suramin (500 \( \mu \)M) completely blocked the ATP-induced \( I_{\text{Cl,swell}} \) current. NPPB does not alter purinergic \( I_{\text{Cl,swell}} \). We then reversed the concentration of ATP to 5 mM did not activate a larger current. Therefore the maximum ATP-induced current was significantly less than the HOS-activated \( I_{\text{Cl,swell}} \). We then compared the percent depression induced by suramin, the wide spectrum purinergic antagonist. A supra-maximal concentration of suramin (500 \( \mu \)M) completely blocked the ATP-induced \( I_{\text{Cl,swell}} \) current. NPPB does not alter purinergic \( I_{\text{Cl,swell}} \).

We next examined the sensitivity of the HOS-activated \( I_{\text{Cl,swell}} \) to blockers of two transporters that are postulated to play a role in ATP release from other cell types. \( p \)-glycoprotein, the product of the MDR1 gene and MRP are both members of the ABC transport family that have been identified as potential modulators of \( I_{\text{Cl,swell}} \) in other cell types (Hainsworth...

Probenicid blocks activity of the MRP transporter at relatively high concentrations (Courtois et al. 1999; Payen et al. 1999). We observed significant but reversible block of $I_{\text{Cl,swell}}$ at 5–10 mM probenicid (Fig. 4, 103/11006 6% block at 10 mM, n = 4, Fig. 4C). In some cells, there appeared to be some block of a resting $\text{Cl}^{-}$ current in addition to the block of $I_{\text{Cl,swell}}$.

Indomethacin, another inhibitor of MRP-mediated transport (Courtois et al. 1999; Payen et al. 1999), reversibly blocked HOS-activated $I_{\text{Cl,swell}}$ at 500 μM (90 ± 11% depression, n = 5, Fig. 4C). Indomethacin only blocks MRP-mediated transport at this high concentration (Courtois et al. 1999; Payen et al. 1999). At lower concentrations (e.g., < 100 μM), indomethacin inhibits cyclo-oxygenase (COX). However, at 200 μM, the effects of indomethacin were substantially reduced. As an added control to ensure that the effect of indomethacin was due to blocking MRP and not due to COX inhibition, we tested the effect of acetysalicylic acid (ASA), another COX inhibitor. ASA did not inhibit $I_{\text{Cl,swell}}$ (100 μM, −4 ± 1%, n = 3), supporting our conclusion that the block of $I_{\text{Cl,swell}}$ by indomethacin was independent of its effect on COX. In contrast to the potent inhibition by MRP inhibitors, verapamil, which blocks transport by $p$-glycoprotein, caused little reduction of $I_{\text{Cl,swell}}$ at a supra-maximal concentration (1 mM, 19 ± 3%, n = 4, Fig. 4C).

To further substantiate MRP involvement in the activation of $I_{\text{Cl,swell}}$, we tested the effect of MK-571, another MRP transport inhibitor that potently and selectively blocks this transporter (Gekeler et al. 1995; Vernhet et al. 1999). MK-571 (100 μM) reduced HOS-activated $I_{\text{Cl,swell}}$ by 96 ± 13% (n = 6) (Fig. 4C).

Finally we examined ATP efflux from astrocytes to determine if HOS, which induces cellular swelling, can induce the release of ATP. It is known that $\text{Ca}^{2+}$ wave propagation is associated with ATP release from astrocytes, which has been measured in cell culture (Wang et al. 2000). We analyzed ATP release by measuring the photons produced by the luciferin-luciferase mediated degradation of ATP. Increased efflux of ATP will be associated with increased light output if ATP is released during HOS stimulation (e.g., Feranchak et al. 2000). Changing the extracellular solution from control to HOS increased the light output from astrocyte cultures indicating that ATP efflux was increased (Fig. 5). MK-571 totally blocked the HOS-mediated increase (n = 5 cultures). Although this technique did not allow the quantification of the local ATP concentration outside of the cellular membrane, it did provide...
support that ATP efflux occurred during HOS stimulation of cultured astrocytes

**DISCUSSION**

The results of this study suggest that $I_{Cl,swell}$ was activated by ATP that was released from astrocytes during swelling. We have shown that the HOS-activated $I_{Cl,swell}$ was depressed by apyrase, an enzyme that degrades ATP and ADP. An NPPB-sensitive Cl$^-$ current that was similar to $I_{Cl,swell}$ was activated by application of ATP. Other agonists of the P2Y1 receptor (ADP and ADPβS) also induced a Cl$^-$ current, whereas UTP, a P2U and P2Y$_{2,3,4}$ agonist and α,β-MeATP, a P2X agonist, were ineffective. The P2X7 agonist, BzATP was not as effective in activating $I_{Cl,swell}$ indicating that P2X7 receptors were likely not involved. Both the nonspecific purinergic receptor antagonist, suramin and the selective P2Y antagonist, RB2 blocked the HOS-activated $I_{Cl,swell}$. PPADS that inhibits P2Y1 receptors also depressed the HOS-activated $I_{Cl,swell}$. Supporting a role for P2Y1 receptor activation. Several pharmacological antagonists of the MRP transporter blocked the HOS-induced $I_{Cl,swell}$. Verapamil, the potent blocker of p-glycoprotein transport had no effect on the HOS-activated $I_{Cl,swell}$ suggesting that this transport pathway was not involved. Finally, HOS-induced the efflux of ATP measured as an increase in light output using luciferin-luciferase reaction to assay ATP efflux. The HOS induced efflux of ATP was reversibly inhibited by MK-571, which was also effective in inhibiting the HOS-activated $I_{Cl,swell}$. These results suggest that MRP causes the efflux of ATP during swelling which in turn acts in an autocrine manner to activate purinergic receptors and subsequently $I_{Cl,swell}$.

In epithelial cells and hepatocytes, the activation of $I_{Cl,swell}$ depends on the activation of extracellular purinergic receptors because the response to HOS can be depressed by purinergic receptor antagonists (Mitchell et al. 1998; Roman et al. 1999; Schwiebert et al. 1995; Wang et al. 1996). The present study demonstrates that astrocytes also regulate their volume through a similar mechanism. Our results provide evidence that ATP is one of the native messenger molecules that bind P2Y receptors and thereby activate $I_{Cl,swell}$. The luciferin-luciferase experiment indicates that ATP itself is released. However, we cannot rule out a potential contribution of ADP release in addition to ATP. UTP, which may be involved in the response to HOS in other cells (Harden and Lazarowski 1999), was not involved in this process in astrocytes because we could not observe any Cl$^-$ current activation by UTP. Exogenously applied ATP evoked a current with properties similar to $I_{Cl,swell}$. Apyrase, the enzyme that degrades ATP, inhibited the HOS-activated $I_{Cl,swell}$. We found that the P2 purinergic antagonists, suramin, RB2, and PPADs, inhibited $I_{Cl,swell}$: Suramin is nonselective against P2Y and P2X, but RB2 is a specific P2Y receptor antagonist (Abbracchio and Burnstock 1994; Burnstock and Warland 1987; Najbar et al. 1996), thus implying P2Y purinergic regulation of $I_{Cl,swell}$. PPADs inhibits P2Y1 receptors in addition to P2X receptors (Charlton et al. 1996; King et al. 1998), suggesting involvement of P2Y1 receptors. However, P2X receptors are not involved because we did not observe activation of Cl$^-$ currents by α,β-MeATP, a P2X agonist. It is likely that P2X7 receptors are not involved because the P2X7 agonist, BzATP was less effective than ATP in inducing $I_{Cl,swell}$. Recently Neary et al. (1999) showed that signaling from P2Y receptors to Erk involved a [Ca$^{2+}$] independent isofrom of PKC. This pathway provides a mechanism by which the HO$S$-mediated activation of purinergic receptors could activate the Erk MAP kinase cascade in a Ca$^{2+}$-indepdent manner and thereby activate $I_{Cl,swell}$ (Crepel et al. 1998).

A critical issue is the mechanism by which ATP is released into the extracellular space. In hepatocytes, the MDR1 gene product, p-glycoprotein functions as an ATP transporter (Abraham et al. 1993; Roman et al. 1997; Vanoye et al. 1999) and as an intimate regulator of $I_{Cl,swell}$ and RVD (Hardy et al. 1995; Luckie et al.
p-glycoprotein is expressed in the astrocyte endfoot processes of the brain microvasculature (Golden and Pardridge 1999) and both MRP1 and p-glycoprotein are expressed in cultured astrocytes (Decleves et al. 2000). In hepatocytes and fibroblasts, verapamil potently blocked p-glycoprotein activity and the activation of $I_{\text{Cl,swell}}$ and thereby inhibited volume recovery (Roman et al. 1997; Valverde et al. 1992). However, in the present study, verapamil did not significantly inhibit $I_{\text{Cl,swell}}$, even when applied at 1 mM. Although these results do not completely rule out some involvement of p-glycoprotein in the activation of $I_{\text{Cl,swell}}$ in astrocytes, they do suggest a very minimal role, if any, for this transporter.

Hainsworth et al. (1996) reported that hypotonicity-induced anion fluxes were significantly larger in MRP-over expressing cells. This finding and reports that MRP is expressed in the brain (Stride et al. 1996; Zaman et al. 1993) and in astrocytes (Decleves et al. 2000) led us to hypothesize that MRP may also transport ATP in response to hypotonicity. Therefore we measured the effect of MRP inhibitors, probenicid, indomethacin, and MK-571, on $I_{\text{Cl,swell}}$. Maximum block has been reported at concentrations ranging from 1 to 10 mM probenicid, 10–500 μM indomethacin, and 50–100 μM MK-571 (Courtois et al. 1999; Draper et al. 1997; Huai-Yun et al. 1998; Payen et al. 1999; Vernhet et al. 1999). In the present study, we report a complete block of $I_{\text{Cl,swell}}$ by 10 mM probenicid, 500 μM indomethacin, and 100 μM MK-571. Indomethacin had no effect at lower concentrations that would be expected to inhibit COX. As a control we also tested ASA, a potent inhibitor of COX, and found no effect indicating that COX is not involved in activation of $I_{\text{Cl,swell}}$. Therefore these results suggest that $I_{\text{Cl,swell}}$ in cultured astrocytes depends on the activity of MRP transporters.

The impact of ATP release from astrocytes and the activation of $I_{\text{Cl,swell}}$ could be profound in the CNS. Measurements of intrinsic optical signals and the extracellular space directly have shown that brain tissue swells in response to activity (Andrew and MacVicar 1994; Grinvald et al. 1986; Holthoff and Witte 1996; Lieke et al. 1989; MacVicar and Hochman 1994; Roman et al. 1997; Valverde et al. 1992). p-glycoprotein is expressed in the astrocyte endfoot processes of the brain microvasculature (Golden and Pardridge 1999) and both MRPI and p-glycoprotein are expressed in cultured astrocytes (Decleves et al. 2000). In hepatocytes and fibroblasts, verapamil potently blocked p-glycoprotein activity and the activation of $I_{\text{Cl,swell}}$ and thereby inhibited volume recovery (Roman et al. 1997; Valverde et al. 1992). However, in the present study, verapamil did not significantly inhibit $I_{\text{Cl,swell}}$ even when applied at 1 mM. Although these results do not completely rule out some involvement of p-glycoprotein in the activation of $I_{\text{Cl,swell}}$ in astrocytes, they do suggest a very minimal role, if any, for this transporter.

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In summary, we conclude that the activation of $I_{\text{Cl,swell}}$ is dependent on the stimulation of purinergic receptors because this current was blocked by the purinergic antagonists suramin and RB2, inhibited by the nucleotidase, apyrase and mimicked by exogenously applied ATP. We propose that the ATP needed to stimulate the purinergic receptors is released via a transporter having a pharmacological sensitivity similar to that of the MRP transporter family.

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