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Effect of Volume Changes on Ouabain-Insensitive Net Outward Cation Movements in Human Red Cells

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Summary. The effect of cell volume changes in human red cells on ouabain-insensitive net outward cation movements through 1) the Na−K and Li−K cotransport, 2) the Li−Na countertransport system and 3) the furosemide-insensitive Na, K and Li pathway was studied. Cell volume was altered by changing a) the internal cation content (isosmotic method) or b) the external osmolarity of the medium (osmotic method). Na−K and Li−K cotransport were measured as the furosemide-sensitive Na or Li and K efflux into (Na, Li and K)-free (Mg-sucrose replacement) medium from cells loaded to contain approximately equal concentrations of Na and K, or a constant K/Li concentration ratio of 9:1, respectively. Li−Na countertransport was assayed as the Na-stimulated Li efflux from Li-loaded cells and net furosemide-insensitive outfluxes in (Na, Li and K)-free media containing 1 mM furosemide. Swelling of cells by the isosmotic, but not by the osmotic method reduced furosemide-sensitive Na and Li but not K efflux by 80 and 86%, respectively. Changes in cell volume by both methods had no effect on Li−Na countertransport. The effects of cell volume changes were measured on the rate constants of ouabain- and furosemide-insensitive cation fluxes and were found to be complex. Isosmotic shrinkage more than doubled the rate constants of Na and Li efflux but did not affect that of K efflux. Osmotic shrinkage increased the K efflux rate constant by 50% only in cells loaded for countertransport. Isosmotic cell swelling specifically increased the K^+ efflux rate constants both in cells loaded for cotransport and countertransport assays while no effect was observed in cells swollen by the osmotic method. Thus, the three transport pathways responded differently to changes in cell volume, and, furthermore, responses were different depending on the method of changing cell water content.

Key Words erythrocytes · countertransport · cotransport · leaks · volume dependence

Introduction

The constancy of cell volume depends on balance between active and passive ion movements across the cell membrane [23]. While the mechanism of active (metabolism-dependent) translocation of Na, K and Ca ions is well-characterized, the passive movement of ions has been shown to be of much more complex nature than can be explained by simple electrodiffusion [3, 10, 11, 13, 15, 18, 19, 21, 24].

Several modes of ouabain-insensitive transport such as the Ca-dependent K efflux, Ca/Na exchange, Na−Li countertransport and Na−K cotransport have been characterized in the past years. Gardos showed that Ca markedly increased the K permeability in the presence of iodoacetic acid and adenosine leading to cell shrinking [11]. The role of this mechanism in volume regulation is still uncertain. In dog and cat red cells the passive permeability to Na is greatly increased in shrunken cells. Conversely, in swollen cells the Na permeability is reduced and Ca-dependent Na extrusion was found to counteract the increase in cell volume [19]. Duck red cells incubated in hypertonic media, after a rapid shrinking gradually returned to their normal volume, a process accompanied by coupled inward movements of Na and K ions (Na+K+2Cl cotransport) [22]. In erythrocytes of the marine teleost *Opsanus tau* [17], there is ouabain-insensitive, chloride-dependent K^+ transport activated by hypotonic stress. In *Amphiuma* red cells the volume-regulatory response to cell swelling was also characterized by net cellular loss of K, Cl and H_2O and the response to shrinkage involved a net cellular uptake of Na, Cl and H_2O [6]. Despite the vast literature on ion transport existing in human red cells, evidence of the ability of these cells to regulate their volume after volume perturbation [9, 20] is contradictory, and the effect of cell volume changes on the most recently characterized ouabain-insensitive cation pathways has not been reported.

We have studied the effect of alterations in cell volume on three modes of ouabain-insensitive cation transport in human red cells: the furosemide-sensitive Na−K cotransport, the phloretin-sensi-

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tive Li−Na countertransport, and the furosemide-insensitive Na, K and Li effluxes. Our results indicate that cell volume changes have different effects on these three ouabain-insensitive transport pathways. Thus, net outward furosemide-sensitive Na and Li fluxes were reduced while net outward furosemide-sensitive K fluxes were unaffected by cell swelling. Li−Na countertransport was not changed by alterations in cell water content. In addition, cell shrinking induced an increase in the passive permeability for Na, Li and K, while cell swelling increased only the passive permeability for K. Preliminary reports have been published elsewhere [1, 2].

Materials and Methods

Materials

KCl, NaCl, LiCl and $MgCl_2$ were purchased from Mallinckrodt, Inc. (St. Louis, Mo.). Sucrose, trimethylaminomethane (Tris), morpholinopropanesulfonic acid (MOPS), *p*-chloromercuribenzenesulfonate (PCMBS), cysteine, adenine and ouabain were purchased from Sigma Chemical Co. (St. Louis, Mo.). Furosemide was a gift of Hoechst Roussel Pharmaceuticals, Inc. (Somerville, N.J.).

Methods

Adjustment of Cell Cation Content

Human red cells from healthy donors were washed 3 times with a solution containing (mM): 75 $MgCl_2$, 95 sucrose, 10 Tris-MOPS, pH 7.4 at 4 °C. Two loading procedures were used: *a*) the "PCMBS technique" and *b*) the Li loading procedure [7].

a) PCMBS Technique. Washed red cells were suspended at 4% hematocrit in solutions containing 0.02 mM PCMBS as described elsewhere [10]. Briefly, cells were incubated for 20 hr at 4 °C in solutions containing the desired cation concentrations. For the recovery step, the cells were incubated for 1 hr at 37 °C in a medium containing (mM): 2 adenine, 3 inosine and 4 cysteine, 10 glucose, 2.5 $NaPO_4$ and 1 $MgCl_2$ and appropriate concentrations of Na and K (*see below*). Subsequently, the cells were washed 6 times with a Mg-sucrose solution of the same composition as above plus 0.1 mM ouabain.

b) Li Loading Procedure. Cells were incubated during 3 hr in (mM): 150 LiCl, 10 Tris-MOPS, pH 7.4, and 10 glucose at 37 °C and 20% hematocrit and then washed 6 times in the same Mg-sucrose solution as above containing ouabain.

Adjustment of Cell Volume for Transport Measurements

Outward Furosemide-Sensitive and -Insensitive Cation Transport

Isosmotic procedure. In this method the cells were incubated with PCMBS in solutions of different osmolalities to adjust the cell cation content. The fluxes were measured afterwards in a medium of normal osmolality (300 mOsm) with an outwardly imposed gradient. Washed red cells were suspended in solutions containing PCMBS for 20 hr in the cold as described above with variable amounts of Na and K determined by trial and error to obtain the desired composition of the cells (Table 1). Subsequently, recovery from the PCMBS treatment was carried out as indicated above in a solution containing in addition (mM): 145 NaCl and 5 KCl.

Table 1. Composition of the PCMBS loading solutions used to adjust cell volume by the isosmotic method

Cells	NaCl	KCl	LiCl	Sucrose	Tris-PO_4 buffer	$MgCl_2$
				(mM)		
Cotransport						
shrunken	60	10	–	160	2.5	1.0
normal	120	30	–	–	2.5	1.0
swollen	220	75	–	–	2.5	1.0
Countertransport						
shrunken	–	93	9	90	2.5	1.0
normal	5	130	15	–	2.5	1.0
swollen	–	280	22	–	2.5	1.0

The osmolalities of the PCMBS loading solutions to obtain shrunken, normal and swollen cells for cotransport assay were (mOsm/liter) ($n=13$): 303 ± 4, 293 ± 1 and 548 ± 12, respectively. For countertransport assay (mOsm/liter) ($n=5$): 286 ± 1, 286 ± 1 and 552 ± 4, respectively. The fluxes were measured in isotonic (300 mOsm) $MgCl_2$-sucrose medium.

Osmotic procedure. Cells of normal volume loaded to contain Na and K by the PCMBS method were incubated in hypo-osmotic, isosmotic and hyperosmotic media (Table 2) for cation efflux measurements. In this method, the cell volume is changed due to the different osmolalities of the incubation media. As explained before, the gradient imposed for cation movements was outwardly directed.

Li Efflux in Absence and Presence of External Na: Li−Na Countertransport

Isosmotic procedure. Washed red cells were loaded to contain the desired cation composition by the PCMBS method as described above (Table 1). The recovery solution contained (mM): 75 LiCl and 75 KCl in addition to the constituents noted above.

Osmotic procedure. Cells of normal volume loaded by the Li loading procedure were incubated in hypo-osmotic, isosmotic and hyperosmotic Na and Mg media (Table 2) for cation efflux measurements.

Measurements of Cation Fluxes

Fifty percent suspensions of washed cells loaded to contain the desired cations as described above were diluted with ice-cold flux media to obtain a 4% hematocrit. Duplicate tubes containing 2 ml of cell suspension were then incubated for 30, 60 and 90 min at 37 °C. After separation from the cells by centrifugation, aliquots of the supernatants were taken for cation analysis.

Na−K cotransport was measured as the maximum activation of the furosemide-sensitive Na and K efflux into (Na and K)-free Mg-sucrose medium containing 0.1 mM ouabain and ±1 mM furosemide as described elsewhere [10].

Li−Na countertransport was measured as the Na-stimu-

Table 2. Composition of the efflux media used to adjust cell volume by the osmotic method

Media	$MgCl_2$	NaCl	Sucrose	Tris-MOPS	Glucose	Ouabain
			(mM)			
Cotransport						
shrunken (hypertonic)	75	–	205	10	10	0.1
normal (isotonic)	75	–	85	10	10	0.1
swollen (hypotonic)	75	–	46	10	10	0.1
Countertransport						
shrunken	60	–	298	10	10	0.1
(hypertonic)	–	120	230	10	10	0.1
normal	60	–	123	10	10	0.1
(isotonic)	–	120	55	10	10	0.1
swollen	60	–	67	10	10	0.1
(hypotonic)	–	120	–	10	10	0.1

To change cell volume cells loaded by the PCMBS method for cotransport and by the Li loading procedure for countertransport assays were incubated during the flux measurement in media of different osmolalities to change cell volume. The osmolalities of the (Na and K)-free (Mg-sucrose replacement) media to obtain shrunken, normal and swollen cells for cotransport assay were (mOsm/liter) ($n=7$): 443 ± 14, 299 ± 2 and 233 ± 4. For countertransport assay (mOsm/liter) ($n=4$): 481 ± 20, 307 ± 1 and 236 ± 7 for Mg media, and 479 ± 17, 306 ± 3 and 242 ± 9 for Na media, respectively.

lated Li efflux from cells containing K and Li in a concentration ratio of about 9:1. All flux media contained 0.1 mM ouabain [7].

Analytical Determinations

Cell Water Content. Packed red cells were weighed before and after incubation at 100 °C for 48 hr. In a preliminary experiment, water content was measured in cells with volume adjusted by the isosmotic method taken after 0, 30, 60 and 90 min of incubation in flux media (*see* legend to Table 3). Since there were no changes in water content between 30 and 90 min, cell water was measured in subsequent experiments only in cell samples taken at 90 min. The values obtained were used to calculate the fluxes in mmol/kg solids × hr.

Cations. The cation content of the supernatants and red cell lysates were measured by atomic absorption spectrophotometry (Perkin-Elmer, 5000) using appropriate standards.

Cells. Red cell cations were measured in appropriate dilutions of the 50% suspension in double-distilled water (1/50 to 1/500) with 0.02% Acationox.

Supernates. The cation concentrations in the supernatants were measured by atomic absorption spectrophotometry without any further dilution.

Statistics

P values in all Tables were estimated versus the mean value of normal cells. The significance level in some cases has been tested by paired *t*-test statistics. Statistical significance was accepted at the level $P<0.01$. Values represent $\bar{X}\pm$SE.

Table 3. Water content of (Na−K) cotransport cells during efflux measurements

Cells	Furosemide	Time (min)			
		0	30	60	90
		Water content (% wt/wt)			
shrunken	0	54.2	53.2	53.8	53.8
	+		53.8	53.8	54.4
normal	0	65.5	64.9	65.2	65.0
	+		65.1	65.1	65.1
swollen	0	68.2	68.8	68.8	69.1
	+		68.7	69.0	69.0

At zero time, triplicate samples were taken from the 50% suspension in the cold. Duplicate tubes containing 10 ml of the 4% suspension with and without furosemide (1 mM) were incubated at 37 °C, transferred to the cold at 30, 60 and 90 min and then spun down for 1 min. The supernatant was aspirated in order to resuspend the packed cells at a hematocrit of about 50%. The suspension was transferred to Eppendorf tubes and spun at $10{,}000\times g$ for 5 min. A sample taken from the bottom was used to determine cell water as described in Materials and Methods. The composition of the Mg-sucrose media for cotransport assay is shown in Table 2.

Results

Effect of Changes in Cell Water Content on Furosemide-Sensitive Na and K Efflux (Cotransport)

Isosmotic Procedure

Table 4 summarizes the composition and the total, furosemide-insensitive and furosemide-sensitive Na and K effluxes from shrunken, normal and swollen cells incubated in Mg-sucrose medium. Although the gradients for Na and K were about two times higher in swollen than in shrunken cells, a 6% increase in intracellular water induced a substantial reduction of furosemide-sensitive Na efflux with no change in the furosemide-sensitive K efflux. Reducing cell volume had no effect on furosemide-sensitive Na or K effluxes.

It should be noted that cell swelling significantly increased the furosemide-insensitive K^+ efflux, suggesting that cell volume changes may also affect K^+ pathways different from those defined by furosemide inhibition.

Osmotic Procedure

Table 5 shows the composition, the total, furosemide-insensitive and furosemide-sensitive Na and

Table 4. Effect of volume changes on Na–K cotransport by the isosmotic method

Cells	*n*	Cellular composition			Effluxes					
		Water	Na	K	Na			K		
					Total	Furosemide-insensitive	Furosemide-sensitive	Total	Furosemide-insensitive	Furosemide-sensitive
		(% wt/wt)	(mmol/kg solids)		(mmol/kg solids × hr)					
shrunken	9	54.6±0.4[c]	89± 4[a]	66± 7[b]	6.3±0.9	4.8±0.6	1.5±0.4	2.6±0.2[a]	1.2±0.1[b]	1.4±0.1
	9	64.0±0.3	172± 8	125±15	4.7±0.3	3.4±0.2	1.4±0.1	3.3±0.2	1.8±0.1	1.4±0.1
normal										
	13	64.1±0.3	179±13	124±11	4.7±0.2	3.2±0.2	1.5±0.1	3.3±0.2	1.7±0.1	1.6±0.1
swollen	13	68.0±0.4[c]	239±14[c]	134±13	4.3±0.2	4.2±0.3[a]	0.3±0.1[c]	5.6±0.4[b]	4.3±0.3[c]	1.3±0.2

[a] $0.001 < P < 0.01$.
[b] $0.0001 < P < 0.001$.
[c] $P < 0.0001$.
The cells were loaded by the PCMBS method in solutions of different osmolalities (*see* Table 1). Efflux medium (mM): 75 $MgCl_2$, 85 sucrose, 10 Tris-MOPS, pH 7.4 at 37 °C, 10 glucose. The cellular water content was determined in the 90-min sample. The cation concentrations were determined from the 50% suspension at 0 time. *P* values were calculated versus the mean value of the normal control cells. Significance level in Tables 4 through 9 was tested by paired *t*-test.

Table 5. Effect of volume changes on Na–K cotransport by the osmotic method

Cells	*n*	Cellular composition			Effluxes					
		Water	Na	K	Na			K		
					Total	Furosemide-insensitive	Furosemide-sensitive	Total	Furosemide-insensitive	Furosemide-sensitive
		(% wt/wt)	(mmol/kg solids)		(mmol/kg solids × hr)					
shrunken	5	56.3±1.0[a]	168±11	136±14	5.9±0.6	4.3±0.4	1.6±0.4	3.6±0.4	2.4±0.4	1.2±0.2
	5	64.0±0.4	168±11	136±14	4.2±0.3	2.9±0.2	1.3±0.2	3.0±0.2	1.8±0.2	1.4±0.3
normal										
	7	64.0±0.3	168±11	136±14	4.1±0.2	2.6±0.2	1.5±0.2	3.4±0.3	1.9±0.1	1.6±0.2
swollen	7	68.6±0.6[a]	168±11	136±14	3.4±0.2	2.3±0.3	1.1±0.1	4.0±0.4	2.8±0.3	1.2±0.1

[a] $0.0001 < P < 0.001$.
Cells loaded with PCMBS were incubated in Mg-sucrose media of different osmolalities (*see* Table 2). *P* values and significance level as described in legend to Table 4.

K efflux from cells incubated in Mg-sucrose media of different osmolalities. As observed in isosmotically shrunken cells, osmotic shrinking did not affect the magnitude of the furosemide-sensitive fluxes of both Na and K. In osmotically swollen cells, the furosemide-sensitive Na and K fluxes were not significantly (statistically) altered by the induced changes in cell volume. It is interesting to note that the response varied from individual to individual. Thus, the furosemide-sensitive Na efflux was found to be between 10 and 54% reduced in 5 out of 7 experiments. The *P* value of this component of Na flux when comparing swollen with normal cells was $P = 0.0425$. The furosemide-sensitive K efflux was reduced more than 10% in only 3 cases under these experimental conditions.

Effect of Cell Volume Changes on Furosemide-Sensitive Li and K Efflux (Li–K Cotransport)

Isosmotic Procedure

Elsewhere we have further described that furosemide-sensitive Li–K cotransport occurs when cells were loaded with Li [8]. Table 6 presents the total, furosemide-insensitive and furosemide-sensitive Li and K efflux (mmol/kg solids × hr) into Mg medium from cells made shrunken, normal or

Table 6. Effect of volume changes on Li−K cotransport by the isosmotic method

Cells	Cellular composition				Effluxes					
	Water	Na	Li	K	Li			K		
					Total	Furosemide-insensitive	Furosemide-sensitive	Total	Furosemide-insensitive	Furosemide-sensitive
	(% wt/wt)	(mmol/kg solids)			(mmol/kg solids × hr)					
shrunken	58.7±0.8[b]	6±1	18±2[a]	190±15[b]	0.67±0.14	0.44±0.01	0.23±0.06	2.9±0.2[b]	2.3±0.3[b]	0.66±0.16
normal	65.7±0.3	7±1	28±0.4	283±12	0.53±0.03	0.33±0.04	0.20±0.01	4.6±0.2	3.9±0.4	0.65±0.22
swollen	69.6±0.1[c]	4±0.5[b]	40±5	352±12[c]	0.51±0.06	0.47±0.11	0.03±0.04[a]	8.1±0.2[b]	8.5±0.3[c]	−0.33±0.34

[a] $0.001 < P < 0.01$.
[b] $0.0001 < P < 0.001$.
[c] $P < 0.0001$.
$n = 5$.
Cells loaded with PCMBS in solutions of different osmolalities for countertransport assay (*see* Table 1) were incubated for flux measurement in Mg-sucrose medium. Na-free (Mg-sucrose replacement) medium (mM): 75 $MgCl_2$, 85 sucrose, 10 Tris-MOPS, pH 7.4 at 37 °C, 10 glucose. Cellular water content, cation concentrations, P values and significance levels as in legend to Table 4.

Table 7. Effect of volume changes on Li−K cotransport by the osmotic method

Cells	Cellular composition				Effluxes					
	Water	Na	Li	K	Li			K		
					Total	Furosemide-insensitive	Furosemide-sensitive	Total	Furosemide insensitive	Furosemide-sensitive
	(% wt/wt)	(mmol/kg solids)			(mmol/kg solids × hr)					
shrunken	55.7±0.6[a]	16±2	16±1	260±7	0.51±0.11	0.37±0.09	0.17±0.06	4.4±0.1	4.5±0.2	0.03±0.26
normal	64.1±0.2	16±2	16±1	260±7	0.34±0.05	0.26±0.05	0.12±0.01	3.4±0.2	2.8±0.3	0.87±0.23
swollen	68.3±0.6[a]	16±2	16±1	260±7	0.29±0.04	0.23±0.03	0.09±0.03	3.1±0.2	2.5±0.1	0.80±0.20

[a] $0.001 < P < 0.01$.
The cells were loaded by the Li loading procedure for countertransport assay and incubated for flux measurements in Mg-sucrose media of different osmolalities (*see* Table 2). $n = 3$ for furosemide-insensitive and furosemide-sensitive Li and K fluxes, for the rest, $n = 4$. P values and significance level as in legend to Table 4.

swollen by adjusting the cation content with PCMBS. Furosemide, at a concentration of 1 mM, inhibited more than 30% of the total Li efflux in both normal and shrunken cells. In Na^+ medium 20% of the total Li efflux was inhibited in those cells (*data not shown*). The absolute magnitude of the furosemide-sensitive Li fluxes and the pattern of variation with increasing cell volume was similar in both media, i.e. unaltered in shrunken cells and reduced in swollen cells. The Table shows that cell swelling reduced furosemide-sensitive Li efflux by almost 70%, a volume effect similar to that seen previously for the furosemide-sensitive Na efflux (Table 4), suggesting that Li can replace Na in the cotransport system. Furthermore, in shrunken and normal cells, furosemide inhibited about 20 and 10% of the total K efflux in both media. These results are similar to those reported for furosemide-inhibited Li efflux and also suggest the presence of Li−K cotransport.

Corroborating the findings of Table 4, it can be seen that cell swelling also increased substantially furosemide-insensitive K^+ efflux in cells loaded with Li for countertransport assay.

Osmotic Procedure

Table 7 shows the cell composition, the total, furosemide-insensitive and furosemide-sensitive Li and K efflux from cells loaded by the Li procedure. Li efflux was again inhibited more than 30% by furosemide in Mg medium and about 20% in Na medium (*results not shown*). However, under these conditions cell swelling did not significantly affect

Table 8. Effect of volume changes on Li–Na countertransport by the isosmotic method

Cells	Cellular composition				Li Efflux					
	Water	Na	Li	K	Total			Furosemide-insensitive		
					Na medium	Mg medium	Δ	Na medium	Mg medium	Δ
	(% wt/wt)	(mmol/kg solids)			(mmol/kg solids × hr)					
shrunken	58.7±0.8[b]	6±1	18±2[a]	190±15	1.3±0.1	0.67±0.14	0.60±0.05	1.0±0.1	0.44±0.01	0.53±0.05
normal	65.7±0.3	7±1	28±0.4	283±12	1.2±0.1	0.53±0.03	0.70±0.10	0.9±0.1	0.33±0.04	0.62±0.08
swollen	69.6±0.1[c]	4±0.5[b]	40±5	352±12[c]	1.2±0.1	0.57±0.06	0.65±0.10	1.2±0.1	0.47±0.11	0.69±0.06

[a] $0.001 < P < 0.01$.
[b] $0.0001 < P < 0.001$.
[c] $P < 0.0001$.
Cells loaded with PCMBS in solutions of different osmolalities (*see* Table 1) were incubated in Mg-sucrose and Na media for flux measurements. Mg-sucrose medium (mM): 75 $MgCl_2$, 85 sucrose, 10 Tris-MOPS, pH 7.4 at 37 °C, 10 glucose. Na medium (mM): 150 NaCl, 10 Tris-MOPS, pH 7.4 at 37 °C, 10 glucose.

Table 9. Effect of volume changes on Li–Na countertransport by the osmotic method

Cells	Cellular composition				Li Efflux					
	Water	Na	Li	K	Total			Furosemide-insensitive		
					Na medium	Mg medium	Δ	Na medium	Mg medium	Δ
	(% wt/wt)	(mmol/kg solids)			(mmol/kg solids × hr)					
shrunken	55.7±0.6[b]	16±2	16±1	260±7	0.97±0.11	0.51±0.11	0.47±0.01	0.79±0.10	0.37±0.09	0.44±0.02[a]
normal	64.1±0.2	16±2	16±1	260±7	1.05±0.09	0.34±0.05	0.72±0.05	0.87±0.06	0.26±0.05	0.62±0.03
swollen	68.3±0.6[a]	16±2	16±1	260±7	1.18±0.12	0.29±0.04	0.89±0.09	0.98±0.12	0.23±0.03	0.73±0.11

[a] $0.001 < P < 0.01$.
[b] $0.0001 < P < 0.001$.
Cells loaded by the Li loading procedure (no PCMBS) were incubated in Mg-sucrose and Na media of different osmolalities (*see* Table 2).

the furosemide-sensitive Li efflux in either medium.

Effect of Changes in Cell Volume on Li–Na Countertransport

Isosmotic Procedure

Table 8 summarizes the composition and the total and furosemide-insensitive Li efflux into Na and Mg media from cells loaded by the PCMBS method. In both shrunken and swollen cells the total and furosemide-insensitive Li effluxes were not significantly different from those of cells with normal volume in both media. In consequence, Li–Na countertransport was not affected by an increase or decrease in the water content of cells by the isosmotic method.

Osmotic Procedure

Na-stimulated Li efflux was also studied in cells loaded with Li without PCMBS treatment (*see* Materials and Methods). Table 9 shows the cell composition and the total furosemide-insensitive Li efflux into Mg and Na media of different osmolalities. Li efflux in Na medium was the same irrespective of the cell water content. In contrast, there was a tendency for Li efflux to increase when the cells were suspended in Mg-sucrose medium of increasing osmolalities both in the absence and presence of furosemide. Thus, the total and furosemide-insensitive Li–Na countertransport increased 90 and 66%, respectively, when comparing swollen with shrunken cells. These results are different from those observed when the cell volume

Table 10. Effect of volume changes on the passive permeability for Na and K in cells loaded for cotransport assays by the isosmotic and osmotic methods

Cells	Rate constants of furosemide-insensitive efflux (hr^{-1})					
	Isosmotic method			Osmotic method		
	n	k_{Na}	k_K	n	k_{Na}	k_K
shrunken	9	0.050 ± 0.004[b]	0.019 ± 0.001	5	0.025 ± 0.003	0.020 ± 0.002
	9	0.018 ± 0.002	0.015 ± 0.002	5	0.016 ± 0.001	0.014 ± 0.001
normal						
	13	0.018 ± 0.001	0.014 ± 0.001	7	0.015 ± 0.001	0.014 ± 0.001
swollen	13	0.018 ± 0.002	0.034 ± 0.004[a]	7	0.014 ± 0.001	0.019 ± 0.002

[a] $0.0001 < P < 0.001$.
[b] $P < 0.0001$.
The rate constants were calculated as the ratio between the furosemide-insensitive Na or K efflux and the cellular cation concentration (data from Tables 4 and 5). Significance level was calculated by t-test.

was changed by the isosmotic procedure (*see* Table 8).

Effect of Changes in Cell Water Content on Na and K Passive Permeabilities in Cells Loaded for Cotransport

Isosmotic Procedure

Red cells loaded for outward cotransport measurements were incubated in Mg-sucrose medium in the presence of both 10^{-4} M ouabain and 10^{-3} M furosemide. The rate constants for Na and K efflux were calculated as the ratio between the furosemide-insensitive Na and K effluxes measured in the units mmol/(kg solids × hr) and the concentrations of the ions measured in units mmol/kg solids. The rate constants were expressed in hr^{-1} and the results were taken as a measure of the passive cation permeabilities. The rate constants of cation fluxes are a good approximation of the passive cation permeabilities provided the flux is linear as a function of the internal cation concentration and the latter does not change during the period of flux measurement. In the present study, the second requirement is fulfilled since the fluxes in human red cells are relatively small and in our experiments they were measured at initial rates. In contrast, although previous observations showed a linear relationship between furosemide-insensitive cation fluxes and concentration in normal red cells [10], this relationship needs to be tested in shrunken and swollen cells. The results shown in Table 10 are calculated from Table 4 where the cellular water content was increased by increasing the cell cation content (PCMBS method). The rate constant for outward furosemide-insensitive Na flux was found almost three times larger in shrunken cells. The rate constant for K but not for Na outward furosemide-insensitive transport was increased in swollen cells, suggesting that there was indeed a permeability change for K ions. The values of furosemide-insensitive cation fluxes calculated according to electrodiffusion are 1.7, 3.4 and 4.3 mmol/kg solids × hr for Na and 1.0, 1.8 and 2.3 for K in shrunken, normal and swollen cells, respectively (from data in Table 4). Hence, neither furosemide-insensitive Na efflux in shrunken cells nor the corresponding K flux in swollen cells can be explained by assuming simple electrodiffusion.

Osmotic Procedure

Red cells, loaded by the PCMBS method to measure the maximum velocity of Na−K cotransport, were incubated in solutions of different osmolalities in the presence of ouabain and furosemide. Results summarized in Table 10 show that the rate constants of furosemide-insensitive Na and K effluxes were not significantly altered by changing the osmolality of the efflux medium. Furthermore, the estimated values of furosemide-insensitive cation fluxes by electrodiffusion were 3.9, 2.9 to 2.6 and 2.3 mmol/liter cells × hr for Na and 2.5, 1.8 to 1.9 and 1.6 for K in shrunken, normal and swollen cells, respectively (calculated on the basis of data from Table 5). While Na seems to move according to electrodiffusion, furosemide-insensitive K efflux in swollen cells was twice the value expected from its electrochemical gradient.

In summary, the present results indicate that

Table 11. Effect of volume changes on the passive permeability for Li and K in cells loaded for countertransport assays by isosmotic and osmotic methods

Cells	Rate constants of furosemide-insensitive efflux (hr^{-1})			
	Isosmotic method		Osmotic method	
	k_{Li}	k_K	k_{Li}	k_K
shrunken	0.023 ± 0.003 [a]	0.013 ± 0.002	0.023 ± 0.004	0.017 ± 0.001 [a]
normal	0.011 ± 0.001	0.014 ± 0.002	0.016 ± 0.002	0.010 ± 0.001
swollen	0.011 ± 0.001	0.024 ± 0.001 [b]	0.014 ± 0.002	0.010 ± 0.000

$n = 5$, isosmotic.
$n = 3$, osmotic.
[a] $0.001 < P < 0.01$.
[b] $0.0001 < P < 0.001$.
The rate constants were calculated as the ratio between the furosemide-insensitive Li or K efflux and the cellular cation concentration (data from Tables 6 and 7). Significance level was calculated by *t*-test.

passive Na permeability was increased in isosmotically shrunken cells but unaffected by isosmotic cell swelling, or by modifications in cell volume by the osmotic method. Passive K permeability was increased in cells swollen by the isosmotic method but was not affected by changing cell volume with the osmotic method.

Effect of Cell Volume Changes on Li and K Passive Permeability in Li-Loaded Cells

Isosmotic Procedure

Table 11 shows the rate constants of the furosemide-insensitive Li and K efflux into Mg-sucrose medium (300 mOsm) from cells loaded with PCMBS for countertransport assay (data presented in Table 6). The rate constant for furosemide-insensitive Li efflux was increased in cells shrunken by the isosmotic procedure but was unaffected by cell swelling. The rate constant for furosemide-insensitive K efflux was increased by cell swelling but unaffected by shrinking of the cells. Moreover, the estimated fluxes contributed by electrodiffusion to the furosemide-insensitive cation fluxes were 0.21, 0.33 and 0.47 mmol/kg solids $\times$ hr for Li and 2.6, 3.9 and 4.8 for K in shrunken, normal and swollen cells, respectively. Hence, neither Li in shrunken cells nor K in swollen cells moved according to their electrochemical gradients.

Osmotic Procedure

Table 11 also summarizes the rate constants of Li and K efflux into Mg-sucrose media of different osmolalities, from cells loaded without PCMBS. The rate constant for furosemide-insensitive Li efflux was not affected when cell volume was changed by the osmotic method while the rate constant for furosemide-insensitive K efflux was increased in shrunken cells. In addition, the furosemide-insensitive Li and K effluxes in shrunken, normal and swollen cells were 0.37, 0.26 and 0.21 mmol/kg solids $\times$ hr and 4.0, 2.8 and 2.3, respectively, as expected by electrodiffusion. Only K efflux in shrunken cells was found slightly increased.

Discussion

The Na$-$K cotransport system, defined as furosemide-sensitive Na and K efflux into Mg-sucrose medium, was uncoupled by isosmotic swelling. In Na- and K-free medium cell swelling was associated with a reduction of furosemide-sensitive Na, but not K efflux. The outward Na$-$K cotransport system in human red cells seems to be very sensitive to cell volume perturbations because in some cases a 2 to 3% increase in cell water content inhibited the furosemide-sensitive Na efflux. This result has been confirmed in duck red cells [12]. Isosmotic swelling also inhibited the small component of furosemide-sensitive Li efflux which seems to be part of a Li$-$K cotransport system (Table 4, Ref. 8). Cell shrinking did not significantly activate Na$-$K cotransport in the absence of external Na and K as has been found in duck red cells [22].

Due to the fact that the furosemide-sensitive Na efflux is reduced by cell swelling, changes in cell volume should be avoided when assaying the rate of Na$-$K cotransport. This is particularly important in clinical studies where the PCMBS loading method is used to prepare cells for assay.

Our studies also indicate that Li−Na exchange is insensitive to isosmotic changes in cell volume. In contrast, osmotic shrinking produced an increase in the furosemide-insensitive Li efflux into Mg medium and a decrease of Li−Na countertransport. Thus, these experiments provide additional evidence that the Li−Na countertransport and the Na−K cotransport mechanisms behave as independent transport pathways rather than a single system with two modes of Na transport.

Isosmotic shrinking promoted an increase in the passive permeability for Na. Isosmotic swelling induced a significant increase in the passive permeability for K. In the isosmotic procedure, the volume of the cells loaded for cotransport was increased by increasing the concentration of NaCl in the loading solution, while KCl was increased in those loaded for countertransport assay (Table 1). Under both conditions, the most significant volume-sensitive changes in cation efflux were observed for K (Tables 4 and 6). The isosmotic gain of water markedly increased K efflux through a ouabain- and furosemide-insensitive pathway. These changes in outward K transport were observed in red cells loaded to attain both maximal activation of Na−K cotransport and Li−Na countertransport. The relationship of the selective, volume-dependent K efflux from human red cells with the Ca-dependent K efflux discovered by Gardos [11] is still unclear. Kregenow and Hoffman [16] reported that Ca alone promotes an increase in K permeability when the cells are depleted of metabolic energy, and that 3 to 7 μmol Ca/liter cells is sufficient to produce the increased K loss. During the isosmotic procedure, the concentration of Ca as contaminant of NaCl and KCl could have been as high as 3 and 26 μM, respectively. In addition, other factors such as temperature of incubations, PCMBS, adenine and the ionic composition of the solutions [14] during the loading and the recovery of the cells could have created the adequate conditions for the Gardos effect to be elicited. However, in preliminary experiments in which isosmotically and osmotically swollen cells were prepared in the presence and absence of 1 mM EGTA, the volume-dependent K efflux was not affected by the Ca chelator. Furthermore, the Gardos effect requires the presence of external K [5]. Therefore, when we measured the volume-dependent K efflux into Mg medium containing less than 30 μM K, this mechanism should have been inoperative. It is also claimed that the Gardos effect is furosemide-sensitive [4] while the effect that we are discussing is an increase in the furosemide-insensitive K efflux. Taken together, these considerations suggest that the selective increase in K efflux was not induced by contaminant calcium. Further studies are required to settle this point.

The changes in the passive permeabilities for Na and K by the isosmotic method are in the direction for the cells to recover normal volume provided those changes are also existent under physiological conditions. Thus, the increase in the passive permeability for K in swollen cells would allow the cells to lose their primary intracellular osmotically active particle with a consequent reshrinking to a normal volume. Conversely, the increase in passive permeability for Na in shrunken cells would allow the cells to gain the main extracellular cation and reswell to a normal volume.

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