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# 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 ( $[Ca^{2+}]$ ) 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 activation of  $I_{Cl,swell}$  and  $K^+$  currents. We used whole cell patch-clamp recordings in cultured astrocytes to investigate the autocrine role of ATP in the activation of  $I_{Cl,swell}$  by hypo-osmotic solution (HOS). Apyrase, an ATP/ADP nucleotidase, inhibited HOS-activated  $I_{Cl,swell}$ , whereas ATP and the P2Y agonists, ADP $\beta$ S and ADP, induced  $Cl^-$  currents similar to  $I_{Cl,swell}$ . Neither the P2U agonist, UTP nor the P2X agonist,  $\alpha,\beta$ -methylene ATP, were effective. BzATP was less effective than ATP, suggesting that P2X7 receptors were not involved. P2 purinergic antagonists, suramin, RB2, and pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) reversibly inhibited activation of  $I_{Cl,swell}$ , suggesting that ATP-activated P2Y1 receptors. Thus ATP release mediates  $I_{Cl,swell}$  in astrocytes through the activation of P2Y1-like receptors. The multidrug resistance protein (MRP) transport inhibitors probenecid, indomethacin, and MK-571 all potently inhibited  $I_{Cl,swell}$ . ATP release from astrocytes in HOS was observed directly using luciferin-luciferase and MK-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  $[Ca^{2+}]$  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, cellular swelling causes ATP release, which acts in an autocrine manner on P2 purinergic receptors to modulate swelling activated  $Cl^-$  currents ( $I_{Cl,swell}$ ) (Roman et al. 1999; Schwiebert et al. 1995; Wang et al. 1996). The swelling-induced release of ATP in hepatoma cells may be through ATP-binding cassette proteins (Schwiebert 1999) such as *p*-glycoprotein (Hazama et al. 2000; Roman et al. 1997). It is possible that similar mechanisms are present in astrocytes. For example, astrocytes exhibit a  $I_{Cl,swell}$

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  $K^+$  ( $[K^+]_{ext}$ ) (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 (Decleves et al. 2000). Finally, purinergic P2Y receptors are expressed on astrocytes both in cell culture (Centemeri et al. 1997; Cotrina et al. 1998a; 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- $[Ca^{2+}]$ -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 released 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

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(with meninges and pia mater removed) was dissociated by mechanical trituration and transferred to tissue culture flasks (1 cortex/flask) containing glial media for 2–3 wk [Dulbecco's modified Eagle medium (DMEM) with 58 mM NaHCO<sub>3</sub>, 20 mM HEPES, 50 U/ml pen/strep, 10% fetal calf serum at 37°C and 5% CO<sub>2</sub>].

### Electrophysiological recordings

Astrocytes were plated onto poly-ornithine-coated glass coverslips  $\geq 1$  day prior to electrophysiological recordings. Coverslips with astrocytes were placed in a 200- $\mu$ l recording chamber on an inverted microscope with phase-contrast optics (Axiovert, Zeiss) and superfused at 1–2 ml/min (20–22°C) with the following solution, which we used previously to isolate the Cl<sup>-</sup> currents (Crepel et al. 1998), containing (in mM): 70 Trizma-HCl, 100 sucrose, 1.5 CaCl<sub>2</sub>, 10 HEPES, 10 glucose, 5 TEA-Cl, and 5 BaCl<sub>2</sub> adjusted to pH 7.3 with CsOH. The osmolarity of this solution was 290 mosM (Crepel et al. 1998), and the hypo-osmotic solution (HOS) was the standard extracellular solution without added sucrose. Patch-clamp pipettes had a resistance of 4–7 M $\Omega$  when filled with electrode solution containing (in mM) 60 Trizma-HCl, 70 Trizma-base, 70 aspartic acid, 15 HEPES, 0.4 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1 ATP, 0.5 GTP, and 1 EGTA, adjusted to pH 7.25 with CsOH. Membrane currents were recorded under voltage clamp ( $V_H = -70$  mV) using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). Cells with a stable holding current and access resistance (<20 M $\Omega$ ; typical capacitance >30 pF) were recorded from for the subsequent experiments. These conditions were the same as we previously used to examine Cl<sup>-</sup> currents in astrocytes (Crepel et al. 1998). We previously showed under these conditions that the liquid junction potential (LJP) was small (1–3 mV) and varied only slightly when the intracellular [Cl<sup>-</sup>] was changed (Crepel et al. 1998). Thus the membrane potential was not corrected for the LJP.

### Data acquisition and analysis

We used two Digidata 1200 Interface boards (Axon Instruments) to simultaneously digitize membrane currents onto two separate computers using Clampex 7 or 8 (Axon Instruments). One computer measured a continuous gap-free recording of membrane current for each experiment (holding potential: -70 mV) and the other measured the current resulting from a 2-s voltage ramp from -120 to +60 mV applied every 30 s.

In all experiments with antagonists, we applied HOS twice to ensure reproducibility of the HOS-induced  $I_{Cl,swell}$ . The result in the experimental solution was compared statistically to the second HOS current normalized to the first HOS current. This is shown as the 0 concentration value in some graphs. Statistical analysis was done using ANOVA. post hoc multiple comparisons were performed using Tukey's ( $P < 0.05$ ). Values are presented as mean  $\pm$  SE.

### Luciferin-luciferase assays

ATP release was examined using luciferin-luciferase (Sigma, St. Louis, MO) that was added to the extracellular solution at 10–20 mg/ml. Measurements were made using a photomultiplier tube with a current-to-voltage converter (Hamamatsu, Hamamatsu, Japan). The output was low-pass filtered (100 Hz) and digitized using the same system as described in the preceding text for voltage-clamp recordings.

### Materials

Culture reagents were obtained from Canadian Life Technologies (Burlington, Ontario), aspartic acid from Fisher (Edmonton, Alberta), and sucrose, glucose, BaCl<sub>2</sub>, MgCl<sub>2</sub>, and CaCl<sub>2</sub> 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<sup>-</sup> current in astrocytes termed  $I_{Cl,swell}$  with properties identical to what we previously reported (Crepel et al. 1998). Figure 1 (A–C) shows the typical HOS activation of  $I_{Cl,swell}$  and the subtraction of the ramp currents that we used to quantify the magnitude of  $I_{Cl,swell}$ . We examined the actions of apyrase, which metabolizes ATP and ADP, on the magnitude of  $I_{Cl,swell}$ . A reduction of  $I_{Cl,swell}$  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  $I_{Cl,swell}$  (Fig. 1, D–F). The preparation with the highest ATPase/ADPase ratio (G–VI) depressed  $I_{Cl,swell}$  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 $I_{Cl,swell}$

If ATP release is important in the response to swelling, then purinergic receptor activation should be necessary for the activation of  $I_{Cl,swell}$  by HOS. Therefore we examined the response of the HOS activated  $I_{Cl,swell}$  to antagonists of purinergic receptors (Fig. 2). In all experiments, we first tested the reproducibility and consistency of the activation of  $I_{Cl,swell}$  by HOS. To do this, we activated  $I_{Cl,swell}$  twice with HOS before receptor antagonists were applied. The second application of HOS consistently induced  $I_{Cl,swell}$  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  $I_{Cl,swell}$  (Fig. 2, A–C; maximum depression at 100  $\mu$ M, 61  $\pm$  2%,  $n = 4$ ). RB2 (50  $\mu$ M), a relatively selective P2Y antagonist, also blocked HOS activated  $I_{Cl,swell}$  to a similar extent as suramin (64  $\pm$  5%,  $n = 4$ ; Fig. 2E). Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (100  $\mu$ M), an antagonist to P2Y1 as well as P2X receptors but not P2U (Charlton et al. 1996; King et al. 1998), also depressed the HOS-activated  $I_{Cl,swell}$  (39.5  $\pm$  6.2%,  $n = 3$ ; Fig. 2E).

### ATP activates a Cl<sup>-</sup> current

The next step was to determine whether ATP itself and purinergic receptor agonists induced a Cl<sup>-</sup> current with properties similar to the HOS-activated  $I_{Cl,swell}$ . We applied ATP to ensure that the putative ligand mimicked the actions of HOS in activating a Cl<sup>-</sup> current. ATP activated a Cl<sup>-</sup> current that reversed at potentials (1 mM ATP; reversal at  $-6.8 \pm 5.4$  mV;  $n = 4$ ) not significantly different from the Cl<sup>-</sup> equilibrium potential ( $-8.6$  mV) and the  $I_{Cl,swell}$  reversal potential ( $-16 \pm 8$  mV,  $n = 4$ ,  $P > 0.3$ ; Fig. 3, A and B). 5-Nitro-2-(3-

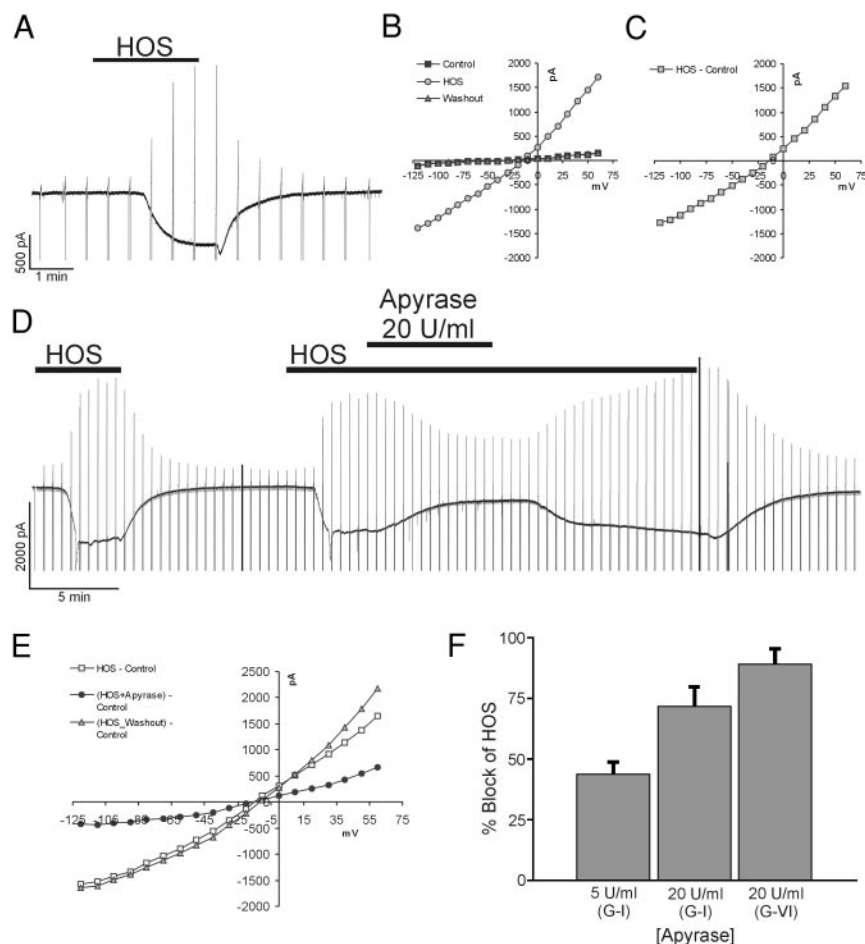


FIG. 1. Hypo-osmotic stimulation (HOS)-activated  $I_{Cl,swell}$  that was reversibly inhibited by apyrase. *A*: gap-free trace of the membrane current recorded before, during, and after HOS (3 min). Voltage-ramp command steps from  $-120$  to  $+60$  mV (2-s duration) were performed every 30 s. The holding potential between ramps was maintained at  $-70$  mV. *B*: individual current-voltage ( $I$ - $V$ ) relations obtained before HOS application (control), during HOS application (HOS), and after HOS application (washout). *C*: the HOS-induced current obtained by subtracting the current recorded before HOS application from that obtained during HOS application. *D*: the membrane current recorded during the superfusion of HOS was depressed by 20 U/ml grade I apyrase. *E*:  $I$ - $V$  relations of the HOS-induced currents obtained before, during, and after application of 20 U/ml grade I apyrase. *F*: illustration of inhibition of the HOS-induced current by 5 and 20 U/ml of grade I apyrase and 20 U/ml of grade VI apyrase.

phenylpropylamino) benzoic acid (NPPB, 1 mM;  $n = 3$ ), which blocks  $I_{Cl,swell}$  (Crepel et al. 1998), also totally blocked the ATP-activated  $Cl^-$  current. NPPB does not alter purinergic receptors directly (Feranchak et al. 2000; Mitchell et al. 1998).

There were differences in the magnitude of the  $Cl^-$  current activated by ATP and the HOS-activated  $I_{Cl,swell}$ . Figure 3C demonstrates that the current was maximally activated by 1 mM ATP; corresponding to current amplitude that was  $53 \pm 17\%$  ( $n = 4$ ) 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  $I_{Cl,swell}$ . 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  $Cl^-$  current but only reduced the HOS-activated  $I_{Cl,swell}$  by  $\sim 50\%$  (Fig. 3D). These results imply that another agent is released in addition to ATP to evoke HOS-activated  $I_{Cl,swell}$ . However, we have not yet identified the nature of this other agent.

We examined the activation of the  $Cl^-$  current by other purinergic receptor agonists to further define the receptor subtype involved (Fig. 3E). These experiments also addressed the possibility that the differences in the magnitude of the current amplitude indicated the involvement of a purinergic receptor that was activated more effectively by another agonist such as UTP. All agonists were tested and compared at 100  $\mu$ M. UTP, which activates P2Y<sub>2,3,4</sub> and P2U receptors (Ralevic and Burn-

stock 1998), did not induce a  $Cl^-$  current ( $n = 5$ ) nor did  $\alpha,\beta$ -methylene ATP ( $\alpha,\beta$ -MeATP;  $n = 4$ ), an agonist at P2X receptors. ADP and ADP $\beta$ S both evoked currents that reversed close to the  $Cl^-$  equilibrium potential (Fig. 3; ADP-induced current reversed at  $-9.6 \pm 2.0$  mV,  $n = 4$ ; ADP- $\beta$ S-induced current reversed at  $-0.4 \pm 1.4$  mV,  $n = 5$ ;  $Cl^-$  equilibrium potential,  $-8.6$  mV). Both ADP and ADP $\beta$ S were slightly more efficacious at inducing a  $Cl^-$  current than was ATP (Fig. 3E). The P2X7 agonist BzATP induced a current at 100  $\mu$ M that was, however, of lesser amplitude than that evoked by 100  $\mu$ M ATP ( $16 \pm 3\%$ ,  $n = 3$ ). This indicates that ATP was not working through P2X7 receptors, which should be preferentially activated by BzATP (Panenka et al. 2001; Ralevic and Burnstock 1998). Our results with the agonists suggest that the purinergic receptor was the P2Y1 subtype (Ralevic and Burnstock 1998). This was consistent with the receptor antagonist profile that we described in the preceding text.

#### *Inhibitors of multidrug resistance protein but not p-glycoprotein blocked HOS-activated $I_{Cl,swell}$ and ATP release*

We next examined the sensitivity of the HOS-activated  $I_{Cl,swell}$  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  $I_{Cl,swell}$  in other cell types (Hainsworth

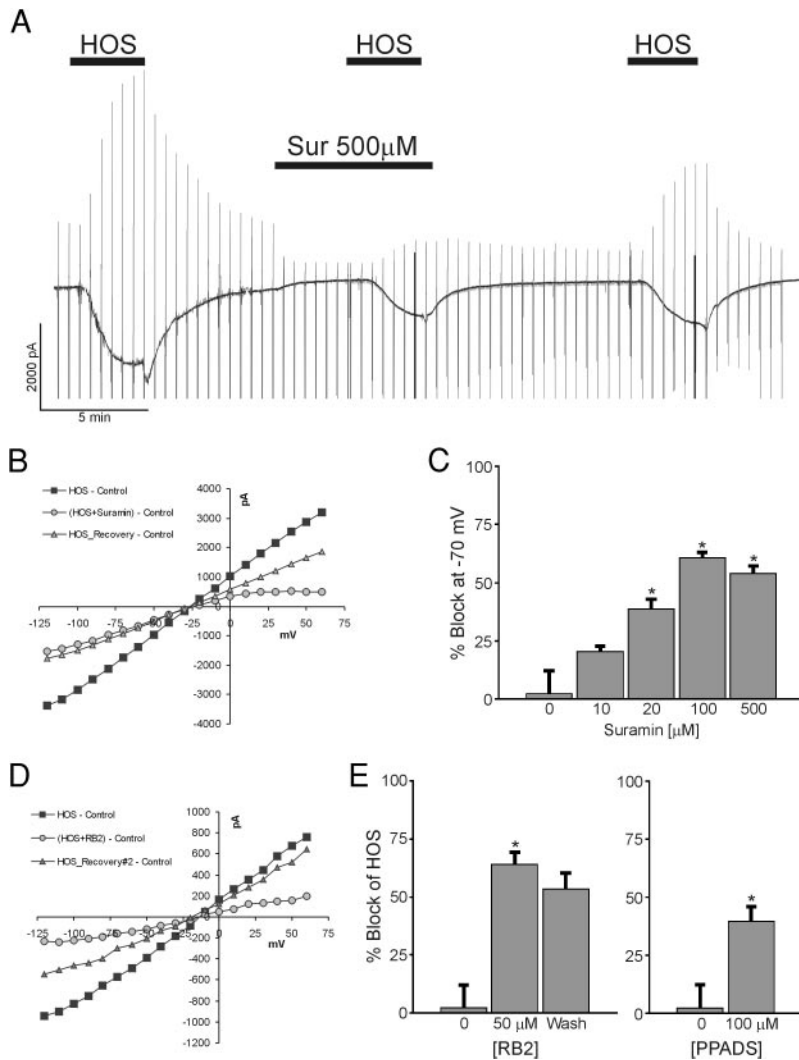


FIG. 2. Suramin, RB2, and pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) inhibited HOS-induced  $I_{Cl,swell}$ . *A*: membrane current evoked by 3 successive HOS stimulations (3 min in duration) illustrating the reversible block of  $I_{Cl,swell}$  by suramin (500  $\mu$ M). *B*: HOS-induced  $I_{Cl,swell}$  (obtained by subtracting the current recorded before each HOS application from that obtained during each HOS application) in the presence and absence of suramin. *C*: dose response for the suramin block of  $I_{Cl,swell}$ . Maximal block was observed at 100  $\mu$ M. The value at 0 concentration in this and the following graphs represents the 2nd HOS-induced current normalized with respect to the 1st application. *D*: RB2 also blocked  $I_{Cl,swell}$  as shown in the *I-V* relations of HOS-induced currents obtained before, during, and after the superfusion of 50  $\mu$ M RB2. *E*: bar graph depicting the degree of inhibition of the HOS-induced current by 50  $\mu$ M RB2 and by 100  $\mu$ M PPADS. \*,  $P < 0.05$ .

et al. 1996; Hardy et al. 1995; Luckie et al. 1994; Roman et al. 1997; Valverde et al. 1992).

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  $I_{Cl,swell}$  at 5–10 mM probenicid (Fig. 4,  $103 \pm 6\%$  block at 10 mM,  $n = 4$ , Fig. 4C). In some cells, there appeared to be some block of a resting  $Cl^-$  current in addition to the block of  $I_{Cl,swell}$ . Indomethacin, another inhibitor of MRP-mediated transport (Courtois et al. 1999; Payen et al. 1999), reversibly blocked HOS-activated  $I_{Cl,swell}$  at 500  $\mu$ M ( $90 \pm 11\%$  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.  $< 100 \mu$ M), indomethacin inhibits cyclo-oxygenase (COX). However, at 200  $\mu$ M, 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 acetylsalicylic acid (ASA), another COX inhibitor. ASA did not inhibit  $I_{Cl,swell}$  (100  $\mu$ M,  $-4 \pm 1\%$ ,  $n = 3$ ), supporting our conclusion that the block of  $I_{Cl,swell}$  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

$I_{Cl,swell}$  at a supra-maximal concentration (1 mM,  $19 \pm 3\%$ ,  $n = 4$ , Fig. 4C).

To further substantiate MRP involvement in the activation of  $I_{Cl,swell}$ , we tested the effect of MK-571, another MRP transporter inhibitor that potently and selectively blocks this transporter (Gekeler et al. 1995; Vernhet et al. 1999). MK-571 (100  $\mu$ M) reduced HOS-activated  $I_{Cl,swell}$  by  $96 \pm 13\%$  ( $n = 6$ ) (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  $Ca^{2+}$  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

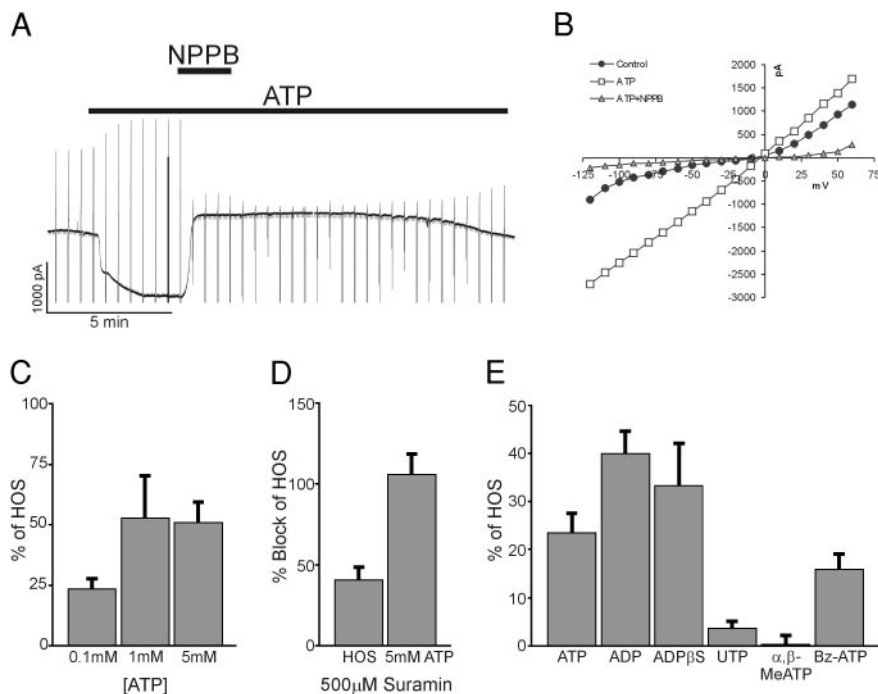


FIG. 3. ATP and P2Y1 agonists activated  $\text{Cl}^-$  currents in cultured astrocytes. **A**: recording of the membrane current evoked by ATP (5 mM) showing substantial block by 1 mM 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB). **B**: individual non-subtracted  $I$ - $V$  relations of the control current and of the currents activated by 5 mM ATP and 5 mM ATP with 1 mM NPPB. The ATP-activated currents reversed at the  $\text{Cl}^-$  equilibrium potential. **C**: bar graphs of the magnitude of the ATP-induced current compared with the HOS-induced  $I_{\text{Cl,swell}}$  recorded in the same cells. The maximal  $\text{Cl}^-$  current was observed at 1 mM ATP and was  $\sim 50\%$  of the HOS current amplitude. **D**: bar graph showing the degree of inhibition invoked by 500  $\mu\text{M}$  suramin on the HOS-induced current and on the 5 mM ATP-induced current. Approximately 50% of the HOS-evoked current was blocked by suramin whereas 100% of the ATP-evoked current was blocked. **E**: bar graph showing the relative amplitudes of the  $\text{Cl}^-$  current evoked by various purinergic receptor analogs (at 100  $\mu\text{M}$ ) as compared with the amplitude of the HOS-induced  $I_{\text{Cl,swell}}$ . The P2Y1 agonists ADP and ADP $\beta\text{S}$  evoked substantial currents, whereas the P2U agonist UTP and the P2X agonist  $\alpha,\beta$ -MeATP were ineffective. The P2X7 agonist, BzATP evoked a current of smaller amplitude than ATP at the same concentration (100  $\mu\text{M}$ ).

support that ATP efflux occurred during HOS stimulation of cultured astrocytes

## DISCUSSION

The results of this study suggest that  $I_{\text{Cl,swell}}$  was activated by ATP that was released from astrocytes during swelling. We have shown that the HOS-activated  $I_{\text{Cl,swell}}$  was depressed by apyrase, an enzyme that degrades ATP and ADP. An NPPB-sensitive  $\text{Cl}^-$  current that was similar to  $I_{\text{Cl,swell}}$  was activated by application of ATP. Other agonists of the P2Y1 receptor (ADP and ADP $\beta\text{S}$ ) also induced a  $\text{Cl}^-$  current, whereas UTP, a P2U and P2Y $_{2,3,4}$  agonist and  $\alpha,\beta$ -MeATP, a P2X agonist, were ineffective. The P2X7 agonist, BzATP was not as effective in activating  $I_{\text{Cl,swell}}$ , 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  $I_{\text{Cl,swell}}$ . PPADS that inhibits P2Y1 receptors also depressed the HOS-activated  $I_{\text{Cl,swell}}$ , supporting a role for P2Y1 receptor activation. Several pharmacological antagonists of the MRP transporter blocked the HOS-induced  $I_{\text{Cl,swell}}$ . Verapamil, the potent blocker of  $p$ -glycoprotein transport had no effect on the HOS-activated  $I_{\text{Cl,swell}}$ , 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  $I_{\text{Cl,swell}}$ . 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  $I_{\text{Cl,swell}}$ .

In epithelial cells and hepatocytes, the activation of  $I_{\text{Cl,swell}}$  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;

Schiebert 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  $I_{\text{Cl,swell}}$ . 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  $\text{Cl}^-$  current activation by UTP. Exogenously applied ATP evoked a current with properties similar to  $I_{\text{Cl,swell}}$ . Apyrase, the enzyme that degrades ATP, inhibited the HOS-activated  $I_{\text{Cl,swell}}$ . We found that the P2 purinergic antagonists, suramin, RB2, and PPADS, inhibited  $I_{\text{Cl,swell}}$ . 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  $I_{\text{Cl,swell}}$ . 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  $\text{Cl}^-$  currents by  $\alpha,\beta$ -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  $I_{\text{Cl,swell}}$ . Recently Neary et al. (1999) showed that signaling from P2Y receptors to Erk involved a  $[\text{Ca}^{2+}]$  independent isoform of PKC. This pathway provides a mechanism by which the HOS-mediated activation of purinergic receptors could activate the Erk MAP kinase cascade in a  $\text{Ca}^{2+}$ -independent manner and thereby activate  $I_{\text{Cl,swell}}$  (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  $I_{\text{Cl,swell}}$  and RVD (Hardy et al. 1995; Luckie et al.

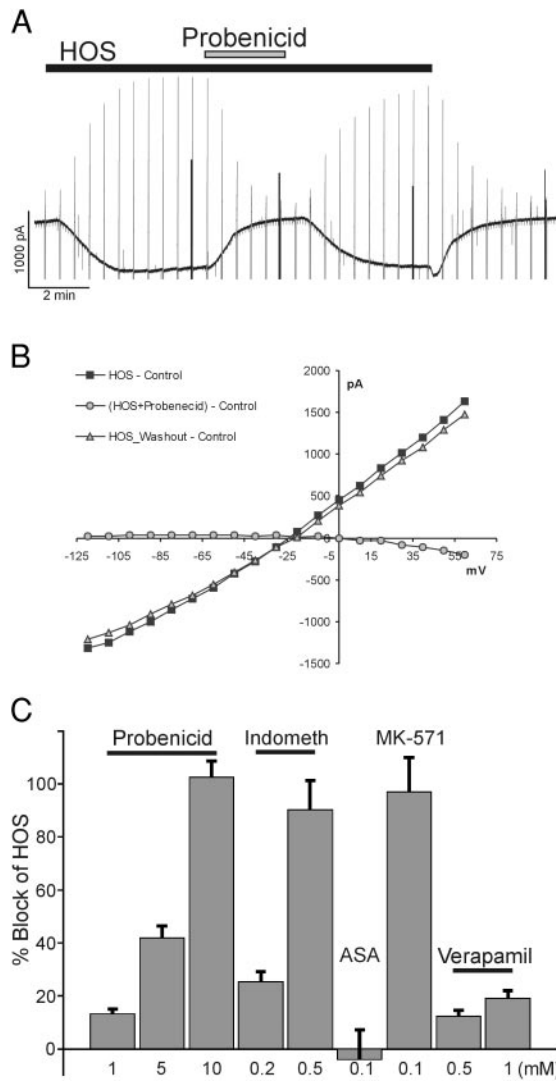


FIG. 4. Antagonists to the MRP transporter activity depressed HOS-induced  $I_{Cl,swell}$ . A: a prolonged application of HOS-activated  $I_{Cl,swell}$  that was reversibly blocked by probenecid (10 mM). B:  $I$ - $V$  relations of the currents induced by HOS alone and HOS with 10 mM probenecid showing profound and reversible block. C: graph summarizing the effects of the various drugs used to test for the mechanism of ATP release. Probenecid, indomethacin, and MK-571 all inhibit the MRP transporter and all were equally potent in their block of  $I_{Cl,swell}$ . Indomethacin did not block the current at lower concentrations (200  $\mu$ M), which blocks cyclo-oxygenase enzyme activity. Also acetylsalicylic acid (ASA), which effectively blocks cyclo-oxygenase at 100  $\mu$ M but has no effect on MRP activity, did not inhibit this current. Verapamil, which is a potent  $p$ -glycoprotein transport inhibitor, only slightly inhibited  $I_{Cl,swell}$ .

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 MRP1 and  $p$ -glycoprotein are expressed in cultured astrocytes (Declèves et al. 2000). In hepatocytes and fibroblasts, verapamil potently blocked  $p$ -glycoprotein activity and the activation of  $I_{Cl,swell}$  and thereby inhibited volume recovery (Roman et al. 1997; Valverde et al. 1992). However, in the present study, verapamil did not significantly inhibit  $I_{Cl,swell}$ , even when applied at 1 mM. Although these results do not completely rule out some involvement of  $p$ -glycoprotein in the activation of  $I_{Cl,swell}$  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 (Declèves 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, probenecid, indomethacin, and MK-571, on  $I_{Cl,swell}$ . Maximum block has been reported at concentrations ranging from 1 to 10 mM probenecid, 10–500  $\mu$ M indomethacin, and 50–100  $\mu$ M 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  $I_{Cl,swell}$  by 10 mM probenecid, 500  $\mu$ M indomethacin, and 100  $\mu$ M 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  $I_{Cl,swell}$ . Therefore these results suggest that  $I_{Cl,swell}$  in cultured astrocytes depends on the activity of MRP transporters.

The impact of ATP release from astrocytes and the activation of  $I_{Cl,swell}$  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

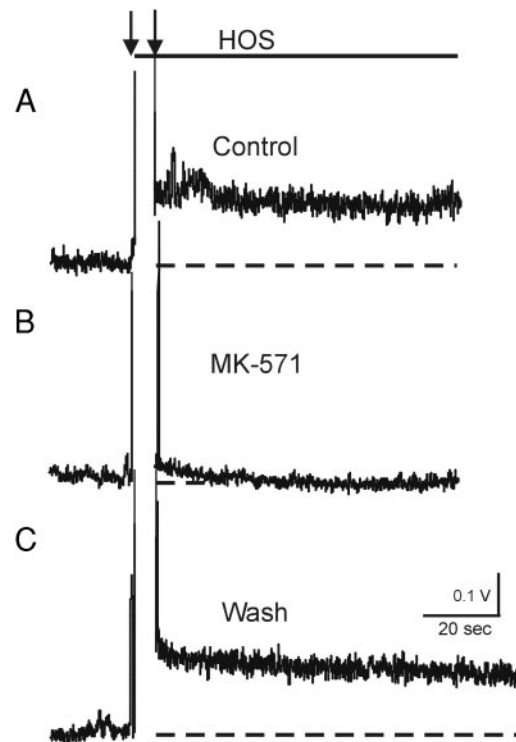


FIG. 5. ATP release from astrocytes, measured using luciferin-luciferase, showed increased efflux in HOS that was blocked by the MRP inhibitor, MK-571. A: the output from the photomultiplier system shows the level of light production in astrocyte cultures with luciferin-luciferase was increased when cultures were exposed to HOS solution. The traces represent the output from a photomultiplier tube that detected photons generated by the luciferase catalyzed reaction of luciferin with ATP. ↓, a gap of ~40 s during the solution change. The light output is proportional to the ATP efflux and shows that after the switch to HOS solution, the efflux was significantly enhanced. In MK-571 (100  $\mu$ M), the efflux of ATP in HOS was blocked and the inhibition by MK-571 was reversible after wash.

1991). Although in the intact CNS it is difficult to rigorously delineate which cell type swells preferentially in response to activity, we have shown that in the optic nerve astrocytes swell in response to high external  $[K^+]$  (MacVicar et al. 2002). The profound swelling that accompanies spreading depression is also associated with activation of  $Cl^-$  channels and the release of glutamate through NPPB-sensitive  $Cl^-$  channels (Basarsky et al. 1998, 1999). If the release of ATP from astrocytes occurs during swelling in vivo, this could provide a novel mechanism by which purinergic receptors could be activated to provide negative feedback by ATP itself at high concentrations (Armstrong et al. 2002) or through activation of inhibitory adenosine receptors by the metabolism of ATP (Dunwiddie and Masino 2001). Alternatively ATP release could contribute to the generation of calcium waves in astrocytes (Guthrie et al. 1999).

In summary, we conclude that the activation of  $I_{Cl,swell}$  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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