Calcium Dependence of Hypoosmotically Induced Potassium Release in Cultured Astrocytes

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A major mechanism in cell volume regulation after hypoosmotic stress is K⁺ release. Our studies show that in astrocytes, K⁺ release during hypoosmotic stress is a Ca²⁺-dependent process. Agents that increase intracellular Ca²⁺, such as ionomycin and ouabain, potentiated hypoosmotically stimulated K⁺ release, while compounds that block Ca²⁺ entry during hypoosmotic stress, such as nimodipine, bepridil, and MK-801, inhibited hypoosmotically stimulated K⁺ release. Similarly, chelation of intracellular Ca²⁺ blocked hypoosmotically induced K⁺ release. Caffeine and U-73122 also inhibited K⁺ efflux under hypoosmotic conditions, suggesting that intracellular Ca²⁺ release from Ca²⁺-induced Ca²⁺ release stores and inositol trisphosphate-sensitive intracellular Ca²⁺ stores play a role in the mechanism of K⁺ release. Blocking the activity of calmodulin, and of CaM kinase, attenuated hypoosmotically induced K⁺ release. Our findings indicate that entry of extracellular Ca²⁺ and Ca²⁺ release from intracellular stores play a key role in the activation of K⁺ release under hypoosmotic conditions and thus in cell volume regulation.

[Key words: astrocytes, calcium, hypoosmotic stress, potassium, volume regulation]

When exposed to hypoosmotic stress, astrocytes initially swell and then reduce their volume to approximately their original size, a phenomenon referred to as regulatory volume decrease (RVD). The release of ions such as K⁺, Cl[−], and of various amino acids may play a key role in the mechanism of RVD (Kimelberg et al., 1992). Since RVD is a Ca²⁺-dependent process in various cell types (McCarthy and O'Neil, 1992), including astrocytes (O'Connor and Kimelberg, 1993), the release of K⁺ may also be dependent on Ca²⁺. The influx of Ca²⁺ during hypoosmotic stress has been observed (Bender et al., 1992b; O'Connor and Kimelberg, 1993), which may be the result of hypoosmotically induced depolarization (Kimelberg and O'Connor, 1988). Voltage-dependent Ca²⁺ channels are present in astrocytes (Barres, 1991; Howell et al., 1991), and the L-type Ca²⁺ channel appears to be the one mediating Ca²⁺ influx

(O'Connor and Kimelberg, 1993; Bender et al., 1994). Ca²⁺ influx may also occur as a result of the reversal of the Na⁺/Ca²⁺ exchanger (Kim-Lee et al., 1992; Bender et al., 1993b), which is likewise present in astrocytes (Fatatis and Brennehan, 1990; Blaustein et al., 1991).

The extracellular Ca²⁺ that enters the cell during hypoosmotic stress may serve as a source for the increase in the intracellular Ca²⁺ observed in astrocytes (O'Connor and Kimelberg, 1993). Increase in intracellular Ca²⁺ is important in the mechanism of RVD, since inhibition of hypoosmotically stimulated phosphoinositide hydrolysis inhibits RVD (Bender et al., 1993a). Agents that deplete intracellular Ca²⁺ also inhibit RVD (Hazama and Okada, 1990; Bender et al., 1991). Intracellular Ca²⁺ may affect the transduction system that regulates RVD, for example, Ca²⁺/calmodulin-dependent protein kinase (CaM kinase) and protein kinase C (PKC). We have shown that in astrocytes calmodulin antagonists inhibit RVD (Bender et al., 1992a).

The purpose of this study was to investigate the role of Ca²⁺ in hypoosmotically stimulated K⁺ release. Agents that facilitate Ca²⁺ entry into the cells, as well as agents that prevent Ca²⁺ entry and interfere with Ca²⁺-dependent processes, were tested for their effects on hypoosmotically stimulated K⁺ release. Our results provide evidence for the Ca²⁺ dependence of K⁺ release under hypoosmotic conditions in astrocytes and the possible involvement of CaM kinase and PKC in modulation of K⁺ release.

Part of this work has been presented in preliminary form (Bender and Norenberg, 1993).

Materials and Methods

Materials. Bepridil hydrochloride, caffeine, ω -conotoxin GVIA, ethylene-bis-(oxyethylenetriamino)-tetra-acetic acid (EGTA), nickel chloride, nimodipine, ouabain, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Company (St. Louis, MO). Ionomycin and bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid, tetra(acetoxymethyl)-ester (BAPTA/AM) were obtained from Calbiochem Corporation (San Diego, CA). U-73122 (1-[6-[[17 β -3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione) was a gift from Dr. John E. Bleasdale from The Upjohn Company (Kalamazoo, MI). KN-62 (1-[*N,O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine) and (+)MK-801 (dizocilpine maleate) were purchased from Research Biochemicals Incorporated (Natick, MA). W-7 hydrochloride (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride) was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). ⁸⁶Rubidium chloride (1.72 mCi/mg) and 3-*O*-[methyl-³H]- α -glucose (87.6 Ci/mmol) were purchased from New England Nuclear Research Products (Boston, MA). All other reagents were of analytical grade.

Agents affecting Ca²⁺ homeostasis used in this study affect intracellular [Ca²⁺], in various ways under isoosmotic conditions. Ionomycin,

Received Aug. 20, 1993; revised Dec. 10, 1993; accepted Dec. 31, 1993.

This work was supported by the Department of Veterans Affairs, GRECC, and USPHS Grants NS-30291 and DK-38153. We thank Dr. Jocelyn H. Bruce for critical reading of this manuscript; Dr. John E. Bleasdale, Department of Cell Biology, The Upjohn Company, Kalamazoo, MI, for his generous gift of U-73122; and Ms. Janina Blicharska for her excellent technical assistance.

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being a Ca^{2+} ionophore, and ouabain (Schlue, 1991) will increase $[\text{Ca}^{2+}]_i$, whereas BAPTA/AM will chelate intracellular Ca^{2+} and EGTA will chelate extracellular Ca^{2+} . Caffeine is known to release Ca^{2+} from intracellular stores and thus it will initially increase $[\text{Ca}^{2+}]_i$. Other agents, such as U-73122, bepridil, nickel, nimodipine, PMA, KN-62, MK-801, W-7, and ω -conotoxin GVIA, are not known to affect $[\text{Ca}^{2+}]_i$ under isoosmotic conditions.

Cell culture. Astrocytic cell cultures were prepared from neonatal (1–2 d old) rat cerebral cortices and maintained in primary culture, as previously described by Booher and Sensenbrenner (1972) and modified by Ducis et al. (1990). Briefly, the tissue was dissociated and 0.5×10^6 cells were plated in 35 mm dishes. The cells were grown for at least 3–4 weeks at 37°C in a humidified atmosphere containing 5% $\text{CO}_2/95\%$ air before being utilized for biochemical studies. After 2 weeks, the cells were maintained with 0.5 mM dibutyryl cyclic AMP. Based on immunohistochemistry of glial fibrillary acidic protein and glutamine synthetase, at least 95% of the cell population were astrocytes.

Cellular volume measurements. Cell volume of astrocytes was determined using the method of Kletzien et al. (1975) adopted to astrocytic cultures by Kimelberg and Walz (1988), which measures the transport of 3-O-methyl- $[\text{H}]\text{-D-glucose}$.

K^+ uptake assay. Uptake studies were done as described by Kimelberg and Walz (1988). The assays were performed in bicarbonate-buffered media, pH 7.4, containing 122 mM NaCl, 3 mM KCl, 25 mM NaHCO_3 , 1.3 mM CaCl_2 , 1.2 mM KH_2PO_4 , 0.4 mM MgSO_4 , and 10 mM glucose (total osmolarity, 300 mOsm). The cells were preincubated in this medium for 20 min at 37°C in 5% $\text{CO}_2/95\%$ air to achieve steady state. Uptake experiments were initiated by adding 2 $\mu\text{Ci}/\text{ml}$ $^{86}\text{Rb}^+$ (analog of K^+) as RbCl to the media for various time periods ranging from 0.5 to 60 min. In cultured glia cells $^{86}\text{Rb}^+$ resembles $^{42}\text{K}^+$, and is extensively employed for the characterization of K^+ fluxes, as an analog for K^+ , instead of $^{42}\text{K}^+$, in view of its longer half-life (Kimelberg et al., 1979). The uptake was terminated by washing the cells rapidly seven times with ice-cold 0.32 M sucrose solution. For uptake assays under hypoosmotic conditions, the NaCl content was reduced to 22 mM to make the media hypoosmotic (total osmolarity, 110 mOsm).

K^+ efflux assay. ^{86}Rb efflux assays were done as described by Kimelberg et al. (1979) and Holopainen et al. (1989). Cultured cells were incubated in bicarbonate-buffered medium of the same composition as described in the uptake assays, containing 8 $\mu\text{Ci}/\text{ml}$ $^{86}\text{Rb}^+$ for 1 hr at 37°C . Subsequently, the cells were washed four times with isoosmotic medium. The release experiments were performed by washing cells on the substratum. The efflux of $^{86}\text{Rb}^+$ was determined during repeated incubations, and desaturation of the cells was carried out every 2 min for 30 or 40 min at 37°C in 0.5 ml fresh nonradioactive buffer. At the end of each interval, the media were removed and placed into plastic test tubes and new media were added to the plates. The cells were exposed to hypoosmotic stress (40 mM NaCl; 120 mOsm) from the 10th until the 30th or 40th min, in the absence or presence of various agents that affect Ca^{2+} . The radioactivities of the collected 2 min fractions and those remaining in the cells at the end of the experiment were measured in a Packard Auto-Gamma 5660 γ counter. Cell viability under various hypoosmotic conditions was measured by the trypan blue exclusion method and was 99%.

Kinetic analyses of $^{86}\text{Rb}^+$ efflux. The sum of radioactivity in each wash and the radioactivity left in the cells at the end of the experiment was considered as the total radioactivity present in astrocytes at the beginning of the desaturation procedure. The radioactivity in the cells at each time point was obtained by sequentially subtracting the radioactivity of each wash. The results were expressed as nanomoles of K^+ /mg protein or as percentage of $^{86}\text{Rb}^+$ left in the cells. Desaturation curves of the radioactivity released from the cells as a function of time were analyzed to determine the kinetic components and the release rate constants for $^{86}\text{Rb}^+$ efflux, using weighted nonlinear least-squares fitting (Marino et al., 1992). The intervals used in the calculations were 0–40 min for isoosmotic conditions and 10–40 min for hypoosmotic conditions. Statistical significance was assessed by ANOVA and Bonferroni post hoc comparisons between experimental and control cultures.

Results

Effect of hypoosmotic stress on K^+ homeostasis

Figure 1 shows the time course for $^{86}\text{Rb}^+$ uptake under isoosmotic and hypoosmotic conditions. The $^{86}\text{Rb}^+$ uptake equilibrated at the level of ~ 850 nmol/mg protein under isoosmotic

Figure 1. Time course of K^+ (as $^{86}\text{Rb}^+$) uptake under isoosmotic and hypoosmotic (110 mOsm) conditions. Astrocytes were exposed to isoosmotic (control; $\square$) or hypoosmotic ($\blacksquare$) bicarbonate-buffered media containing 2 $\mu\text{Ci}/\text{ml}$ $^{86}\text{RbCl}$ for various time periods ranging from 0.25 to 60 min, and $^{86}\text{Rb}^+$ uptake was determined. Results are expressed as mean $\pm$ SEM of six separate determinations.

conditions and at ~ 400 nmol/mg protein under hypoosmotic conditions. However, the initial rate of $^{86}\text{Rb}^+$ uptake within the first 10 min was $\sim 50\%$ higher under hypoosmotic conditions than under isoosmotic conditions.

Figure 2A shows that hypoosmotic stress stimulates $^{86}\text{Rb}^+$ efflux. After 2 min of hypoosmotic stress there was a rise in $^{86}\text{Rb}^+$ release by ~ 200 nmol/mg protein, which further increased to ~ 300 nmol/mg protein after 10 min. The rate constant for $^{86}\text{Rb}^+$ release under isoosmotic conditions ($0.0464 \pm 0.0008 \text{ min}^{-1}$; $n = 4$) increased by 100% (to $0.0969 \pm 0.0021 \text{ min}^{-1}$) under hypoosmotic conditions. The stimulation of $^{86}\text{Rb}^+$ release by hypoosmotic stress is probably responsible for the decrease in equilibration levels of $^{86}\text{Rb}^+$ uptake under hypoosmotic conditions.

The time course for stimulation of $^{86}\text{Rb}^+$ efflux correlates well with the time course of RVD (see Fig. 2B), and thus K^+ release is likely an important factor in volume regulation of astrocytes during osmotic swelling.

Effect of osmolarity on hypoosmotically stimulated $^{86}\text{Rb}^+$ efflux

Table 1 shows the dependence of $^{86}\text{Rb}^+$ efflux on osmolarity, which was decreased from 310 mOsm to 70 mOsm, by reducing the NaCl concentration in the medium from 122 mM to 0 mM. The rate constants for $^{86}\text{Rb}^+$ efflux ranged from $0.0435 \pm 0.0003 \text{ min}^{-1}$ ($n = 4$) for control (310 mOsm) to $0.1183 \pm 0.0069 \text{ min}^{-1}$ ($n = 4$) when osmolarity was reduced to 70 mOsm. Statistically significant difference from control (isoosmotic) conditions in $^{86}\text{Rb}^+$ efflux was first observed when osmolarity was reduced to 230 mOsm ($p < 0.05$; $n = 4$) and became even more pronounced with decreasing osmolarity ($p < 0.001$).

When hypoosmotic media were made isoosmotic with sucrose, LiCl, or choline chloride, there was no significant stim-

Table 1. Effect of NaCl substitution and osmolarity on K^+ efflux under hyposmotic conditions

Osmolarity of medium (mOsm)	Concentration of NaCl (mM)	Substitution (mM)	Rate constant for Rb^+ efflux (min^{-1})
310	122	None	0.0484 ± 0.0007
110	22	None	$0.0861 \pm 0.0009^{***}$
310	22	Sucrose (200)	0.0504 ± 0.0007^{NS}
310	22	LiCl (100)	0.0487 ± 0.0004^{NS}
310	22	Choline Cl (100)	0.0512 ± 0.0006^{NS}
270	102		0.0462 ± 0.0003^{NS}
230	82		$0.0520 \pm 0.0004^*$
190	62		$0.0540 \pm 0.0003^*$
150	42		$0.0728 \pm 0.0009^{**}$
110	22		$0.1086 \pm 0.0019^{***}$
70	0		$0.1183 \pm 0.0069^{***}$

Astrocytes were loaded with $8 \mu\text{Ci/ml}$ Rb^+ for 1 hr and then were desaturated in isoosmotic (122 mM NaCl) and hyposmotic (22 mM NaCl) conditions, and in media where 100 mM of NaCl was equimolarly substituted with sucrose, LiCl, or choline chloride. The desaturation curves were constructed and rate constants for $^{86}\text{Rb}^+$ efflux under various osmolarities were obtained using weighted nonlinear least-squares fitting (Marino et al., 1992). The results are expressed as means $\pm$ SEM of four separate determinations. The statistical significance between control (122 mM NaCl) and various substitutions or osmolarities was assessed by ANOVA and Bonferroni post hoc comparisons. NS, statistically not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ulation of $^{86}\text{Rb}^+$ release (Table 1), suggesting that the reduction in osmolarity rather than the decrease of NaCl concentration was responsible for stimulation of $^{86}\text{Rb}^+$ release.

Effect of agents that facilitate or inhibit Ca^{2+} influx on hyposmotically stimulated K^+ release

Figure 3 shows that agents such as ionomycin ($0.5 \mu\text{M}$), a Ca^{2+} ionophore, and ouabain ($500 \mu\text{M}$), an Na^+/K^+ ATPase inhibitor that increases intracellular levels of Ca^{2+} by activating the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Blaustein et al., 1991), significantly potentiated hyposmotically stimulated $^{86}\text{Rb}^+$ efflux in astrocytes. Under hyposmotic conditions, the rate constant for K^+ efflux was significantly higher in the presence of ionomycin ($0.3464 \pm 0.0245 \text{ min}^{-1}$; $n = 4$; $p < 0.001$) or ouabain ($0.1868 \pm 0.0071 \text{ min}^{-1}$; $n = 4$; $p < 0.05$) than in the absence of either agent ($0.1182 \pm 0.0032 \text{ min}^{-1}$; $n = 4$).

Figure 3 also shows that nimodipine, an inhibitor of L-type Ca^{2+} channels, significantly inhibited hyposmotically stimulated $^{86}\text{Rb}^+$ release. The rate constant for $^{86}\text{Rb}^+$ efflux under hyposmotic conditions was significantly reduced from $0.1386 \pm 0.0072 \text{ min}^{-1}$ ($n = 4$) to $0.0795 \pm 0.0020 \text{ min}^{-1}$ ($n = 4$; $p < 0.001$) in the presence of $10 \mu\text{M}$ nimodipine. Nickel ($100 \mu\text{M}$), a T-type Ca^{2+} channel blocker, and ω -conotoxin GVIA ($0.5 \mu\text{M}$), an N-type Ca^{2+} channel blocker, had no effect on hyposmotically induced K^+ release. Bepridil ($50 \mu\text{M}$), a nonspecific inhibitor of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which also inhibits Ca^{2+} channels (Stys et al., 1991), also reduced hyposmotically stimulated $^{86}\text{Rb}^+$ release to $0.0720 \pm 0.0031 \text{ min}^{-1}$ ($n = 4$; $p < 0.001$) (see Fig. 3). Additionally, MK-801 ($500 \mu\text{M}$), which is known to inhibit $\text{Na}^+/\text{Ca}^{2+}$ exchanger in astrocytes (Cornell-Bell and Kim, 1993), also inhibited hyposmotically induced K^+ release. The rate constant for Rb^+ release under hyposmotic conditions ($0.0914 \pm 0.0004 \text{ min}^{-1}$) was reduced in the presence of MK-801 to $0.0765 \pm 0.0006 \text{ min}^{-1}$ ($n = 4$; $p < 0.001$).

Figure 2. Effect of hyposmotic stress on K^+ (as $^{86}\text{Rb}^+$) release (*A*) and on cell volume (*B*). To determine $^{86}\text{Rb}^+$ efflux, astrocytes were incubated for 1 hr in the presence of $8 \mu\text{Ci/ml}$ $^{86}\text{RbCl}$, washed four times, and then desaturated. Cells were exposed to hyposmotic (110 mOsm) stress at the 10th min and collections continued until the 40th min. The results represent means $\pm$ SEM of six separate determinations. To examine the cell volume changes under hyposmotic stress (*B*), astrocytes were pretreated for 20 min with isoosmotic bicarbonate-buffered media, and then exposed to hyposmotic (110 mOsm) media containing 1 mM 3-*O*-methyl- α -D-glucose and $0.5 \mu\text{Ci/ml}$ 3-*O*-methyl- ^3H - α -D-glucose for various time intervals from 0 to 30 min. The accumulated radioactivity was converted to $\mu\text{l/mg}$ protein. Results are expressed as means $\pm$ SEM of six separate determinations.

Effect of extra- and intracellular Ca^{2+} on hyposmotically stimulated K^+ release

Removal of extracellular Ca^{2+} from the medium enhanced the release of $^{86}\text{Rb}^+$ stimulated by hyposmotic conditions; rate constant for $^{86}\text{Rb}^+$ efflux reached $0.1288 \pm 0.0040 \text{ min}^{-1}$ ($n =$

Figure 3. Effect of stimulation of Ca^{2+} influx and inhibition on hypoosmotically stimulated K^+ release. Astrocytes were incubated for 1 hr in the presence of $8 \mu\text{Ci/ml}$ $^{86}\text{RbCl}$, washed four times, and then desaturated. Cells were exposed to hypoosmotic stress in the absence or presence of $0.5 \mu\text{M}$ ionomycin (Δ) or $500 \mu\text{M}$ ouabain ($\circ$) and $10 \mu\text{M}$ nimodipine ($\bullet$) or $50 \mu\text{M}$ bepridil ($\triangle$) at the 10th min, and collections continued until the 30th min. A single representative study of four identical experiments is shown; SEMs were less than 5%.

4) from $0.0914 \pm 0.0024 \text{ min}^{-1}$ ($n = 4$; $p < 0.001$). When 0.1 mM EGTA, in the absence of extracellular Ca^{2+} , was added to completely remove extracellular Ca^{2+} , the release of $^{86}\text{Rb}^+$ under hypoosmotic conditions did not change significantly from that when only extracellular Ca^{2+} was removed (rate constant was $0.1294 \pm 0.0050 \text{ min}^{-1}$; $n = 4$; $p < 0.001$). In the absence of extracellular Ca^{2+} and presence of EGTA, when intracellular Ca^{2+} was chelated by BAPTA/AM ($50 \mu\text{M}$), the release of $^{86}\text{Rb}^+$ under hypoosmotic conditions did not change (rate constant was $0.1316 \pm 0.0043 \text{ min}^{-1}$; $n = 4$; $p < 0.001$). However, chelation of intracellular Ca^{2+} with BAPTA/AM ($50 \mu\text{M}$), in the presence of extracellular Ca^{2+} , blocked K^+ release by 45% under hypoosmotic conditions within 4 min of the hypoosmotic exposure ($29,302 \pm 1470$ vs $16,344 \pm 1144 \text{ cpm}$; $n = 4$; $p = 0.0004$).

Role of intracellular Ca^{2+} stores in hypoosmotically stimulated K^+ release

Figure 4 shows that caffeine (10 mM) and U-73122 ($10 \mu\text{M}$) inhibited $^{86}\text{Rb}^+$ efflux under hypoosmotic conditions. Caffeine, which affects Ca^{2+} -induced Ca^{2+} release (CICR) intracellular Ca^{2+} stores, reduced the rate constant of $^{86}\text{Rb}^+$ efflux under hypoosmotic conditions from $0.1219 \pm 0.0039 \text{ min}^{-1}$ ($n = 4$) to $0.0934 \pm 0.0016 \text{ min}^{-1}$ ($n = 4$; $p < 0.01$). On the other hand, U-73122, an inhibitor of phospholipase C (Bleasdale et al., 1990) that subsequently prevents phosphoinositide hydrolysis and inositol trisphosphate (IP_3) formation, also reduced the rate constant of $^{86}\text{Rb}^+$ release to $0.0825 \pm 0.0028 \text{ min}^{-1}$ ($n = 4$; $p < 0.001$). These results suggest that IP_3 -sensitive Ca^{2+} stores and CICR Ca^{2+} stores may be involved in the mechanisms of hypoosmotically stimulated K^+ release.

Figure 4. Effect of caffeine and U-73122 on hypoosmotically stimulated K^+ release. Astrocytes were incubated for 1 hr in the presence of $8 \mu\text{Ci/ml}$ $^{86}\text{RbCl}$, washed four times, and then desaturated. Cells were exposed to hypoosmotic stress in the presence or absence of caffeine (10 mM) or U-73122 ($10 \mu\text{M}$) at the 10th min and collections continued until the 30th min. A single representative study of four identical experiments is shown; SEMs were less than 5%.

Involvement of Ca^{2+} -dependent protein kinases in the modulation of hypoosmotically stimulated K^+ release

Figure 5A shows that W-7 ($50 \mu\text{M}$), a calmodulin antagonist, inhibited hypoosmotically induced $^{86}\text{Rb}^+$ efflux. The rate constant under hypoosmotic conditions ($0.1329 \pm 0.0031 \text{ min}^{-1}$; $n = 4$) was reduced to $0.045 \pm 0.0019 \text{ min}^{-1}$ ($n = 4$; $p < 0.0001$) in the presence of W-7. The selective inhibitor of Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase) KN-62 ($20 \mu\text{M}$) (Tokumitsu et al., 1990; Ishii et al., 1991; Tansey et al., 1992) also reduced rate constant under hypoosmotic conditions to $0.0801 \pm 0.0038 \text{ min}^{-1}$ ($n = 4$; $p < 0.001$).

Figure 5B shows that activation of protein kinase C (PKC), after a 30 min treatment with 100 nM PMA, significantly reduced hypoosmotically stimulated $^{86}\text{Rb}^+$ release. The rate constant of $0.0991 \pm 0.0023 \text{ min}^{-1}$ ($n = 4$) was reduced to $0.0796 \pm 0.0011 \text{ min}^{-1}$ ($n = 4$; $p < 0.001$). When cells were treated with 100 nM PMA for 13 hr to downregulate PKC, the rate constant for $^{86}\text{Rb}^+$ release under hypoosmotic conditions was $0.0832 \pm 0.0018 \text{ min}^{-1}$ ($n = 4$; $p < 0.01$).

Discussion

Our results indicate that one of the responses of astrocytes to hypoosmotically induced swelling is an increase in K^+ permeability. Evidence for a marked increase in K^+ permeability was obtained from measurements of K^+ uptake and K^+ efflux. During osmotically induced cell swelling the initial increase in the rate of K^+ influx is followed by a loss of intracellular K^+ , frequently measured as the unidirectional efflux of $^{86}\text{Rb}^+$. The initial increase in the rate of K^+ influx could be possibly due to activation of Na^+/K^+ -ATPase by hypoosmotic stress; such activation has been observed in the skeletal muscle cell (Venosa, 1991). Hypoosmotic stress activates a two- to threefold increase in the rate of $^{86}\text{Rb}^+$ efflux over the control (isoosmotic) rate, as indicated by the rate constants for K^+ efflux. This loss in intra-

Figure 5. Effect of W-7 and KN-62 (*A*) and PMA (*B*) on hypoosmotically stimulated K^+ release. Astrocytes were incubated for 1 hr in the presence of $8 \mu\text{Ci/ml}$ $^{86}\text{RbCl}$, washed four times, and then desaturated. Cells were exposed to hypoosmotic stress in the absence or presence of $50 \mu\text{M}$ W-7 or KN-62 ($20 \mu\text{M}$) at the 10th min, and collections continued until the 30th min. Cells treated with 100 nM PMA for 30 min to activate PKC or for 13 hr to downregulate PKC were also exposed to hypoosmotic stress. A single representative study of four identical experiments is shown; SEMs were less than 5%.

cellular $^{86}\text{Rb}^+$ (K^+) roughly parallels the time course of the volume recovery. If coupled to the concomitant efflux of an anion (e.g., Cl^-), the loss of K^+ results in the osmotically obliged movement of water to account for the observed RVD. These findings are consistent with the important role of K^+ release in volume regulation in other cell types (Haddad and Graf, 1989; Dickman and Goldstein, 1990; Dube et al., 1990; MacLeod et al., 1992; Wehner et al., 1992; Farrugia and Rae, 1993).

K^+ release was dependent on osmolarity. Stimulation of K^+ efflux was observed when osmolarity was reduced to 230 mOsm . When media with reduced NaCl content were made isoosmotic with sucrose, choline chloride, or lithium chloride, there was

no stimulation of K^+ efflux, suggesting that reduction in osmolarity and not Na^+ omission was responsible for the stimulation of K^+ efflux under hypoosmotic conditions.

The signals that initiate this K^+ release could be the influx of extracellular Ca^{2+} and the release of Ca^{2+} from intracellular stores. Astrocytes under hypoosmotic stress are able to stimulate Ca^{2+} uptake and Ca^{2+} release from intracellular stores (O'Connor and Kimelberg, 1993; Bender et al., 1994). The present study supports the contention that hypoosmotically induced K^+ release is dependent on the influx of extracellular Ca^{2+} . Agents that are known to inhibit hypoosmotically stimulated influx of Ca^{2+} , such as nimodipine, bepridil, and MK-801 (Cornell-Bell and Kim, 1993; O'Connor and Kimelberg, 1993; Bender et al., 1994), also inhibited the K^+ efflux under hypoosmotic conditions. By contrast, agents that stimulate Ca^{2+} influx, such as ionomycin and ouabain, potentiated hypoosmotically induced K^+ release.

Intracellular Ca^{2+} stores could also play an important role in the mechanism of K^+ release and RVD. We have previously shown that inhibition of phospholipase C and the consequent blockage of hypoosmotically stimulated inositol phosphate hydrolysis by U-73122 resulted in inhibition of RVD (Bender et al., 1994). In this study we show that U-73122 significantly inhibited K^+ efflux under hypoosmotic conditions, indicating that Ca^{2+} from IP_3 -sensitive intracellular Ca^{2+} stores may play a role in activating K^+ release during hypoosmotic stress. It was previously shown that CICR intracellular Ca^{2+} stores are involved in volume regulation in human epithelial cells (Hazama and Okada, 1990). Caffeine, which affects these Ca^{2+} stores, inhibited RVD in astrocytes (Bender et al., 1991). Hypoosmotically stimulated K^+ release in astrocytes is also sensitive to caffeine. Therefore, CICR also may play a role in the mechanism of K^+ efflux under hypoosmotic stress. When intracellular Ca^{2+} is chelated with BAPTA/AM, there was also a 45% inhibition of hypoosmotically stimulated K^+ release, further supporting the contention that intracellular Ca^{2+} is important in the mechanism of K^+ efflux under hypoosmotic conditions.

Our results indicate that influx of extracellular Ca^{2+} and Ca^{2+} released from IP_3 -sensitive and CICR Ca^{2+} stores may play a role in the mechanism of K^+ release under hypoosmotic stress. However, when extracellular Ca^{2+} is omitted from the media there is an increase in the hypoosmotically stimulated K^+ release. Furthermore, adding EGTA (to chelate the remaining extracellular Ca^{2+}) or BAPTA/AM (to chelate intracellular Ca^{2+}) did not significantly affect hypoosmotically stimulated K^+ release in the absence of extracellular Ca^{2+} . In astrocytes from rat cerebellum, omission of Ca^{2+} in the presence of EGTA caused stimulation of K^+ release under isoosmotic conditions, but not under hypoosmotic conditions (Sanchez-Olea et al., 1993). Dependence on external Ca^{2+} for K^+ influx and efflux was described in cultured glial cells from chick embryonic brain where a decrease of extracellular Ca^{2+} below 0.1 mM resulted in a decrease of intracellular K^+ (Latzkovits et al., 1982). Extracellular Ca^{2+} is required for membrane stability, and thus removal of extracellular Ca^{2+} perhaps makes membranes more permeable to K^+ .

Hypoosmotically stimulated K^+ efflux is Ca^{2+} dependent and may be mediated through Ca^{2+} -activated K^+ channels. Such channels have been described in astrocytes (Quandt and MacVicar, 1989; Barres et al., 1990; Tse et al., 1992). Ca^{2+} -activated K^+ channels are activated by increases in intracellular Ca^{2+} levels and by membrane depolarizations. In primary cul-

tured proximal tubule cells hypoosmotic stress activated maxi K^+ channels, which required external Ca^{2+} , although some residual single-channel activity was measured in the absence of extracellular Ca^{2+} (Dube et al., 1990). Such channels could also be involved in mediating K^+ release under hypoosmotic conditions in astrocytes.

The hypoosmotically stimulated K^+ release is modulated by Ca^{2+} -dependent protein kinases such as CaM kinase and PKC. An inhibitor of CaM kinase, KN-62 (Tokumitsu et al., 1990; Ishii et al., 1991; Tansey et al., 1992), partially inhibited the K^+ release under hypoosmotic conditions, whereas W-7, a calmodulin antagonist, almost completely inhibited K^+ release, suggesting that perhaps calmodulin-dependent enzymes besides CaM kinase (e.g., Ca^{2+} /calmodulin-dependent phosphatase, calcineurin) may modulate K^+ release. Previously, we have shown that RVD is blocked by W-7 (Bender et al., 1992a). PKC may also be implicated in the modulation of K^+ release under hypoosmotic conditions, since acute and prolonged treatment with PMA, to activate and downregulate PKC, respectively, also inhibited K^+ efflux, a finding that is consistent with the inhibition of RVD by PMA (Bender et al., 1992a). Also, PMA treatment inhibits K^+ uptake in mouse cultured astrocytes (Bender et al., 1988).

In summary, our results suggest an important role of Ca^{2+} in K^+ release following hypoosmotic challenge and thus in cell volume regulation. Extracellular Ca^{2+} influx and intracellular Ca^{2+} release during hypoosmotic stress appears to initiate K^+ release, possibly by activating Ca^{2+} -dependent K^+ channels or perhaps by affecting Ca^{2+} -dependent kinases and phosphatases.

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