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Furosemide Stimulates K Transport in HCD57 Erythroid Cells

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Abstract. We examined the influence of serum and furosemide on K movement and cell volume in HCD57 cells, a murine erythroleukemia cell line, which require erythropoietin (EPO) for survival. We found that maintenance of cell volume depends on the concentration of serum in the culture medium. In isotonic medium containing 20% serum, HCD57 cells maintain their steady-state volume. In contrast, the cells shrink progressively as medium serum content is reduced. In serum-free medium, raising external K to 75 mM prevents cell shrinkage and a further increase in K to 145 mM results in swelling, revealing a role for K permeability in the regulation of cell volume. Of particular interest has been a serendipitous finding with furosemide. Below an external K concentration of 2.1 ± 0.3 mM in medium containing 2% serum, furosemide inhibits K uptake, probably stemming from its well known inhibitory action on KCl cotransport. However, above that K concentration, furosemide stimulates K uptake in a dose-dependent manner. Moreover, furosemide potentiates cell shrinkage induced by serum withdrawal. These findings suggest that the transport machinery mediating cellular shrinkage, once primed by serum depletion, becomes receptive to a second stimulus.

Key words: Furosemide — K transport — Serum-deprivation — Cell shrinkage — HCD57 cells

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Introduction

Much of the metabolic energy expended in animal cells is directed toward encapsulating impermeant, charged solutes that impart osmotic force, thereby causing the cells to swell. Our current understanding of the mechanism by which animal cells sustain volume homeostasis has been guided by the "pump and leak" concept postulated by Tosteson and Hoffman (1960), and Leaf (1959) nearly four decades ago. Central to this view is the presence of an ATP-driven Na^+K pump (Skou, 1957) that keeps cellular Na and K ions away from their electrochemical equilibrium. "Leak" refers to the passive movement of Na and K down their activity gradients across membranes. Simply stated, the model posits that cell volume is maintained by the balance of ion movements between the pump and leak pathways. In the years following these seminal publications, much has been learned about the molecular structure of transporters. In particular, the subunit structure and kinetics of the Na^+K pump have been extensively investigated (Post, 1989). Moreover, what was once thought to be a simple leak is now known to be mediated by diverse molecular entities such as ion channels, tightly coupled cotransporters, and exchangers. Thus, it has been well established that the homeostasis of cell volume is regulated by a self-sufficient cellular program that depends on the intricate coordination of a variety of transport pathways within the cells (Hallows & Knauf, 1994*a;* Parker, 1994; Hoffmann & Dunham, 1995). It has also become increasingly evident in recent years that a variety of extracellular stimuli influence cells through receptor-mediated signal transduction pathways which, among other things, regulate ion transport and cell volume (Kim, 1991; Kim et al., 1996; Sarkadi & Parker, 1991).

In some cases, external stimuli direct the cells to

shrink even in isotonic media, presumably by disrupting the coordination of transporter activities. The targeted cells display a set of hallmark biochemical and morphological changes and undergo programmed cell death or apoptosis, which was first described by Kerr, Wyllie & Currie (1972) nearly three decades ago.

In this communication, we report the results of our investigation of HCD57 cells, a murine erythroleukemia cell line (Ruscetti et al., 1990), which undergo shrinkage in response to serum depletion. In addition, we found that furosemide, apart from its well-known inhibitory action on KCl cotransport (Lauf, 1984), also stimulates K movement and potentiates shrinkage in low serum media.

Materials and Methods

Cell Culture

HCD57 cells were grown in suspension in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum (heat treated for 30 min at 56°C), 100 μg/ml streptomycin, 100 U/ml penicillin, 0.9% bovine serum albumin (BSA), 5 μl monothioglycerol/L, and 1.0 unit/ml erythropoietin. HCD57 cells were grown in suspension culture at 37°C in a humidified 5% CO_2 incubator. Every 2 to 6 days, an aliquot of the cells was diluted into fresh medium to maintain the culture. Cells were passed in this way for 15 times and then discarded.

Cell Volume Measurements

Cell volume was measured using a Coulter counter and channelizer by the method described previously (Kim et al., 1996). Briefly, cultured cells were washed once with isotonic medium containing (in mM) 140 NaCl, 3.2 KCl, 1.2 $MgSO_4$, 1 $CaCl_2$, 10 glucose, 0.8 K_2HPO_4, 0.2 KH_2PO_4, HEPES · Tris buffer (pH 7.4) with 0.1% bovine serum albumin (BSA) either with or without serum. The cells were then suspended in the same medium containing serum at 37°C unless otherwise stated and were taken for Coulter counter measurement. The magnitude of cell volume, as reported by the Coulter counter varied, among other things (Kim et al., 1996), with the density of cells in the suspension medium. Thus, the results are expressed as relative cell volume, in which the cell volume measured over time was normalized to the initial cell volume in each experiment.

K Uptake

K uptake was measured using ^{86}Rb as K congener by the procedure described previously (Kim et al., 1996). Briefly, HCD57 cells taken from ongoing culture were washed and suspended at 37°C in the same isotonic medium used for cell volume measurements but containing ^{86}Rb (1.7 μCi/ml). ^{86}Rb uptake was carried out in the presence of 10.8 ± 2.0 × 10^6 cells/ml (mean ± SEM, $n = 8$), which was determined by a hemocytometer. ^{86}Rb uptake was terminated by adding ice-cold 166 mM NaCl containing 0.1% BSA followed by centrifugation at 4°C. The cells were washed three times and solubilized with 0.5 M NaOH. Radioactivity was determined by liquid scintillation spectroscopy.

For the determination of KCl cotransport activity, ^{86}Rb uptake was measured in isotonic media in which Na was replaced by N-methyl-D-glucamine salts (NMG-Cl) and Cl was replaced by NO_3 salts (NMG-NO_3).

K Efflux

K efflux was measured according to the modified method described previously (Kim et al., 1996). Briefly, HCD57 cells were incubated for 2 hr or more at 37°C in isotonic medium containing 20% serum and 1.7 μCi/ml ^{86}Rb. After preloading, the cells were washed 3 times in ice-cold medium containing 20% serum and resuspended in isotonic medium containing 2 or 20% serum at 37°C. The number of cells used for ^{86}Rb efflux was 5.0 ± 0.4 × 10^6 cells/ml (mean ± SEM, $n = 7$). At frequent intervals 1 ml of the suspension was removed, centrifuged and 0.9 ml of the supernatant was used for the determination of radioactivity. An aliquot of the cell suspension was counted for the measurement of total radioactivity.

Northern Blot Analysis

Total RNA was isolated from HCD57 cells by the guanidinium thiocyanate/phenol/chloroform method (Chomczynski & Sacchi, 1987). RNA samples were fractionated by electrophoresis in a formaldehyde 1% agarose gel and transferred to a nitrocellulose membrane. The UV-crosslinked membrane was preincubated for 3–5 hr at 60°C in Express Hyb hybridization solution (Clontech, Palo Alto, CA) and hybridized for 18–24 hr at 60°C with 4 × 10^6 cpm/ml of ^{32}P-random-primed cDNA probe corresponding to a 1.5 kb Bgl II restriction fragment of the rat KCC1 cDNA (Gillen et al., 1996). The membrane was washed three times with 2× SSC (1× SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0) containing 0.1% SDS at room temperature, and three times with 0.2× SSC containing 0.1% SDS at 60°C. The dried membrane was exposed to x-ray film (Kodak) at −70°C with intensifying screens.

X-Ray Microanalysis

Na, K, Ca and Cl content of HCD57 cells were measured by x-ray microanalysis using the procedure described previously (Kirk & Lee 1988; Lee, Kirk, & Reasor, 1993). Briefly, HCD57 cell samples taken at various time points were packed by centrifugation in a microfuge. An aliquot of packed cells was deposited on top of a small wooden dowel and quickly frozen in liquid propane precooled in liquid nitrogen to −189°C. Ultrathin sections (90–100 nm) were cut at −120°C with a diamond cryoknife in an ultramicrotome (Reichert Ultracut E41) equipped with a cryochamber (FC4D) and an antistatic probe (Static-Line II), and placed on nickel grids. Sections were freeze-dried in a vacuum chamber, and used for elemental analysis by x-ray microanalysis. X-rays were collected with a 30-mm^2 energy dispersive detector (Kevex) and a spectrometer (Kevex 7000 series) in conjunction with an electron microscope (JEOL 100CX) in a scanning transmission mode. X-ray spectra were acquired in 50 sec at an accelerating voltage of 80 kV and a beam current of 1 nA. The multiple least-square method (Schamber, 1977) was used for quantification. Elemental concentrations were calcualted as mmol/kg dry weight according to the following equation (Hall, 1971):

$$C_x = (I_x/I_b) \cdot W_x$$

where C_x is the concentration of the element x, I_x is the intensity of the characteristic x-ray of element x, I_b is the background x-ray intensity, and W_x is a calibration constant that is determined empirically by using a KCl standard in 20% deionized bovine serum albumin.

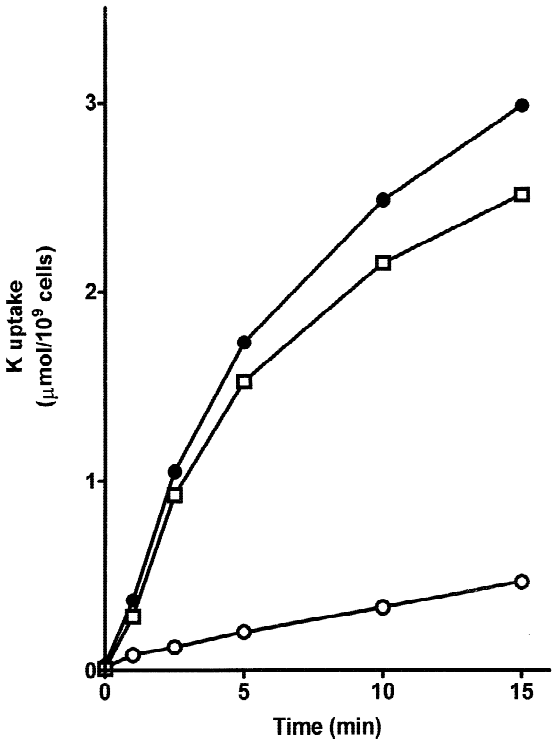

Fig. 1. Cl-dependent K uptake by HCD57 cells. The cells were preincubated in serum-free medium for 20 min at 37°C. K uptake was determined using ^{86}Rb as a K congener in serum-free NMG-Cl (●) or NMG-NO_3 (○) medium containing 1 mM ouabain. Cl-dependent K uptake (□) is calculated as the difference between K uptake measured in NMG-Cl and NMG-NO_3 media. The result shown is one of two experiments performed.

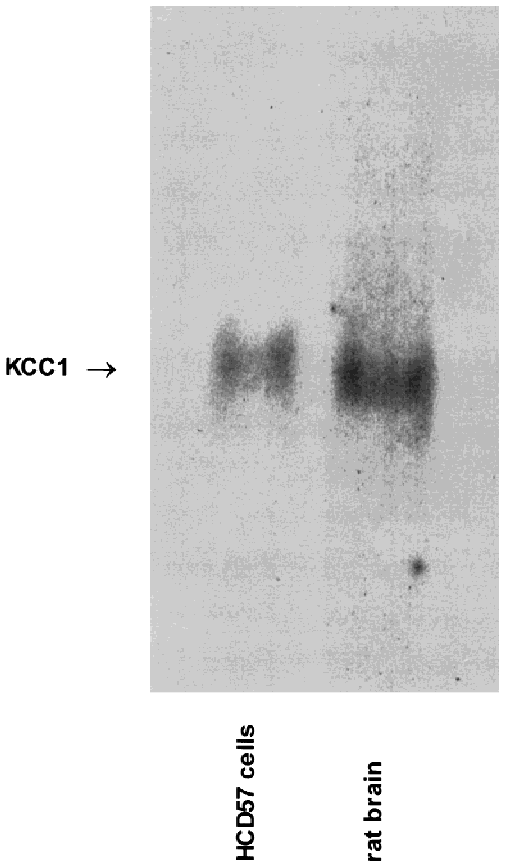

Fig. 2. Expression of the KCl cotransporter (KCC1) mRNA. Total RNA from HCD57 cells and rat brain were analyzed by northern blot hybridization. A single mRNA transcript of about 3.8 kb was detected. The result shown is from a single experiment representative of two others for rat brain and four others for HCD57 cells.

Sources of Materials

Furosemide was purchased from Aldrich Chemical Company, Milwaukee, WI. Stock solutions were prepared fresh daily by solubilizing furosemide in 1 M Tris with a final pH of approximately 7. We tested other vendors of furosemide including: Sigma Chemical, Research Biochemical, and ICN Biomedicals. All were efficacious in inducing cell shrinkage in low serum media. DIDS was purchased from Sigma Chemical, St. Louis, MO. The cDNA probe for KCCl was a gift from Dr. Eric Delpire. HCD57 cells were a gift from Dr. Stephen Sawyer.

Results

HCD57 Cells Possess a KCl Cotransporter

Figure 1 shows K uptake by HCD57 cells suspended in NMG-Cl or NMG-NO_3 medium. It is evident that K uptake is much larger in Cl medium than in NO_3 medium. It seems likely that the difference of K uptake measured in Cl and NO_3 media is mediated by a KCl cotransporter. The possible presence of a KCl cotransporter was further examined by Northern blot analysis. We found that a single transcript with the expected size of the KCC1 mRNA hybridizes with a cDNA probe for rat KCC1 under stringent hybridization conditions, as shown in Fig. 2. Taken together, these findings suggest the presence of at least one type of KCl cotransporter in HCD57 cells.

Furosemide Stimulates K Uptake

Figure 3 shows that K uptake by HCD57 cells is linear for the first few min and approaches a plateau by about 20 min. Surprisingly, furosemide added at 10 min (as indicated by the arrow) stimulates K uptake. At the end of 30 min, furosemide-treated cells exhibit K levels severalfold higher than control cells. If Cl is substituted by NO_3, furosemide fails to stimulate K uptake (*data not shown*). Of interest was the finding that, like the KCl

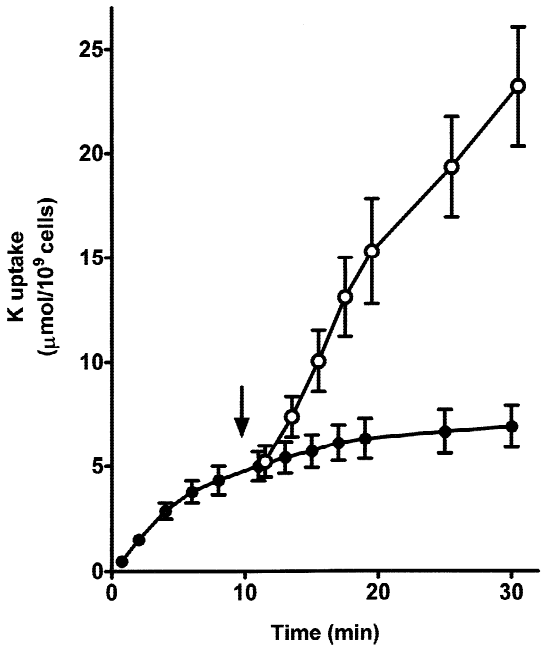

Fig. 3. Stimulation of K uptake by furosemide. Cells were preincubated in serum-free NMG-Cl medium for 20 min at 37°C. K uptake was measured in serum-free and NMG-Cl medium containing 1 mM ouabain (●). At the time indicated by the arrow, furosemide (4 mM) was added (○). Values represent mean ± SEM, $n = 3$.

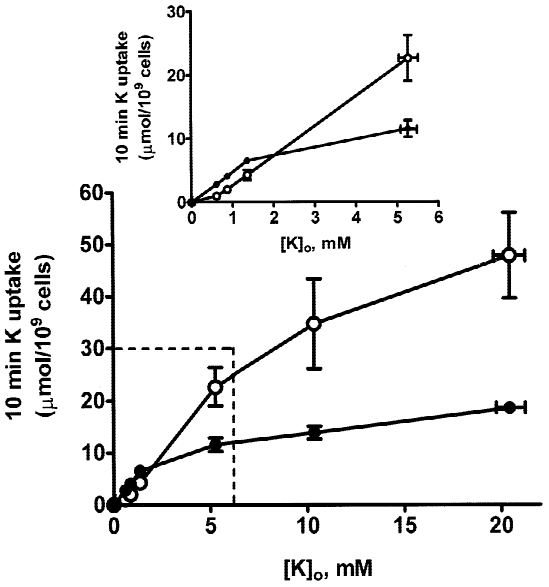

Fig. 4. Effects of furosemide on K uptake in the presence of various external K concentrations. K uptake was measured for 10 min in media containing 2% serum either with 4 mM furosemide (○) or without (●). The area marked by the dotted line is enlarged and shown in the upper panel. Values are mean ± SEM, $n = 3$.

cotransporter that is stimulated by cell swelling (Kim et al., 1989; Lauf et al., 1992), there is a lag time of 1.7 ± 0.3 min (mean ± SEM, $n = 3$) before furosemide activation of K uptake ensues.

Figure 4 shows the complex effects of furosemide on K uptake as a function of the external K concentration in medium containing 2% serum. At the external K concentration of 2.1 ± 0.3 mM (mean ± SEM, $n = 3$), which we refer to as the crossover point, furosemide neither inhibits nor activates K uptake. Below this K concentration, furosemide inhibits K uptake (Fig. 4, upper panel), consistent with its well known pharmacological action on the KCl cotransporter in other cells. However, furosemide stimulates K uptake at K concentrations above the crossover point. It seems likely that furosemide inhibits the KCl cotransporter over the entire range of external K concentrations tested, but the inhibition is masked by the activation of a separate high capacity K transport pathway. In any case, these findings reveal for the first time an inhibitory as well as a stimulatory action of furosemide on K movements.

Furosemide Stimulates K Efflux

Figure 5 depicts the influence of furosemide concentration on K efflux using ^{86}Rb as a K congener. K efflux was measured by the appearance of ^{86}Rb in the medium from preloaded cells and is expressed as the percent of ^{86}Rb remaining in the cells. Furosemide promotes K efflux in a dose-dependent manner (Fig. 5*a*). In several such experiments, a dose-response relationship was defined for furosemide by normalizing K efflux occurring at 20 min in the presence of furosemide to K efflux observed in the absence of furosemide (Fig. 5*b*). Assuming that the K efflux seen at 6 mM furosemide represents a near maximal activation, the half-maximal stimulatory concentration of furosemide is about 3 mM in the presence of 2% serum. At 6 mM furosemide, K efflux is stimulated by more than twofold.

Stimulation of K Efflux by Furosemide Depends on Serum Concentration

We found that K efflux from HCD57 cells depends on serum concentration in the medium, as shown in Fig. 6. The magnitude of K efflux is greater in low serum medium than in high serum medium ($P < 0.01$ paired T test). Furosemide potentiates this. Thus, K efflux is greatest when the cells are challenged simultaneously by serum depletion and exposure to furosemide. It should be recalled that the results on K movements depicted above were obtained either in serum-free medium (Figs. 1 and 3) or medium containing 2% serum (Fig. 4). Inasmuch as K movements depend on serum concentration, the aforementioned K movements do not reflect steady-

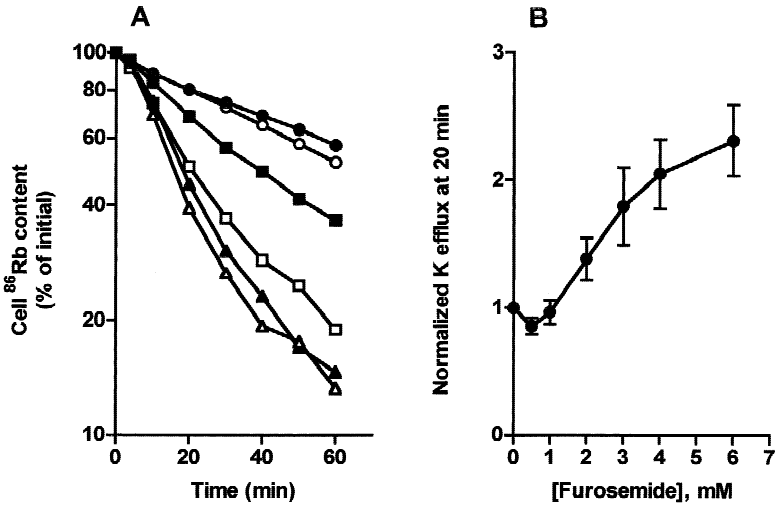

Fig. 5. Stimulation of K efflux by furosemide (*A*). K efflux was monitored in the presence of 2% serum and various furosemide concentrations in (mM): 0, (●); 1, (○); 2, (■); 3, (□); 4, (▲); 6, (△). The result shown is from a single experiment representative of 3 others. Furosemide dose-response curve (*B*). K efflux seen with furosemide at 20 min was normalized against control. Values are mean ± SEM, $n = 3$ to 4.

state K flux. Nonetheless, these qualitative determinations of K movements provide a useful clue for assessing the effects of furosemide.

Serum Deprivation leads to Cell Shrinkage: Potentiation by Furosemide

Figure 7 depicts the influence of serum and furosemide on HCD57 cell volume. As with K efflux, the ability of HCD57 cells to maintain their volume depends on the serum concentration. In medium containing 20% serum, the cells fully maintain their control volume for 2 to 3 hr. At this high serum concentration, furosemide is without effect on cell volume, despite the finding that K efflux has been augmented by the drug (Fig. 6). In medium containing 1 or 5% serum, the cells gradually undergo shrinkage. It is evident that the lower the serum concentration, the more the cells shrink. Moreover, furosemide exerts a more prominent influence on cell volume as the serum concentration is decreased.

In serum-free isotonic medium, HCD57 cells shrink rapidly by 23 ± 2% (mean ± SEM, $n = 19$) after 2 hr. If furosemide is present, the cells shrink even more (34 ± 2%, mean ± SEM, $n = 19$) during the same period.

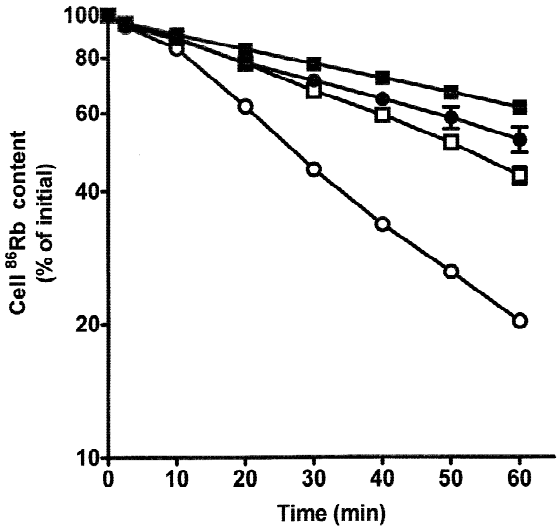

Fig. 6. K efflux potentiated by serum deprivation and/or exposure to furosemide. The experimental conditions were the same as in Fig. 5. K efflux was measured from cells suspended in 20% serum either with 4 mM furosemide (□) or without (■) and in 2% serum either with 4 mM furosemide (○) or without (●). Values represent mean ± SEM, $n = 3$. Error bars in some cases are within the size of symbols.

Prevention of Shrinkage by High External K Concentrations

To further establish the link between K efflux and cell shrinkage, we measured the effects of increased external K on cell volume under conditions (serum-free medium ± furosemide) that elicit maximal K transport. In 5 mM K medium, the cells undergo a rapid shrinkage that is potentiated by furosemide, as depicted in Fig. 8*a*. In medium where the external K concentration is raised to 75 mM, the cells maintain their volume and furosemide still reduces cell volume slightly (Fig. 8*b*). In medium containing 145 mM K, the cells swell in the absence of furosemide (Fig. 8*c*). However, this increase in cell volume is largely attenuated by furosemide. Since in high K media, the K gradient is reversed, K entry is probably driven by both the KCl cotransporter and the putative high capacity K transport system. The smaller cell volume observed with furosemide in high K media may stem from the inhibitory action of the drug on the inwardly directed KCl cotransporter. Taken together these findings corroborate the view that the K electrochemical

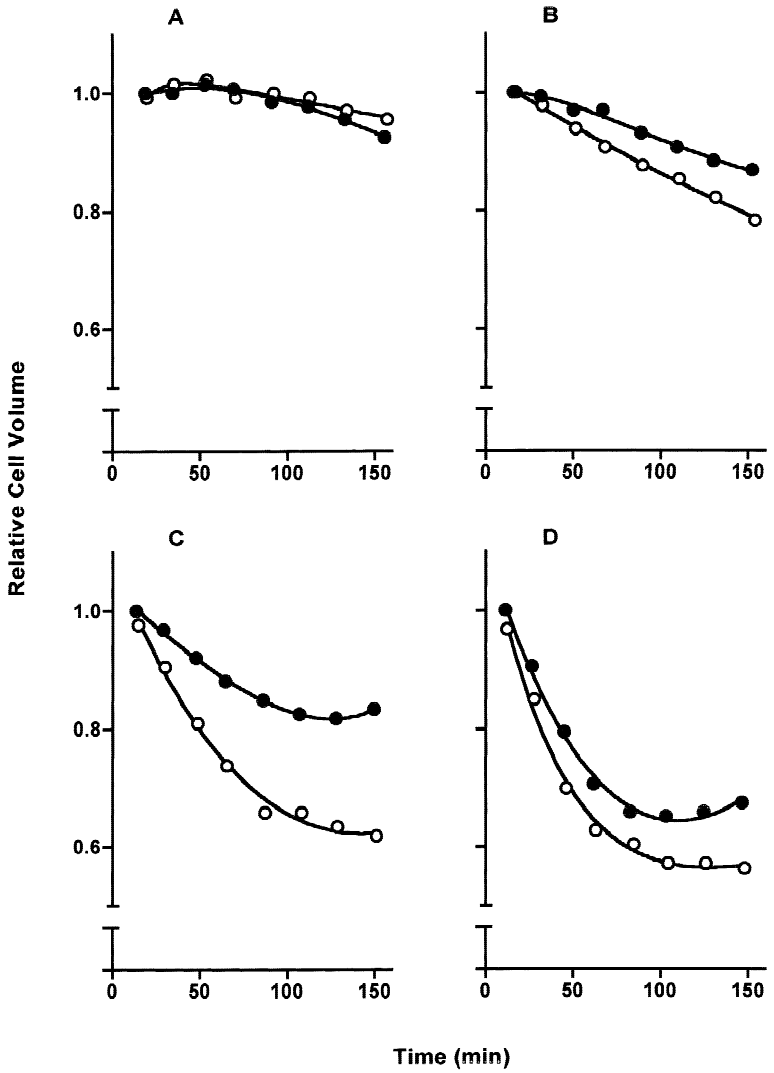

Fig. 7. Cells shrinkage potentiated by serum deprivation and/or exposure to furosemide. Cell volume was monitored in media containing 20% serum (*A*), 5% serum (*B*), 1% serum (*C*) on no serum (*D*), either with 4 mM furosemide (○) or without (●). The result shown is from a single experiment representative of 3 others.

gradient is the driving force for K efflux, which in turn mediates cell shrinkage.

Cl is a Partial Anion Counterpart for K Efflux

Cell shrinkage is caused by the loss of cellular osmolytes. Since animal cells, with only a few exceptions (Tosteson & Hoffman, 1960; Miles & Lee, 1972), have a high K content, acute reduction in cell volume such as the one observed in this study invariably stems from K loss. That a high concentration of external K prevents cell shrinkage (Fig. 8) is consistent with this view.

In an attempt to determine the identity of the anion that is lost concomitantly with K, we examined ^{36}Cl uptake either with or without DIDS. As expected, the bulk of tracer Cl uptake was DIDS-sensitive, regardless of whether the HCD57 cells were suspended in high or low serum medium (*data not shown*). As a result, it has not been possible to delineate the Cl movement accompanying K loss in the presence of a presumably robust anion exchange activity.

However, another approach was the determination of cellular ionic content accompanying cell shrinkage using x-ray microanalysis. The results are summarized in the Table. As expected, cells suspended in 2% serum lose K after 2 hr of incubation. Moreover, K loss is greater in the presence of furosemide. The Cl content also diminishes over time. However, the extent to which the cells lose Cl does not match the magnitude of K loss observed. We also found that Ca content was increased, whereas Na content was decreased during cell shrinkage.

Discussion

The findings concerning the effects of furosemide on K movement are intriguing. Of the diuretic drugs, furose-

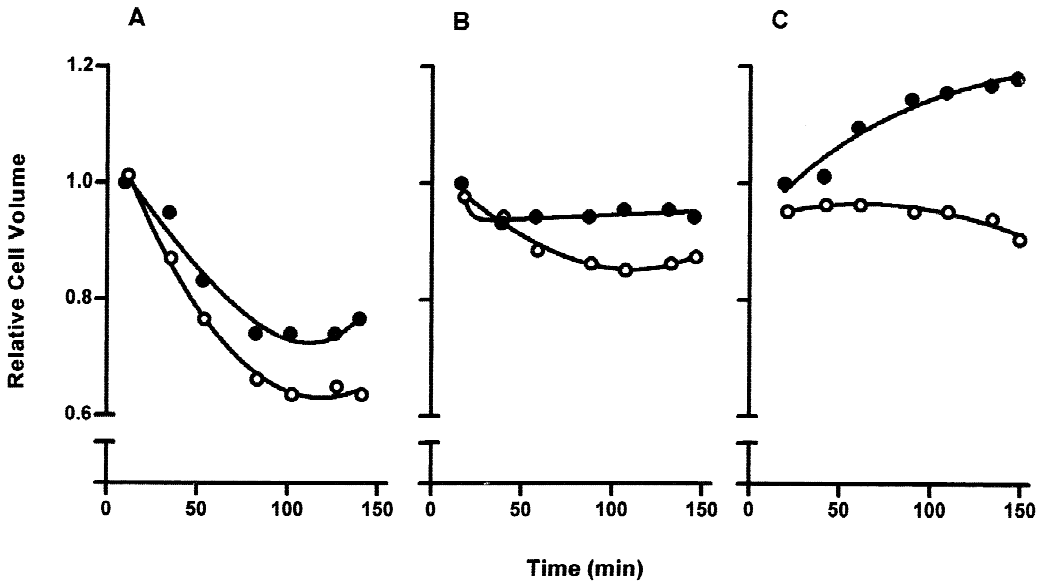

Fig. 8. Effects of high external K concentrations on cell volume. Cell volume was monitored in serum-free media containing 5 mM K (*A*), 75 mM K (*B*), or 145 mM K (*C*), either with 4 mM furosemide (○) or without (●). The result shown is from a single experiment representative of 4 others.

mide and the other loop diuretics are the most widely used (Brater, 1998). The efficacy of the drug has been inferred to stem from its well-known inhibitory action on the Na/K/Cl cotransporter in the renal tubule. Furosemide is also known to inhibit other transporters including chloride channels (Tamaoki, Graf, & Nadel, 1987; Uchida et al., 1995) and the Cl/HCO_3 exchanger (Brown, Dunk, & Turnberg, 1989; Halligan, Shelat, & Kahn, 1991; Tosco, Orsenigo, & Faelli, 1993). The results presented here document yet another effect of the drug. As shown in Fig. 4, furosemide has a dual effect on K uptake. The inhibitory component is likely to reflect the action of furosemide on the resident KCl cotransporter. Conversely, the observation that furosemide stimulates K movement is a novel finding. These results are consistent with the interpretation that the activation of K movement stems from a K transport pathway that is a distinctly different entity from the KCl cotransporter. In this regard, it is of interest that Garay et al. (1984) reported that two families of diuretic drugs, (aryloxy) acetic acid diuretics (ethacrynic acid, tienilic acid and (−)-indacrinone) and furopyridines [(±)-BN 50157 and (±)-cycletanide] stimulate K efflux from human red blood cells. However, a similar stimulation of K movement by furosemide could not be elicited.

HCD57 cells belong to a group of cells that undergo a reduction in cell volume upon withdrawal from serum (Kulkarni & McCulloch, 1994; Orlov et al., 1996). Of particular interest has been the finding that this intriguing feature can be potentiated by furosemide in HCD57 cells. At the present time, the identity of a molecular target for furosemide and/or serum depletion is not known. We interpret the results reported here to mean that the reduction in cell volume stems primarily from the net loss of KCl salt. The shrinkage of HCD57 cells can be fully prevented in the presence of 20% serum, where K and Cl movements across the plasma membrane must be at a steady state. Thus, in the presence of a high serum content, the magnitude of K influx must be matched by that of K efflux, and the same holds true for Cl movements. Moreover, both the K and Cl conductances will have to be increased to bring about cell shrinkage. Although furosemide has been found to stimulate K efflux in the

Table. Effects of furosemide on the ion content of HCD57 cells

	K	Na	Cl	Ca
	(mmol/kg dry weight)			
Control (n = 18)	782 ± 46	133 ± 26	303 ± 14	4 ± 2
Time 0				
2 hr (n = 28)	675 ± 20	70 ± 12	270 ± 11	25 ± 4
2 hr + Furosemide (n = 30)	612 ± 25	85 ± 13	220 ± 12	32 ± 3

HCD57 cells were suspended in isotonic medium containing 2% serum and quickly frozen. The determination of ion content was carried out by X-ray microanalysis as described in Materials and Methods. The values shown are the mean ± SEM; "n" refers to the number of cells analyzed. It should be noted that the K concentrations presented here are higher than anticipated. It is well known that the X-ray microanalysis tends to report measurements that are higher than those determined by conventional flame photometry (Beck et al., 1980; Andrews et al., 1983). This discrepancy was explained as due to the lower estimation of the extracellular space inherent in bulk chemical analysis. Inasmuch as X-ray microanalysis measures the total amount of intracellular elements including free and bound, it is possible that the values in the Table include bound salts that do not participate in the osmotic event.

presence of a 20% serum, it fails to elicit a corresponding reduction in cell volume. There are precedents for this. The treatment of lymphocytes with gramicidin (Rothstein & Bear, 1989) or the exposure of HSG-PA cells with a nucleotide receptor agonist (Kim et al., 1996) results in an increase in K permeability without a concomitant reduction in cell volume. In both cases, the rate-limiting step turns out to be a low anion conductivity, which does not increase in concert with the augmented K permeability. This implies that serum somehow holds the Cl conductance in check, rendering it unresponsive to the influence of furosemide. It should be recalled that both K efflux and the magnitude of shrinkage gradually increase as the serum content is reduced. Thus, it seems conceivable that the augmented K efflux seen in medium containing little or no serum hyperpolarizes the membrane potential, which serves as the driving force for a net anion loss. Clearly, more work is required to discern the underlying mechanism by which HCD57 cells carry out this uncommon feat, i.e., a rapid and extensive shrinkage in isotonic medium.

The serum factors responsible for maintaining volume change are not known. However, dialysis renders serum ineffective with respect to maintaining cell volume. This suggests that either the relevant factors denature during dialysis or are small enough to pass through the dialysis membrane. In this regard, it is of interest that glutamine at a millimolar concentration has been found to block shrinkage of HCD57 cells when added to isotonic medium lacking serum (*data not shown*).

If K loss is mediated by a K channel, it does not seem to be a Gardos type since the treatment of HCD57 cells with charybdotoxin or the addition of EGTA in calcium-free medium has little effect on cell shrinkage which is induced by lowering serum concentration (*data not shown*).

It is known that treatment of epithelia with loop diuretics induces cell shrinkage, caused by an inhibition of Na and Cl influx as a result of blockade of the Na/K/Cl cotransporter (Rick et al., 1987; Persson et al., 1991). Inasmuch as K transport in HCD57 cells is stimulated rather than inhibited by high concentrations of furosemide and is uninfluenced by external Na, the shrinkage triggered by furosemide in HCD57 cells seems unlikely to be mediated by the Na/K/Cl cotransporter.

Much is now known about the way in which ion and osmolyte transport participates in the regulation of cell volume. Our current understanding of this important area comes mostly from studies examining how animal cells respond when challenged by exposure to an iosotonic media (Hoffman & Simonsen, 1989; Law & Burg, 1991; Sarkadi & Parker, 1991; Häussinger, Lang & Gerok, 1994; Hoffmann & Dunham, 1995). In hypotonic media, most animal cells undergo a rapid initial swelling due to water entry followed by a much slower shrinkage, which is mediated by K and other osmolyte loss, toward their original volume. This phenomenon is termed regulatory volume decrease (RVD). Of note is the finding that the extent of K loss turns out to exceed that of Cl during RVD. This disparity has been attributed to a possible reuptake of Cl by the Cl/HCO_3 anion exchanger in Ehrlich Ascites cells (Livne & Hoffman, 1990) and participation of other organic anions in place of Cl in HL-60 cells (Hallows & Knauf, 1994*b*). We report here an analogous situation in HCD57 cells undergoing shrinkage in isotonic media (Table). X-ray microanalysis also revealed that shrinking cells accumulate Ca, which could conceivably reduce the Na content through the activation of a Ca/Na exchanger (Egger & Niggli, 1999). However, it is not known whether HCD57 cells express the exchanger.

The stepwise reduction in HCD57 cell volume by two separate stimuli, serum depletion and furosemide exposure, is reminiscent of the findings we reported with the HSG-PA human submandibular gland duct cell line (Kim et al., 1996). HSG-PA cells, which express $P2Y_2$ nucleotide receptors (Yu & Turner, 1991; Parr et al., 1994), have the capacity to conduct RVD. HSG-PA cells exhibit progressively increased RVD in response to decreased medium osmolarities ranging from 220 mosM to 180 mosM. The addition of the $P2Y_2$ receptor agonist, UTP, potentiates RVD. This suggests that a crosstalk between volume and receptor signals regulates RVD. In this paradigm where separate signals converge, it should be noted that a single stimulus, in and of itself, does not necessarily promote cell shrinkage. In HSG-PA cells, UTP can not promote cell shrinkage in isotonic media even though UTP causes a profound K movement through Ca^{2+}-activated K channels. For UTP to be effective, the cell volume itself needs to be altered first by hypotonic challenge. Similarly, in HCD57 cells suspended in serum, furosemide can not cause cell shrinkage even though furosemide elicits K loss. The initial step of removing serum that initiates cell shrinkage is required for furosemide to be effective. Thus, these examples illustrate a commonality wherein the putative "volume sensor" (Chamberlin & Strange, 1989; Parker, 1993) needs to be turned on first by one stimulus, i.e., hypotonicity or serum withdrawal, before a second stimulus, i.e., UTP or furosemide, can potentiate cellular shrinkage.

We have shown that HCD57 cells respond to serum withdrawal by triggering a signaling pathway that directs cells to shrink in isotonic medium. The shrinking cells display hallmark features of apoptosis (Kim et al., 1998). The rapidity with which HCD57 cells respond to serum withdrawal renders these cells a suitable model for the investigation of apoptosis.

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