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Regulatory Volume Increase in Ehrlich Ascites Tumor Cells Is Mediated by the 1Na : 1K : 2Cl Cotransport System

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Summary. After swelling in hyposmotic solution, Ehrlich ascites tumor cells shrink towards their original volume. Upon restoration of isosmolality (300 mOsM) the cells initially shrink but subsequently recover volume. This regulatory volume increase (RVI) is completely blocked when $[Na^+]_o$ or $[Cl^-]_o$ is reduced by 50% in the presence of normal $[K^+]_o$. With normal $[NaCl]_o$ but less than 2 mM $[K^+]_o$, not only is volume recovery blocked but the cells lose KCl and shrink. When $[K^+]_o$ is increased to 5 mM there is a rapid net uptake of K^+ and Cl^- which results in volume recovery. This suggests that the reswelling phase requires the simultaneous presence of Na^+, K^+, and Cl^-. Although ouabain has no effect on volume recovery, bumetanide completely blocks RVI by inhibiting a cotransport pathway that mediates the net uptake of Na^+, K^+ and Cl^- in the ratio of 1Na : 1K : 2Cl. Na^+ that accumulates is then replaced by K^+ via the Na/K pump.

Key Words Ehrlich tumor cells · volume regulation · ion transport

Introduction

Animal cells when suspended in hyposmotic media (less than 300 mOsM) initially swell osmotically but then exhibit regulatory volume decrease (RVD) whereby the cells return to near normal volume within a few minutes [2, 9, 16]. The decrease in cell volume is due to the loss of solute (primarily K^+ and Cl^-) and water osmotically committed to it. In Ehrlich cells loss of these ions occurs as the result of a transient increase in the conductive K^+ and Cl^- permeabilities which returns to resting level at the termination of RVD [10]. Subsequent resuspension in hyperosmotic media (i.e., 300 mOsM) results first in osmotic shrinkage which is followed by restoration of the cell volume to near the normal isotonic volume. This progression of events, termed regulatory volume increase (RVI), was first described in frog skin epithelial cells [25] but now has been shown to occur in many others [9]. The transport mechanism(s) underlying RVI is unclear. In Ehrlich cells it was proposed that RVI occurs without the direct participation of K^+, mediated by bumetanide-sensitive cotransport of NaCl [12]. Recent observations, however, implicate K^+ in this process [15]. Other studies, however, have shown that Ehrlich cells when incubated at low temperature shrink due to the loss of KCl and water but exhibit RVI when subsequently warmed to 37°C. The restorative process in this case requires extracellular K^+, Na^+ and Cl^- and is mediated by a bumetanide-sensitive cotransport system with a stoichiometry of 2Cl : 1K : 1Na [3, 18, 19]. Since it is unlikely that both types of cotransport systems are operative, the present studies were undertaken to clarify the ionic requirements, particularly the role of K^+, in RVI. Some of the present data have been presented in abstract form [20].

Materials and Methods

Reagents

Bumetanide was kindly supplied by Hoffman-La Roche, Nutley, NJ. Ouabain was a product of Sigma Chemical, St. Louis, MO, while ^{3}H-mannitol (19.1 Ci/mmol) and ^{86}Rb as RbCl (1.1 Ci/mmol) were purchased from New England Nuclear, Boston, MA. All other reagents were of the highest quality obtainable.

Cell Suspension

Experiments were performed with Ehrlich Lettre' ascites tumor cells (hyperdiploid strain) maintained in Ha/IRC white male mice by weekly transplantation. Tumor bearing animals with growths between 8 and 10 days were used. Cells were removed from the peritoneal cavity by aspiration and washed free of ascitic fluid [17]. The standard solution had the following composition (in mM): 150 NaCl, 5 KCl, 10 HEPES-NaOH (pH 7.3–7.45, 290–300 mOsM). This, as well as all other media, was filtered (Gelman Metricel; 0.45 μm) prior to use. Cells were washed twice in this

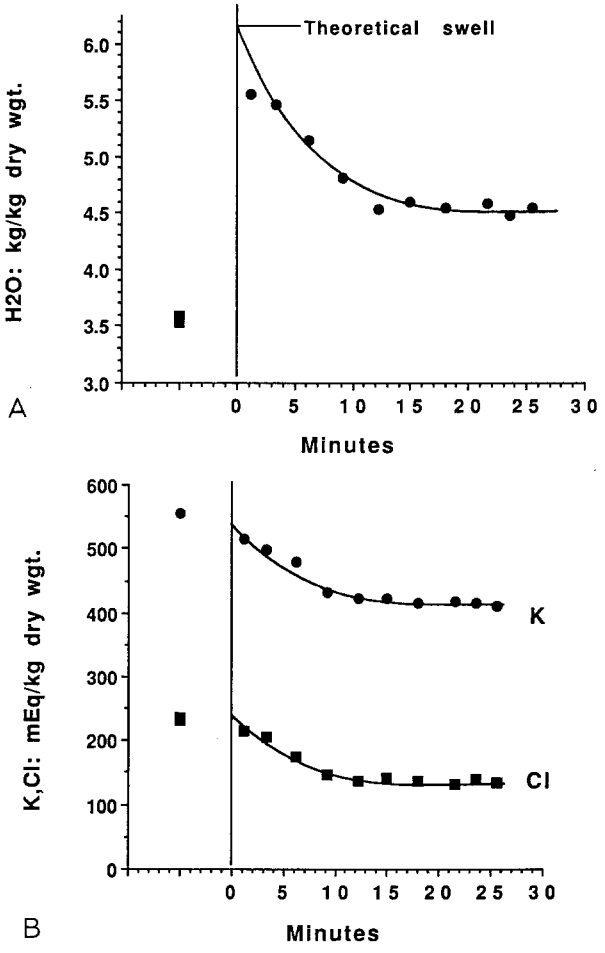

Fig. 1. Regulatory volume decrease (RVD). Cells in standard medium (5 mM KCl, 140 mM NaCl; pH 7.35, 300 mOsM) were centrifuged and at time zero resuspended in buffered hyposmotic saline (80 mM NaCl; 175 mOsM). (*A*) Change in cell volume (water content) was measured during the next 26 min. (*B*) Change in cellular K^+ and Cl^- content over the same time course. Cell K^+, Cl^- and water content in 300 mOsM medium prior to suspension in hyposmotic saline is shown for reference. Representative experiment displayed; five others gave similar results.

solution and subsequently incubated at a density of 7–9 mg dry wt/ml under an air atmosphere for 30 min at 21–23°C.

Regulatory Volume Decrease (RVD)

Following the preincubation period, cell suspension was centrifuged, resuspended and incubated in hyposmotic saline (75 mM NaCl, 10 mM HEPES-NaOH, pH 7.35) for 20–30 min at 23°C. The resulting osmolality was 175 ± 5 mOsM. Samples were removed for the determination of cell water and ion content before and after exposure to the hyposmotic environment.

Regulatory Volume Increase (RVI)

The osmolality of the cell suspension was restored to 300 mOsM by the addition of a small volume (150 μl/10 ml) of a mixture containing 5 M NaCl, 2 M KCl and a tracer amount of ^{86}Rb. Periodically during the next 10 to 15 min, 0.7-ml aliquots of cell suspension were removed and added to preweighted 1.5-ml microcentrifuge tubes containing 0.7-ml ice-cold choline dihydrogen citrate solution (CDHC; [29]) plus 0.25 μCi of ^3H-mannitol. The samples were immediately centrifuged (15 sec at 15,000 × *g*), the supernatant removed and the tubes weighed before the addition of 1 ml of 1% (vol/vol) ice-cold perchloric acid (PCA).

Ion Fluxes

Net fluxes of K^+, Na^+ and Cl^- were estimated as the slopes of the initial time-dependent change in cellular electrolyte content. In general, the slopes were constant during the first 3 min. The unidirectional influx of K^+ was determined from $(d[^{86}Rb]/dt)/SA$ where $d[^{86}Rb]/dt$ is the slope of the time-dependent uptake of ^{86}Rb (cpm/mg dry cell wt · min^{-1}) and SA is the specific activity (cpm/mmol) of the extracellular K^+ (Rb^+). Ion fluxes are reported as: mEq/kg dry wt · min^{-1} while errors as the mean ± SE.

Analytical Methods

The cell pellets were extracted with PCA in an ice-bath for 60 min and subsequently centrifuged 2 min (15,000 × *g*) to remove the PCA-insoluble residue. Aliquots of the PCA extracts and medium were used to determine Na^+ and K^+ by emission flame photometry using Li^+ as an internal standard. Cl^- was assayed with an autotitrator [21]. Aliquots of the PCA extract and the CDHC solution were assayed for ^3H-mannitol and ^{86}Rb with a liquid scintillation spectrometer adjusted so that ^3H activity was confined to only one channel. Correction for Na^+, K^+ and Cl^- trapped in the extracellular space was determined in each experiment from distribution of the ^3H-mannitol. Since mannitol is an impermeant solute [18], the ^3H activity associated with the cell pellet served as a measure of the trapped fluid which typically represented 20–25% of the wet wt of the pellet. Cell volume, which is reported in terms of kg cell water (corrected for ECS) per kg dry cell wt, was measured as described previously [1]. In the steady state in standard solution (295–305 mOsM; pH 7.3–7.4) cell volume was equivalent to 3.55 ± 0.4 (n = 25) kg water/kg dry wt.

Results

A typical RVD response of Ehrlich cells is illustrated in Fig. 1. The initial osmotic swelling induced by suspension in 175 mOsM buffered saline was always less than that predicted for a perfect osmometer. Deviation from "theoretical" behavior can be attributed to incomplete mixing although this may not be the entire explanation [7, 27]. Swelling was followed almost immediately by a return towards normal, isotonic volume. Restoration of cell volume correlates with the loss of KCl and is shown in Fig. 1*B*. Only relatively small changes in Na^+ were observed. These data are consistent with the suggestion that volume increase activates separate conductive pathways for K^+ and Cl^-, allowing loss of KCl driven by the net outward gradient [8]. Water then follows

Table 1. Ionic composition following RVD

	K^+	Na^+ (mEq/kg dry wt)	Cl^-	H_2O (kg/kg dry wt)
Steady-state control	568 ± 12	95 ± 6	228 ± 8	3.55 ± 0.4
	(157.5 ± 2)[a]	(26.4 ± 2)	(63.5 ± 2)	
After RVD	430 ± 15	112 ± 8	136 ± 5	4.86 ± 0.5
	(93.5 ± 1)	(24.1 ± 1)	(29.2 ± 2)	

Ehrlich cell suspension was incubated at 23°C in standard medium (300 mOsM, 5 mM KCl, 150 mM NaCl) or in hyposmotic medium (175 mOsM, 80 mM NaCl) for 20–30 min. Aliquots of cell suspension were then removed from each for the measurement of ion and water content (*see* Materials and Methods).

[a] Ion concentrations are expressed as mEq/kg water. Values reported represent a minimum of 10 separate determinations.

the osmotic gradient. Table 1 summarizes the results of several experiments in which the ionic composition of steady-state cells at normal isotonic volume is compared to that following 20–30 min incubation in hyposmotic media. Note that during RVD the cells lost 24% of K^+ content, 40% of Cl^- content but increased Na^+ content by 18%. The loss of K^+ exceeds that of Cl^- by 46 mEq/kg dry wt. This excess, which is partially offset by Na^+ gain, has been attributed to H^+ uptake through the Jacobs-Stewart cycle [13], resulting in intracellular acidification [22] or possibly from the loss of organic anions [7, 9].

In the following experiments hyposmotic pretreatment was followed by increasing the extracellular osmolality to approximately 300 mOsM. Under these conditions a regulatory volume increase (RVI) may be observed following the initial osmotic shrinkage. Since it has been suggested that RVI following RVD occurs by NaCl cotransport without the direct involvement of K^+ [12], the requirement for extracellular K^+ in this process was investigated. The data illustrated in Fig. 2 shows that after the hyposmotic pretreatment, return of the extracellular osmolality to 306 mOsM by the addition of hyperosmotic NaCl ($[K^+]_o$ = 0.6 mM) resulted in an osmotic shrinkage close to that predicted for a perfect osmometer (4.55 kg water/kg dry wt · 175/306) = 2.6 kg water/kg dry wt. Volume, however, did not increase, but rather slowly decreased (0.02 ± 0.005 kg water/kg dry wt · min^{-1}) during the course of the experiment. The decrease in cell volume can be accounted for by the changes in the cell Na^+, K^+ and Cl^- contents. For example, the initial rate of loss of K^+ and Cl^- was 8.5 ± 0.9 and 2.7 ± 0.5 mEq/kg dry wt · min^{-1}, respectively, while Na^+ increased by 5.8 ± 0.4 mEq/kg dry wt · min^{-1}. Net change therefore, represented a loss of about 6 mOsm/kg dry wt · min^{-1} which is consistent with the rate of shrinkage observed. Clearly, RVI does not occur under conditions of low $[K^+]_o$. By contrast, after the hyposmotic pretreatment, increasing the osmolality of

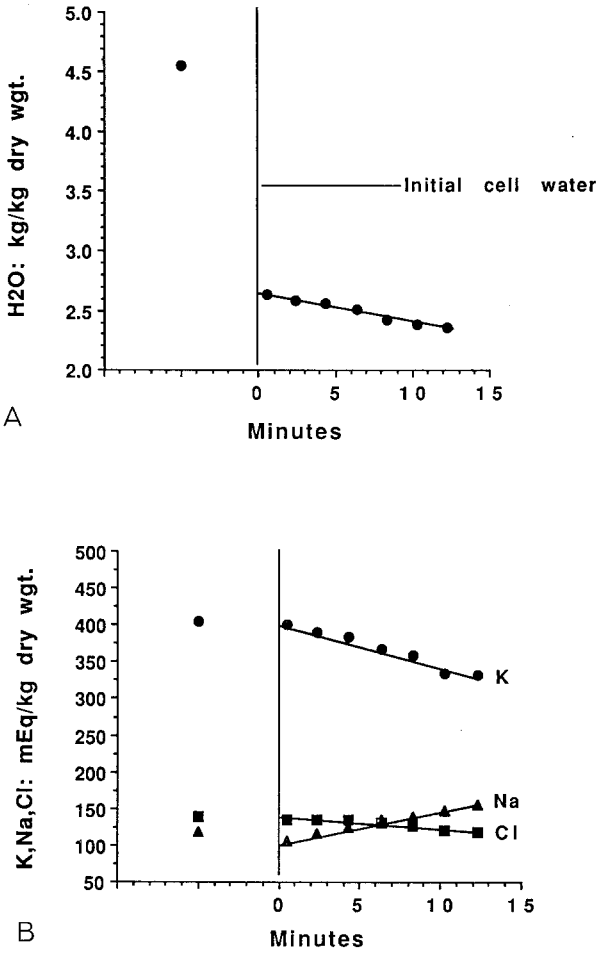

Fig. 2. Inhibition of RVI at low $[K^+]_o$. After exposure to a low external osmolality (175 mOsM saline) for 20 min, the cell suspension was incubated at 37°C and a small volume of 5 M NaCl was added to raise the osmolality to 306 mOsM. Cell water content (*A*), K^+, Na^+, and Cl^- content (*B*) were followed during the next 12 min. During this interval $[K^+]_o$ increased from 0.6 to 1.6 mM. Cell K^+, Na^+, Cl^- and water content in 175 mOsM medium prior to restoration of the osmolality is shown for reference. Initial cell water refers to the water content measured in standard medium (300 mOsM). Representative experiment displayed; four others gave similar results.

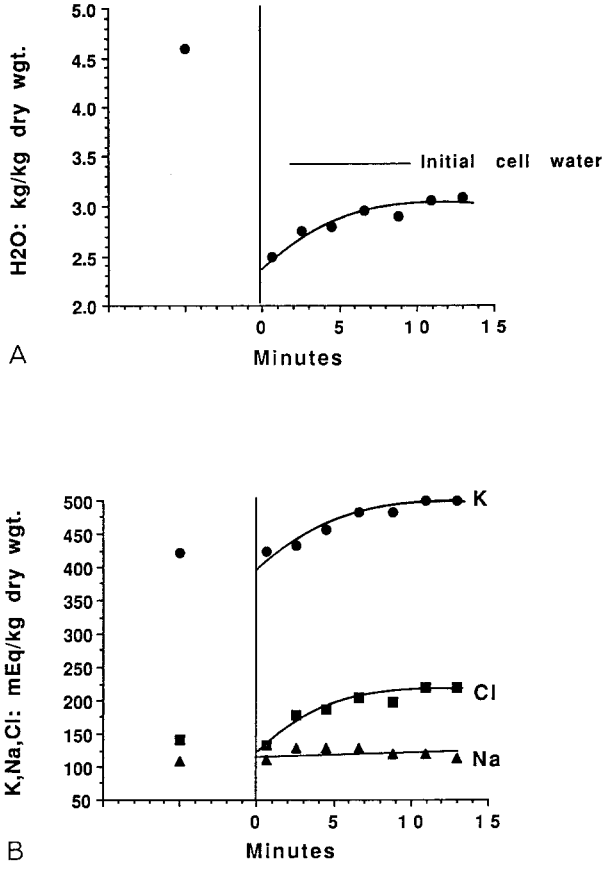

Fig. 3. RVI at 5 mM $[K^+]_o$. After exposure to a low external osmolality (175 mOsM saline) for 20 min, the cell suspension was incubated at 37°C and a small volume of a mix: 5 M NaCl + 2 M KCl + tracer ^{86}Rb was added to raise the osmolality to 300 mOsM (K_o^+ = 5 mM). Cell water content (*A*), K^+, Na^+ and Cl^- content (*B*) were followed during the next 12 min. Cell K^+, Na^+, Cl^- and water content in 175 mOsM medium prior to restoration of the osmolality is shown for reference. Initial cell water refers to the water content measured in standard medium (300 mOsM). Representative experiment displayed; five others gave similar results.

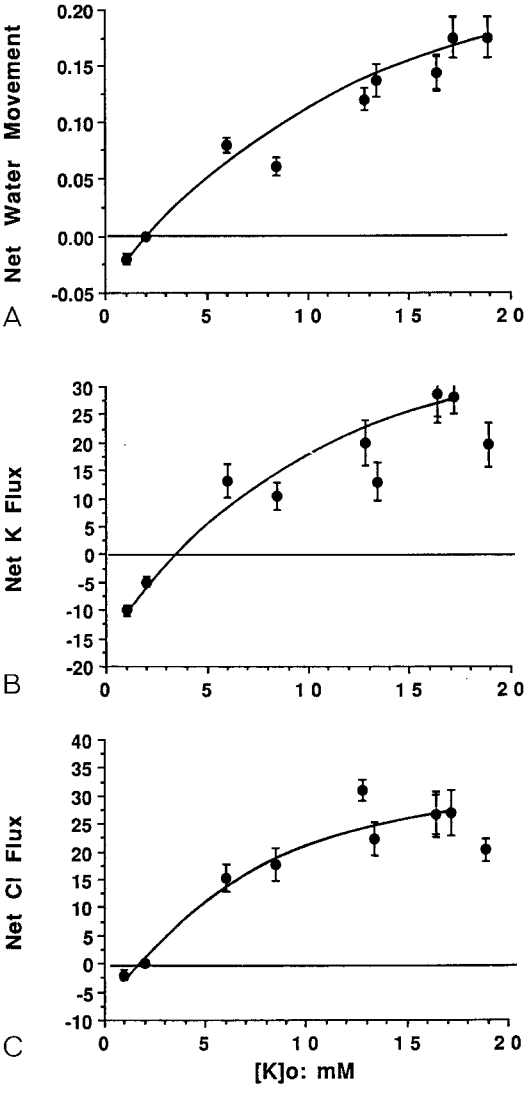

Fig. 4. Effect of $[K^+]_o$ on RVI. After exposure to a low external osmolality (175 mOsM saline) for 20 min, the cell suspension was incubated at 37°C and a small volume of a mix: 5 M NaCl + 2 M KCl was added to raise the osmolality (300 mOsM). Extracellular $[K^+]$ ranged from 0.6 to 18.5 mM. Net water movement (*A*), net K^+ (*B*) and net Cl^- fluxes (*C*) were measured from the initial change in electrolyte content (*see* Materials and Methods). Results from four separate experiments are shown.

the medium to 300 mOsM by the addition of NaCl/KCl (final $[K^+]_o$ = 5 mM) induced an initial osmotic shrinkage followed by a rapid net uptake of water, K^+ and Cl^-. A representative experiment, illustrated in Fig. 3 shows that cell volume increased rapidly (0.08 kg water/kg dry wt · min^{-1}), but by 6 min had stabilized at 85% of the original steady-state volume. The increase in cell volume was driven by uptake of the electrolytes. Initial net fluxes (mEq/kg dry wt · min^{-1}), calculated from the slope of the first part of the uptake curves, were 13.5 and 14.1 for K^+ and Cl^-, respectively. The uptake of Na^+, although measurable, was insignificant (1.5). Unidirectional ^{86}Rb influx (66 mEq/kg dry wt · min^{-1}) which was also measured during RVI greatly exceeded the net K^+ flux, indicating the existence of a significant backflux. The data illustrated in Fig. 4 summarizes the effects of extracellular $[K^+]$ on RVI. At less than 2 mM the Ehrlich cell shrinks, i.e., loses volume. However, with increasing $[K^+]$ there is a progressive increase in the rate of volume restoration. A similar pattern is observed for the net transport of K^+ and Cl^-. At less than 2 mM $[K^+]_o$ there is a loss of cellular K^+ with little change in Cl^-. Electroneutrality is maintained by a corresponding uptake of Na^+ (*see* Fig. 2). However, with increasing $[K^+]_o$ the net flux of both K^+ and Cl^- increase.

Table 2. Effect of reduced Na^+, K^+ or Cl^- on RVI

Experimental condition (mmol/liter)	Initial flux of water[a] (kg H_2O/kg dry wt · min^{-1})
5 KCl, 150 NaCl	0.08 ± 0.01 (n = 6)
<1 K^+, 145 NaCl	−0.022 ± 0.004 (n = 5)
6 KCl, 80 NaCl, 60N-methyl glucamine Cl	−0.01 ± 0.01 (n = 3)
6 KCl, 80 NaCl, 60 Na gluconate	−0.01 ± 0.01 (n = 4)

Cells, incubated 20 min in buffered hyposmotic NaCl (80 mM NaCl, 175 mOsM) to permit RVD, were centrifuged and resuspended in the media (300 mOsM) of reduced [K^+], [Na^+] or [Cl^-]. The change in cell volume (initial flux of water) was then measured.

[a] Direction of net fluxes: (+) refers to uptake and (−) refers to loss.

The results described so far indicate that RVI not only requires extracellular K^+ but that the rate of volume recovery is accelerated with an increase in $[K^+]_o$. The data summarized in Table 2 reaffirm this and also show that RVI is blocked when there is a significant reduction in extracellular Na^+ or Cl^-. Thus, it is reasonable to conclude that volume recovery following RVD requires the simultaneous presence of Na^+, K^+ and Cl^-.

The suggestion that RVI is mediated by an NaCl cotransport originated in part from the observation that when the Na/K pump was inhibited by ouabain an uptake of Na^+ and Cl^- in equimolar amounts could be demonstrated even though the uptake of K^+ was negligible [12]. This is confirmed in the experiment illustrated in Fig. 5. Furthermore, there is no difference in water influx in the presence or absence of ouabain, suggesting that the Na/K pump does not play a direct role in RVI (Table 3). It is important to note that even though there was a small net loss of K^+ in the presence of ouabain, the simultaneously measured unidirectional influx of ^{86}Rb remained high and was almost 80% of the uninhibited value, suggesting the presence of a ouabain-insensitive K^+ uptake pathway. This pathway, however as shown in Table 3, was significantly inhibited by bumetanide, a potent inhibitor of Cl-dependent cation cotransport systems.

The combination of ouabain + bumetanide not only reduced the uptake of NaCl but also completely blocked RVI. Furthermore, these agents almost completely inhibited the unidirectional K^+ (Rb^+) influx and increased the rate of K^+ loss. Figure 6 and Table 3 illustrates these results. The effects of ouabain and bumetanide on the initial net flux of ions and water were used to assess participation of the principal ion transport pathways activated during RVI. This analysis is presented in Table 4. It is

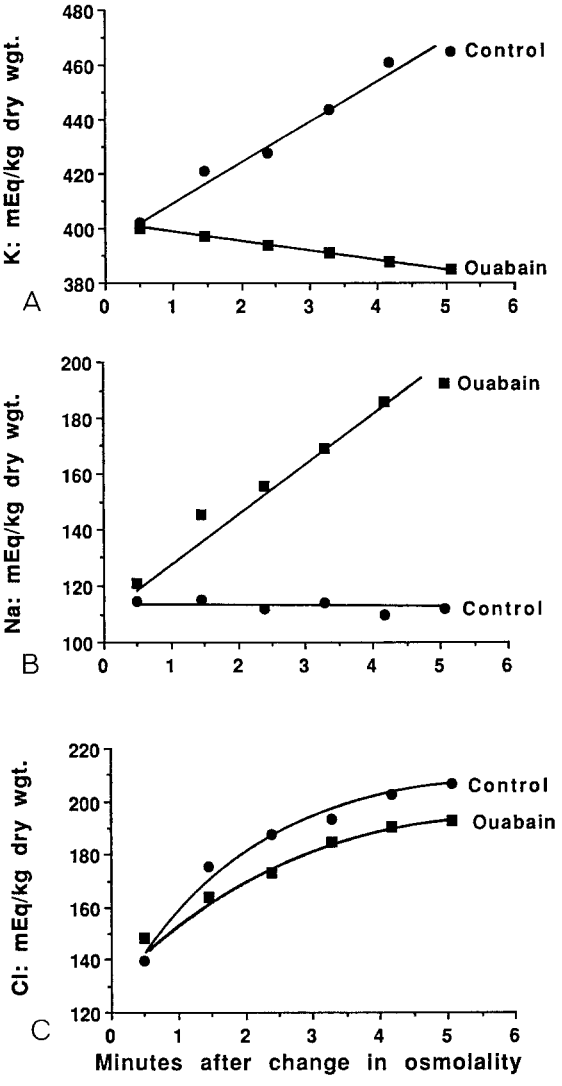

Fig. 5. Effect of ouabain on ion content during RVI. After exposure to a low external osmolality (175 mOsM saline) for 20 min, the cell suspension was incubated at 37°C and a small volume of a mix: 5 M NaCl + 2 M KCl + tracer ^{86}Rb ± ouabain (1 mM) was added to raise the osmolality to 300 mOsM (K_o^+ = 5 mM). Cell water content (*A*), K^+, Na^+ and Cl content (*C*) were followed during the next 5 min. A representative experiment is displayed; five others gave similar results.

assumed that the transport inhibitors at the concentrations used, are specific in that ouabain inhibits only the Na/K pump while bumetanide inhibits only Cl-dependent cation cotransport. The fluxes that persist in the presence of ouabain + bumetanide are attributed to diffusion and represent the leak pathways.

Thus, although the primary event of RVI in ouabain-inhibited cells appears to be uptake of NaCl followed by water, the data summarized in Tables 3 and 4 are more consistent with the idea that volume

Table 3. Initial ion and water fluxes[a] during RVI

	K^+	Na^+	Cl^-	^{86}Rb	H_2O
	(mEq/kg dry wt)				(kg/kg dry wt · min^{-1})
Control	13.2 ± 2	1.2 ± 0.4	15.2 ± 2	68.7 ± 1	0.08 ± 0.005
Ouabain	−2.5 ± 1	16.8 ± 0.7	14.4 ± 0.07	53.5 ± 1	0.08 ± 0.005
Bumetanide	6.4 ± 0.1	−5.6 ± 3	2.2 ± 1	14.3 ± 1	~0
Ouabain + Bumetanide	−10.3 ± 1	10.2 ± 0.8	1.5 ± 1	1.1 ± 0.5	~0

Following RVD the Ehrlich cell suspension was incubated at 37°C in the presence and absence of transport inhibitors (1 mM ouabain, 50 μM bumetanide). The composition of the extracellular medium was restored to normal isosmolality (5 K^+, 150 Na^+, 140 Cl^-, 300 mOsM) by the addition of NaCl + KCl + a tracer amount of ^{86}Rb. Changes in cell water content and electrolytes were measured during the next 12–15 min. Net and unidirectional influxes (mEq/kg dry wt · min^{-1}) were measured during the first 3 min of change (Fig. 3). Values reported ± SE of the mean with a minimum of five separate measurements.

Direction of net fluxes: (+) refers to influx and (−) refers to efflux.

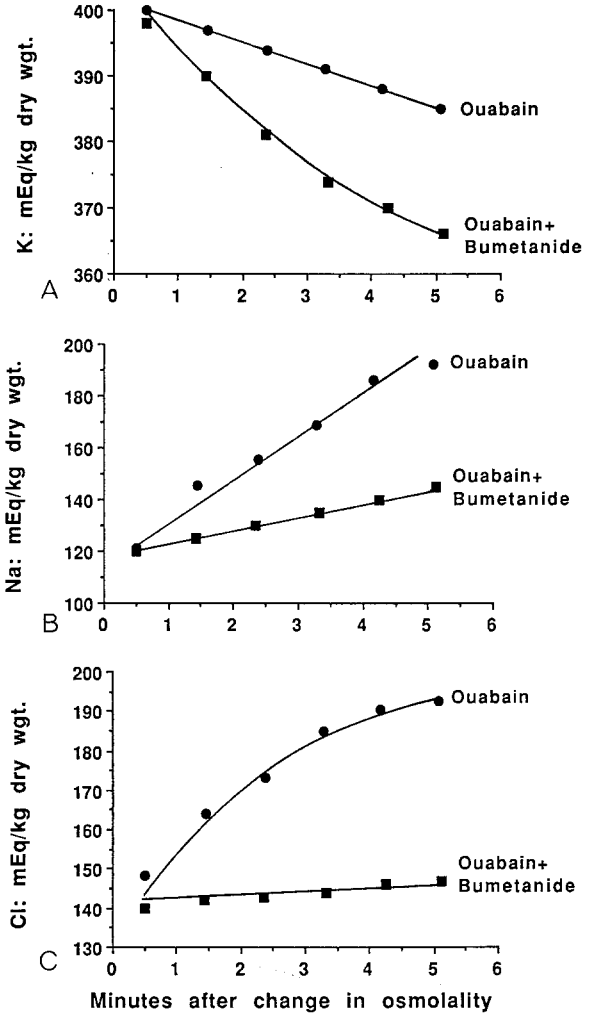

Fig. 6. Effect of bumetanide on ion content during RVI in ouabain-inhibited cells. After exposure to a low external osmolality (175 mOsM saline) for 20 min, the cell suspension was incubated at 37°C and a small volume of a mix: 5 M NaCl + 2 M KCl + tracer ^{86}Rb + ouabain (1 mM) ± bumetanide (50 μM) was added to raise the osmolality to 300 mOsM (K_o^+ = 5 mM). Cell water content (*A*), K^+, Na^+ and Cl^- content (*C*) were followed during the next 5 min. Representative experiment displayed; five others gave similar results.

recovery is accomplished by a bumetanide-sensitive cotransport system that mediates that simultaneous uptake of K^+, Na^+ and Cl^-.

Discussion

The experiments described in this paper were aimed at clarifying the ionic requirements, particularly that of K^+, for RVI. RVI has often been studied in vertebrate cells after a cycle of RVD [9]. In this protocol incubation in a hyposmotic medium (less than 300 mOsM) causes cell swelling, a loss of intracellular ion content, particularly K^+ and Cl^-, and a subsequent return towards normal volume (Fig. 1, Table 1). Reconstituting the extracellular medium to 300 mOsM by the addition of NaCl + KCl results initially in an osmotic shrinkage which is followed by a rapid increase in cell volume, that is, RVI. Although evidence has been presented to suggest that RVI in Ehrlich cells occurs without direct participation of K^+, mediated by a bumetanide-sensitive NaCl cotransport, our data argue against this view and point to an important, if not essential role for K^+. This conclusion is based on the finding that RVI is highly dependent on extracellular $[K^+]$. At low $[K^+]_o$ (less than 2 mM) volume recovery is blocked and in fact, cells lose KCl and shrink (Fig. 2). However, with an increase in $[K^+]_o$ there is a progressive increase in the rate of water influx which is not saturated even at 18 mM K^+ (Fig. 4). The increase in the rate of RVI correlates well with the increase in the net fluxes of K^+ and Cl^- and suggests that it is the reaccumulation of these ions that is responsible for volume recovery.

Although these results establish the importance of $[K^+]_o$ they do not identify the transport pathways involved in this process. Insight into this was obtained by studing the effects of the transport inhibitors, ouabain and bumetanide. Although ouabain is without effect on volume recovery (Table 3), Na^+

Table 4. Transport pathways activated during RVI

Net ion fluxes (mEq/kg dry wt · min^{-1})	
K^+:	Leak = J^K (ouabain + bumetanide) = 10.3
	Pump = J^K (ouabain + bumetanide) + J^K (bumetanide) = 16.7
	Cotransport = J^K (leak) − J^K (ouabain) = 7.8
Na^+:	Leak = J^{Na} (ouabain + bumetanide) = 10.2
	Pump = J^{Na} (ouabain) − J^{Na} (control) = 15.6
	Cotransport = J^{Na} (ouabain) − J^{Na} (leak) = 6.6
Cl^-:	Leak = J^{Cl} (ouabain + bumetanide) = 1.5
	Cotransport = J^{Cl} (ouabain) − J^{Cl} (leak) = 12.9

The net fluxes shown in Table 3 were used to assess the contributions of the Na/K pump, the Cl-dependent cotransport system and nonmediated ("leak") pathways during RVI.

and K^+ transport is markedly affected (Fig. 5, Table 3). While there is a small loss of cellular K^+, a corresponding uptake of Na^+ and Cl^- occurs in almost equivalent amounts (Table 3). Although this is consistent with NaCl cotransport, two observations make this unlikely. First, a substantial fraction (80%) of the total unidirectional K^+ (Rb^+) influx is ouabain-insensitive, indicating K^+ uptake even when the Na/K pump is inhibited. This component, however, is almost completely inhibited by bumetanide which also blocks net Cl^- influx and as a consequence, RVI. Second, more direct evidence for the participation of K^+ in RVI comes from the effect of bumetanide on electrolyte and water transport in ouabain-inhibited cells. Although the rate of K^+ loss is low (2.5 mEq/kg dry wt · min^{-1}), it increases (10.3 mEq/kg dry wt · min^{-1}) with the addition of bumetanide (Fig. 6). This suggests that bumetanide-sensitive K^+ uptake partially offsets the loss of K^+ through the "leak" or diffusional pathway. In a similar manner net Na^+ uptake in the presence of ouabain is high (16.8 mEq/kg dry wt · min^{-1}) but decreases to 10.2 mEq/kg dry wt · min^{-1} with the addition of bumetanide. This is consistent with the idea that with the Na/K pump inhibited Na^+ entry results from the activity of two transport pathways, one driven by the Na^+ electrochemical potential gradient ("leak") and a bumetanide-sensitive component. Inhibition of the bumetanide-sensitive pathway leads to a decrease in net Na^+ uptake. Although Cl^- influx is uneffected by ouabain, bumetanide effectively blocks its uptake (Fig. 6, Table 3). Therefore, the stoichiometry of the bumetanide-sensitive net fluxes activated during RVI is $6.6Na^+ : 7.8K^+ : 12.9Cl^-$ or approximately 1Na : 1K : 2Cl (Table 4).

Volume recovery is also associated with a stimulation of unidirectional K^+ (Rb^+) transport activity. The unidirectional K^+ influx (68.7 mEq/kg dry wt · min^{-1}) greatly exceeds net K^+ influx (13.2 mEq/kg dry wt · min^{-1}) and indicates an exchange of cellular for extracellular K^+ superimposed on the net flux (Table 3). When the cotransport pathway is inhibited K^+/K^+ exchange is zero and under these conditions unidirectional and net K^+ fluxes through the Na/K pump are the same. A K^+/K^+ exchange associated with activation of RVI has previously been reported in the duck red blood cell [26] as well as the Ehrlich cell [18] and may represent a partial reaction of the transport mechanism [23, 24].

The Na/K pump is also activated during RVI presumably because of stimulation by intracellular Na^+ taken up by the cotransport pathway. However, since the coupling of Na^+ to K^+ movement is $16.7K^+ : 15.6Na^+$ or approximately 1 : 1, the Na/K pump does not contribute to net solute accumulation (Tables 3 and 4). Rather, it is the bumetanide-inhibitable, 2Cl : 1Na : 1K cotransport that is solely responsible for volume recovery following RVD.

Although the foregoing clearly establish a role for K^+ in RVI, the energetics of this process are not as clear cut. It has often been asserted that net cotransport of $2Cl^+ : Na^+ : K$ is controlled by the magnitude and direction of their respective ionic gradients and that this process is an example of secondary active transport [6, 14]. However, in the present study this does not appear to apply. For example, using the data from Table 1 for cellular Na^+, K^+ and Cl^- contents following RVD and a water content of 2.55 kg/kg dry wt (Fig. 3) following resuspension of cells in isosmotic (300 mOsM) medium, the ratio: $[Na]_o \cdot [K]_o \cdot [Cl]_o^2/[Na]_i \cdot [K]_i \cdot [Cl]_i^2$ predicts an outwardly directed gradient of 0.65 kcal/mol when $[K^+]_o$ is 2 mM. Yet at this concentration of K^+ there is no net cotransport activity (Fig. 4). At 6 mM $[K^+]_o$ RVI is observed (Fig. 4) although the calculated driving force is approximately zero. This seems to be inconsistent with secondary active transport and raises the possibility for the involvement of other factors, including an alternate energy source.

As noted in the Introduction, a bumetanide-sen-

sitive cotransport system with the stoichiometry of 1Na : 1K : 2Cl mediates volume restoration in Ehrlich cells previously depleted of KCl by low temperature incubation [3, 18, 19]. Since the characteristics of that system are indistinguishable from those activated by hyperosmolality following a cycle of RVD, it seems reasonable to conclude that the same cotransport is activated in both cases. In fact, there is good reason to believe that volume recovery following shrinkage induced by the Ca ionophore, A23187, is also mediated by this system [8]. In each of these cases RVI is activated following a reduction in intracellular Cl^- which has led to the suggestion that this ion plays an important role in the regulation of this cotransport pathway [4, 12, 18, 19, 28]. It is important to note, however, that while these processes have several features in common (ionic requirement, bumetanide sensitivity), they may be distinguished on the basis of the driving forces energizing the transport. Thus, in contrast to the results presented in this study, volume restoration in cells previously depleted of KCl by low temperature incubation appears under certain conditions to be highly dependent on the gradient of chemical potential [19].

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