Volume-sensitive K Transport in Human Erythrocytes

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ABSTRACT Studies have been carried out on human erythrocytes to examine the alterations of K transport induced by swelling or shrinking the cells by osmotic and isosmotic methods. Hypotonic swelling of erythrocytes (relative cell volume, 1.20) resulted in a striking, four- to fivefold augmentation in the ouabain-resistant K influx over the value obtained at a normal cell volume. Shrinking the cells in hypertonic media resulted in a small but statistically significant reduction in K influx. Three different methods of varying cell volume gave similar results. These include the addition of sucrose and of NaCl to hypotonic media and the isosmotic (nystatin) method. The major fraction of the K influx in swollen cells is specific in its requirement for Cl or Br and is not supported by thiocyanate, iodide, nitrate, methylsulfate, or acetate. Bumetanide (0.1 mM), MK-196 (0.2 mM), and piretanide (1 mM) are poorly effective in suppressing K uptake in swollen cells, but at higher concentrations, bumetanide (1 mM) inhibits 80% of the Cl-dependent K influx in swollen cells. The bumetanide concentration required to inhibit 50% of the Cl-dependent K influx is 0.17 mM. The volume-sensitive K influx is independent of both extracellular and intracellular Na, so that the (Na + K + 2Cl) cotransport pathway is not a likely mediator of the volume-sensitive K transport. A variety of inhibitors of the Ca-activated K channel are ineffective in suppressing swelling-induced K influx. Like K uptake, the efflux of K is also enhanced by cell swelling. Swelling-activated K efflux is Cl dependent, is independent of extracellular and intracellular Na, and is observed with both hypotonic and isosmotic methods of cell swelling. The activation of K efflux by cell swelling is observed in K-free media, which suggests that the volume-sensitive K transport pathway is capable of net K efflux. The addition of external K to hypotonic media resulted in an increase in K efflux compared with the efflux in K-free media, and this increase was probably due to K/K exchange. Thus, hypotonic or isosmotic swelling of human erythrocytes results in the activation of a ouabain-resistant, Cl-dependent, Na-independent transport pathway that is capable of mediating both net K efflux and K/K exchange.

INTRODUCTION

In recent years, a number of investigators have focused their attention on the effects of variations in cell volume on monovalent cation transport across cell

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membranes. The recent interest in volume-sensitive cation transport pathways stems from the recognition of the importance of these pathways in regulating cell volume in anisotonic media and in maintaining the constancy of the intracellular milieu. Recently, it has been suggested (Eveloff and Calamia, 1986) that volume-sensitive Na and K transport pathways may also serve an important role in the regulation of salt and water reabsorption in the kidney.

A variety of K transport pathways are activated by swelling or shrinking different cell types. In particular, swelling has been reported to activate Cl-dependent K transport in avian (Kregenow, 1974; Kregenow and Caryk, 1979; Haas and McManus, 1985), sheep (Ellory and Dunham, 1980; Dunham and Ellory, 1981; Lauf, 1984), dog (Parker, 1983), and toadfish erythrocytes (Lauf, 1985), K/H exchange in *Amphiuma* erythrocytes (Cala, 1983), K conductance in human lymphocytes (Sarkadi et al., 1985) and Ehrlich ascites tumor cells (Hoffmann et al., 1984), and K loss through an unidentified mechanism in flounder erythrocytes (Cala, 1977).

There exists a relative paucity of information about the effects of altering cell volume on K transport in human erythrocytes. Almost 50 years ago, Davson (1937) reported that swelling or shrinking was ineffective in producing alterations of K transport in human erythrocytes. The decrease in K influx with cell swelling and the increase in K influx with shrinking described in one study (Poznansky and Solomon, 1972) have not been confirmed in two other studies (Dunham and Benjamin, 1984; Lauf et al., 1985), in which no change was found in K influx with swelling or shrinking. The swelling of human erythrocytes has been reported to increase K efflux (Adragna and Tosteson, 1984), but the anion and cation requirements of this K efflux pathway have not been characterized.

This report describes alterations of K transport with swelling and shrinking of human erythrocytes by the osmotic and isosmotic methods. The properties of the volume-activated K transport pathway with respect to anion and cation dependence and sensitivity to various transport inhibitors have been characterized. A preliminary account of this work has been presented to the Red Blood Cell Club (New Haven, CT, 1985) and to the Society of General Physiologists (Kaji, 1985).

METHODS

Heparinized blood was collected from 14 normal human males (6 Caucasians, 3 Asians, and 5 blacks) and processed within 1 h of collection. After the removal of the plasma and the buffy coat, erythrocytes were washed three times with 10 vol of the same medium in which the K influx was measured.

The hemoglobin, hematocrit, and intracellular Na, K, and water contents were measured as described before (Kaji et al., 1981; Cheng et al., 1984; Kaji and Kahn, 1985). The relative cell volume (RCV) was measured by monitoring the alteration of the mean corpuscular hemoglobin concentration, as described previously (Cheng et al., 1984). The volume of cells in fresh blood was taken as unity.

Alteration of Cell Volume

Cell volume was altered by either the osmotic or the nystatin method.

Osmotic method. In the majority of studies, cell volume was altered by the suspension of cells in anisotonic media. The osmolality of the medium was varied by adding either

sucrose (up to 200 mM) or NaCl (up to 100 mM) to a hypotonic flux medium. This hypotonic medium contained 100 mM NaCl plus KCl, 0.1 mM ouabain, 6.7 mM glucose, and 6.7 mM Tris·MOPS, pH 7.4 at 37°C, osmolality 200–202 mosmol/kg. After suspension, cells were washed at least three times in the same medium before the addition of ^{86}Rb for K influx.

Nystatin method. Washed cells were incubated at 2–4% hematocrit with 30 mg/liter (final) nystatin in a loading solution containing 140 mM NaCl plus KCl, 15–200 mM sucrose, and 2.5 mM HEPES, pH 7.2 at 4°C. After 15 min of incubation at 0°C, the cells were washed once and resuspended in the same medium for an additional 15 min. Cells were then washed four times at 37°C in the same medium but without nystatin and with 0.1% desalted albumin to elute nystatin. Albumin was prepared as a 10% aqueous solution and rendered Na-free by treatment with cation exchange resin (AG 50W-X). The acidic Na-free albumin was titrated to pH 7.4 at 37°C with Tris. Next, cells were washed with the same medium without sucrose, pH 7.4 at 37°C. Finally, cells were washed three more times in a medium containing 150 mM *N*-methylglucamine Cl or NO_3, 10 mM glucose, and 10 mM Tris·MOPS, pH 7.4 at 37°C. After saving aliquots for measurement of Na, K, hemoglobin, and hematocrit, cells were used for K influx as described below.

Anion Equilibration

Cells were equilibrated in media with various monovalent permeant anions substituted for Cl (Dunham et al., 1980). Cells were first washed in the medium with substitute anion and then incubated at least twice at 37°C for 30 min in the same medium; cells were washed and resuspended between incubations. Finally, cells were washed three times in the same medium at room temperature. Washed cells were used for K influx as described below.

K Influx

Unidirectional K influx was measured by using ^{86}Rb as a tracer as described previously (Kaji et al., 1981; Cheng et al., 1984; Kaji and Kahn, 1985). Packed erythrocytes were suspended at 5% hematocrit in media with various compositions as described in the figure legends. When transport inhibitors were used, they were added to prewarmed cell suspensions at least 15 min before the addition of the isotope. Cells were incubated at 37°C with ^{86}Rb for 30–45 min. At the end of incubation, cells were washed three times with ice-cold, unbuffered 115 mM $MgCl_2$. The radioactivity of the cells and supernates was counted in a gamma counter (model 4000, Beckman Instrument Co., Fullerton, CA). Preliminary studies revealed that K influx was linear up to 45 min in normal as well as in swollen cells. Results are expressed as millimoles of K influx per liter of original cells per hour ($_iM_k$), which was calculated as $_iM_k = \bar{x}_c/\bar{x}_m$ (i), where $\bar{x}_c$ is the change in counts per minute per liter original cells per hour and $\bar{x}_m$ is the specific activity of the medium.

K Efflux

Cells were loaded with ^{86}Rb (10 μCi/ml) for 3 h in media containing (mM): 99 NaCl, 1 KCl, 5 glucose, 5 Tris·MOPS, pH 7.4 at 37°C. Cells were washed free of supernatant radioactivity by washing seven times in ice-cold media without isotope. The last three washes were performed in media with the same composition as that used for the efflux experiment. Cells were incubated at 37°C (hematocrit, 5–6%) in media with the desired composition (see figure legends). Efflux was terminated by layering aliquots of suspension over dibutyl phthalate, followed by rapid centrifugation to separate the cells from the supernate. Two aliquots were taken at 5 min and three more were taken at 65 min. Preliminary studies revealed that K efflux was linear at least up to 65 min. The K efflux rate constant (per hour) was calculated from the slope of ln (1 − cpm supernate/cpm

suspension) vs. time (h). K efflux (mmol/liter cells·h) = K efflux rate constant (h^{-1}) × cell K (mmol/liter cells). In hypotonically swollen cells, cell K refers to the content (mmol/liter cells) in hypotonic medium. K efflux (mmol/liter original cells·h) = K efflux (mmol/liter cells·h) × relative cell volume.

Materials

Reagent-grade chemicals were obtained from Eastman Kodak Co. or J.T. Baker & Co. (through VWR Scientific, Inc., Rochester, NY), British Drug House (through Gallard Schlesinger, Inc., Carle Place, NY), or Sigma Chemical Co., St. Louis, MO. Bumetanide was a gift of Dr. Peter Sorter, Hoffmann La Roche, Nutley, NJ. Furosemide and 3-*N*-pyrrolidino-4-phenoxy-5-sulfamoylbenzoic acid (piretanide) were gifts from Hoescht-Roussel Pharmaceuticals, Inc., Somerville, NJ. Amiloride and 6,7-dichloro-2-methyl-2-phenyl-1-oxoindanyloxyl acid (MK-196) were gifts from Merck, Sharpe & Dohme, Rahway, NJ. Inhibitors of the Ca-activated K channel were obtained from Sigma Chemical Co., except 3,3′-dipropylthiadicarbocyanine iodide, which was obtained from Molecular Probes, Inc., Junction City, OR. ^{86}Rb was obtained as the Cl salt from Amersham Corp., Arlington Heights, IL.

Presentation of Data

Results are expressed as means ± standard deviation (SD). Because of variations in the absolute rates of K transport in cells from different donors, the results from experiments on different donors have not been pooled. Instead, representative studies are shown in the figures and tables. In the figure legends, *n* refers to the number of experiments for a single donor. In each case, similar results were obtained in two or more separate experiments with cells from different donors.

RESULTS

Effect of Cell Volume on K Influx

As shown in Fig. 1, hypotonic swelling of normal human erythrocytes (open circles) resulted in a striking increase in the basal ouabain-resistant (OR) K influx, whereas shrinking resulted in a less dramatic but statistically significant decrease in the K transport rate. A 10% increase in RCV resulted in an 80% rise in K uptake, whereas a 10% reduction in cell volume resulted in a smaller (12–15%) but statistically significant ($p < 0.01$) decrease in K influx compared with the transport rate at normal cell volume. When cells were swollen by 20%, K influx increased four- to fivefold over values obtained at normal cell volume.

Cell swelling by the isosmotic (nystatin) method resulted in a similar increase in OR K influx (solid circles). The finding that, for a given cell volume, K influx was higher with the isosmotic (nystatin) method than with the osmotic method (Fig. 1) may be due to the higher external (and internal) Cl concentrations used for the K influx assay with the nystatin method (150 mM external Cl) compared with the concentrations used with the osmotic method (100 mM external Cl). In addition, intracellular K concentrations were higher with the isosmotic method than with the osmotic method.

The increment in K influx in hypotonic media was typical of 17 experiments on cells from donor 1, 15 experiments on cells from donor 2, and 12 other experiments on cells from 12 other donors.

These results are apparently at variance with those obtained by another group of workers (Lauf et al., 1985), who reported that basal Rb influx was unchanged upon lowering the medium osmolality. Whereas the present series of experiments was performed at an external K concentration of 30 mM, the earlier studies were done with an external Rb concentration of 10 mM. To examine whether differences in external concentrations of K (or Rb) could account for the discrepancy in results between the two studies, K influx was measured in anisotonic media with a lower (5 mM) K concentration. Even at this lower

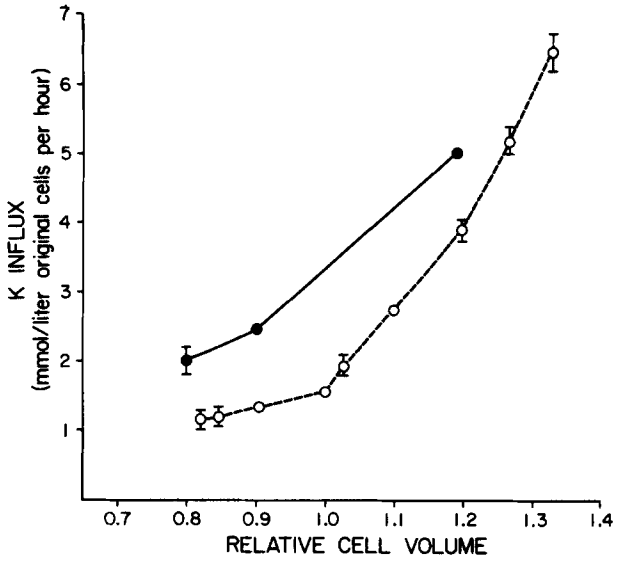

FIGURE 1. Effect of cell volume on the OR K influx in human erythrocytes. When erythrocyte volume was altered by the nystatin method (solid circles), the intracellular Na and K concentrations were left unchanged (10 ± 2 and 132 ± 4 mmol/liter cell water, respectively). K influx in these cells was measured in a medium containing 120 mM NaCl and 30 mM KCl, osmolality 298 ± 2 mosmol/kg. When cell volume was altered by the osmotic method (open circles), cells were incubated in a medium containing 70 mM NaCl, 30 mM KCl, and 0–200 mM sucrose added to adjust the osmolality to between 200 and 400 mosmol/kg. All media contained 0.3 mM ouabain, 6.7 mM glucose, and 6.7 mM Tris·MOPS, pH 7.4 at 37°C. Mean ± SD from four experiments on the same donor. When the SD was smaller than the symbol, the error bar was omitted.

concentration of external K, the hypotonic swelling of cells resulted in a marked augmentation of K influx, whereas shrinking resulted in a smaller but statistically significant decrease in K influx (Table I). Furthermore, the replacement of external K with Rb did not influence the volume dependence of transport (not shown).

Effect of NaCl or Sucrose Addition

The response of OR K influx to cell volume in anisotonic media may be the result of changes in the Cl ratio (internal Cl/external Cl). To examine the role

TABLE I

K Influx

	K influx		
		mmol/liter cells·h	
Relative cell volume	1.20±0.03	0.99±0.02	0.85±0.03
Donor 1 (n = 6)	1.394±0.051	0.630±0.003	0.540±0.021
Donor 2 (n = 6)	1.284±0.064	0.600±0.021	0.567±0.021

Cells were washed and incubated in media containing 95 mM NaCl, 5 mM KCl, 0.3 mM ouabain, 6.7 mM glucose, 6.7 mM Tris·MOPS, pH 7.4 at 37°C with 0, 100, or 200 mM sucrose to vary the osmolality between 200 and 400 mosmol/kg. Cell volume was altered by the osmotic method. In each case, K influx in swollen cells was significantly higher than K influx in cells at normal volume ($p < 0.001$), and K influx in shrunken cells was significantly decreased compared with the transport in normal-volume cells ($p < 0.001$).

of alterations in the Cl ratio (and therefore membrane potential and cell pH), the medium osmolality was varied by the addition of either sucrose or more NaCl to hypotonic media containing 70 mM NaCl and 30 mM KCl. The addition of sucrose to hypotonic media would increase the ratio of internal to external Cl (depolarization), whereas NaCl addition would result in an increase in both external and internal Cl and a small fall in the Cl ratio (slight hyperpolarization) because of the nonideal solute properties of hemoglobin (Freedman and Hoffman, 1979; Dunham and Ellory, 1981). At constant external pH, the increase in the Cl ratio with sucrose would also lead to alkalinization of the cell interior relative to cells in which more NaCl was added to increase medium osmolality. Fig. 2 shows that similar changes in K influx were observed with the sucrose and NaCl methods, which suggests that changes in membrane potential or cell pH did not play a major role in the alteration of K transport with volume changes or in its activation.

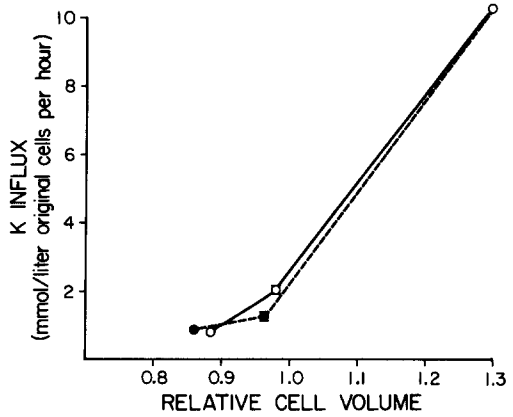

FIGURE 2. OR K influx in human erythrocytes with volume altered by the NaCl or the sucrose method. Cell volume was varied by addition of either NaCl (0–100 mM) or sucrose (0–200 mM) to a standard hypotonic medium containing 70 mM NaCl, 30 mM KCl, 0.3 mM ouabain, 6.7 mM glucose, and 6.7 mM Tris·MOPS, pH 7.4 at 37°C. The SD was smaller than the symbols in all cases (n = 6).

Anion Dependence

To examine the anion dependence of volume-sensitive K influx, K influx was measured as a function of relative cell volume in Cl and in NO_3 media. While swelling induced a dramatic increase in OR K influx in Cl media, similar cell swelling in NO_3 media resulted in only a slight (but statistically significant) increase in K influx (Fig. 3). This finding suggested that Cl may be required for the activation process or the transport function of the volume-sensitive K transport pathway. Alternatively, NO_3 may have exerted a specific inhibitory effect on this transport pathway.

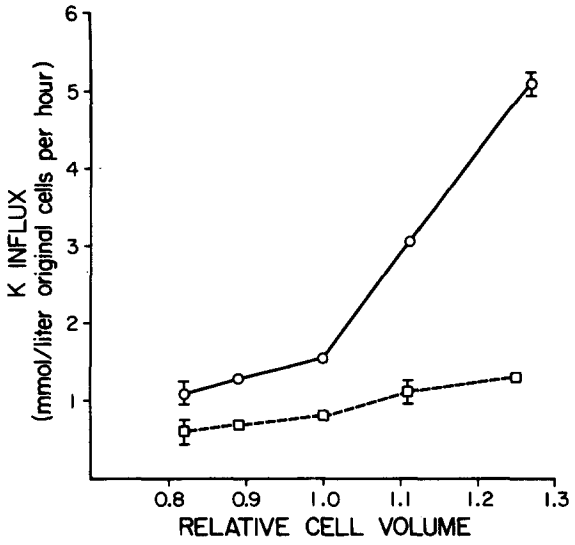

FIGURE 3. OR K influx in human erythrocytes as a function of relative cell volume in Cl (circles) or NO_3 (squares) media. Cell volume was altered by the osmotic method. The Cl media contained 70 mM NaCl, 30 mM KCl, and 0–200 mM sucrose. The nitrate media contained 70 mM $NaNO_3$, 30 mM KNO_3, and 0–200 mM sucrose. All media contained 0.3 mM ouabain, 6.7 mM glucose, and 6.7 mM Tris·MOPS, pH 7.4 at 37°C. Mean ± SD (n = 2).

To examine the anion specificity of volume-sensitive cation transport, K influx was measured in cells that were swollen and anion-equilibrated in hypotonic media. The swelling-activated K influx was found to be specific in its anion requirement for Br or Cl, the order of anion preference being Br > Cl > SCN > I > NO_3 > $MeSO_4$ (Fig. 4).

The requirement of the volume-activated K influx for the Cl ion raised the possibility that volume-sensitive K influx may be mediated by (Na + K + 2Cl) cotransport. To evaluate this possibility, the properties of the volume-sensitive K transport pathway were examined with respect to its inhibition by bumetanide and other loop diuretic agents, its dependence on external Na, and its requirement for intracellular Na.

Effect of Loop Diuretic Agents

Bumetanide and its analogues of the 5-amino benzoic acid class of loop diuretic agents have been shown to be potent inhibitors of the (Na + K + 2 Cl) cotransport pathway in a variety of systems. When employed at concentrations at which they inhibit (Na + K + 2Cl) cotransport completely in human erythrocytes (Dunham et al., 1980), bumetanide (0.1 mM) inhibited 12–15%, MK-196 (0.2 mM) inhibited only 10%, and piretanide (1 mM) produced no inhibition of the OR K influx in swollen cells (Fig. 5), whereas furosemide (1 mM) suppressed 42% of the OR K influx.[1] This lack of inhibition of volume-sensitive K influx by bumetanide (0.1 mM) and its analogues suggested that (Na + K + 2Cl) cotransport pathway was not the mediator of the volume-sensitive K influx. An alternative but unlikely possibility is that the phenomenon of swelling altered the

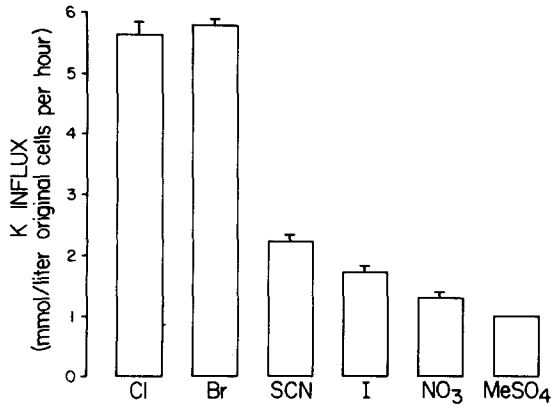

FIGURE 4. OR K influx in swollen human erythrocytes equilibrated in media with various monovalent permeant anions, all at 100 mM. Cells were swollen by incubation in hypotonic media (osmolality = 200 ± 2 mosmol/kg) containing 70 mM Na*X* and 30 mM K*X*, where *X* is the monovalent permeant anion substituted for Cl. The relative cell volume was 1.23 ± 0.02 (n = 4). The media also contained 0.3 mM ouabain, 6.7 mM glucose, and 6.7 mM Tris·MOPS, pH 7.4 at 37°C.

properties of the (Na + K + 2Cl) cotransport pathway and rendered it resistant to low concentrations of bumetanide.

In contrast to the inhibition of the (Na + K + 2Cl) cotransport with a low concentration (10^{-5} M) of bumetanide, inhibition of the putative (K + Cl) cotransport pathway in swollen avian erythrocytes and the *N*-ethylmaleimide–stimulated, putative (K + Cl) transport pathway in human erythrocytes requires a much higher concentration of this diuretic agent (McManus, T., quoted in Siebens, 1985; Lauf et al., 1984). Therefore, it was of interest to examine the effect of higher concentrations of bumetanide on K transport in swollen human

[1] The percent inhibition of the Cl-dependent component of K influx (K influx in Cl media minus K influx in NO_3 media) by these agents was greater than the percent inhibition of the OR K influx. Bumetanide inhibited 23%, MK-196 13%, and furosemide 57% of the Cl-dependent K influx. A small interindividual variation was also observed in the percent inhibition of K influx by the diuretics.

erythrocytes. Fig. 6 shows the inhibition of Cl-dependent K influx (calculated as K influx in Cl media minus K influx in NO_3 media) as a function of the bumetanide concentration. At 1 mM, bumetanide inhibited 80% of the Cl-dependent K influx in swollen cells. The bumetanide concentration required for

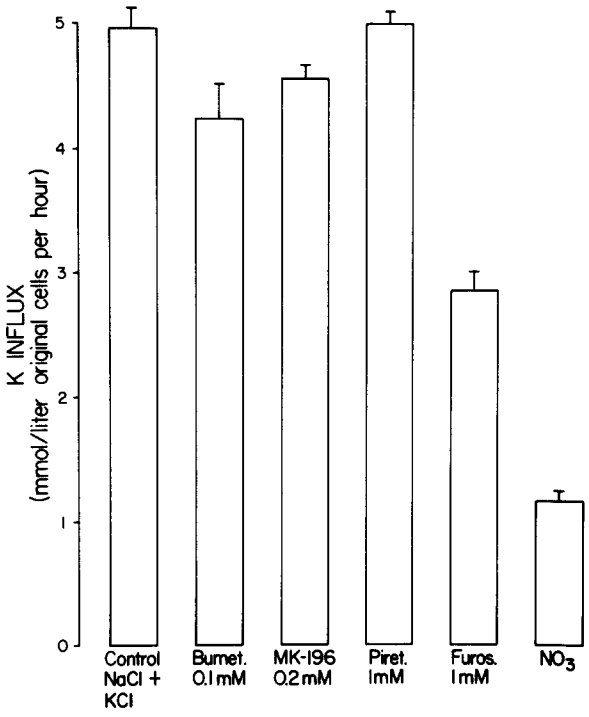

FIGURE 5. Effect of loop diuretics on OR K influx in swollen human erythrocytes. Cells were swollen by incubation in a standard hypotonic medium containing 70 mM NaCl, 30 mM KCl, 0.3 mM ouabain, 6.7 mM glucose, and 6.7 mM Tris·MOPS, pH 7.4 at 37°C. Diuretics were dissolved in a mixture of 1 M Tris and DMSO (equal parts), and the pH of the stock solution was neutralized before its addition to the hypotonic media. The abbreviations are as follows: bumet, bumetanide; MK-196, 6,7-dichloro-2-methyl-2-phenyl-1-oxoindenyloxic acid; piret, 3-*N*-pyrrolidino-4-phenoxy-5-sulfamoylbenzoic acid (piretanide); furos, furosemide. The OR K influx in the NO_3 medium (shown on the far right) was measured in cells equilibrated in the NO_3 media, where NO_3 was substituted for Cl ($n = 6$).

half-maximal inhibition of Cl-dependent K influx in swollen cells was 0.17 mM.[2] This value is comparable to 0.20 mM, the concentration of this drug required for half-maximal inhibition of *N*-ethylmaleimide–stimulated K transport in human erythrocytes (Lauf et al., 1984).

[2] In three subjects, the bumetanide concentrations required for half-maximal inhibition of Cl-dependent K influx were 0.15, 0.17, and 0.22 mM.

Effect of Replacing External Na

To examine the dependence of this transport process on external Na, K influx was measured in erythrocytes suspended in isotonic and hypotonic media with and without Na (Fig. 7). In agreement with results shown earlier (Figs. 1–4), K influx was markedly higher in hypotonic NaCl media than in isotonic NaCl media (open bars on the left, Fig. 7). The replacement of Cl by NO_3 largely abolished the response to cell swelling.

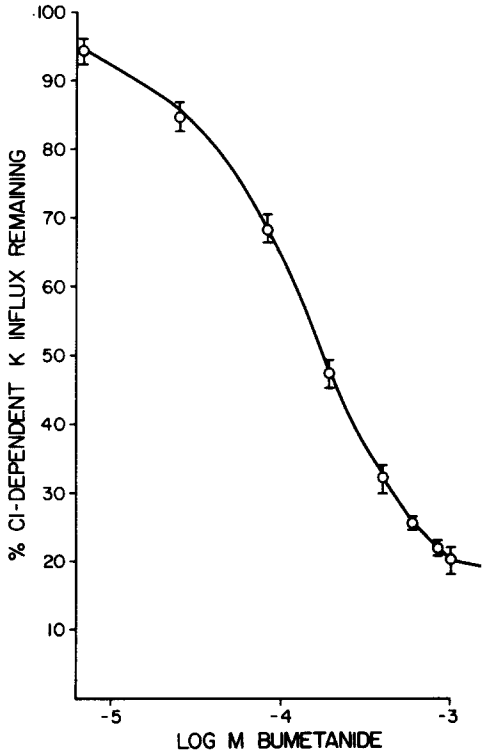

FIGURE 6. Dose-response curve for bumetanide inhibition of Cl-dependent K influx (defined as K influx in Cl media minus K influx in NO_3 media). Erythrocytes were swollen by incubation in hypotonic Cl and NO_3 media with compositions similar to those of the media described in Fig. 5. Half-maximal inhibition of Cl-dependent K influx occurred at 0.17 mM bumetanide (n = 2).

A similar increment in K influx was observed with hypotonic swelling of cells in Na-free Cl media (hatched bars on the left). Thus, swelling-activated K transport was shown to be independent of external Na. A large portion of this rise was abolished in hypotonic Na-free NO_3 media (hatched bars on the right). The latter finding was the result of the removal of Cl rather than external Na, since K influx in $NaNO_3$ media was also decreased. Thus, swelling-activated K transport was shown to be Cl dependent but independent of external Na.

In nonswollen cells, external Na (Na_o) augmented K influx in Cl media (open and hatched bars labeled "ISO" in Fig. 7). This augmentation of K influx by external Na is in agreement with previous findings (Wiley and Cooper, 1974; Wiater and Dunham, 1983; Kaji and Kahn, 1985) and has been interpreted to support the concept of Na-K cotransport. In swollen cells, the Na_o-augmented component of K influx was abolished. Indeed, external Na appeared to exert a small but significant ($p < 0.05$) inhibitory effect on K influx in swollen cells (open and hatched bars labeled "Cl"). Similar findings were observed when cell swelling was achieved by the isosmotic method (not shown).

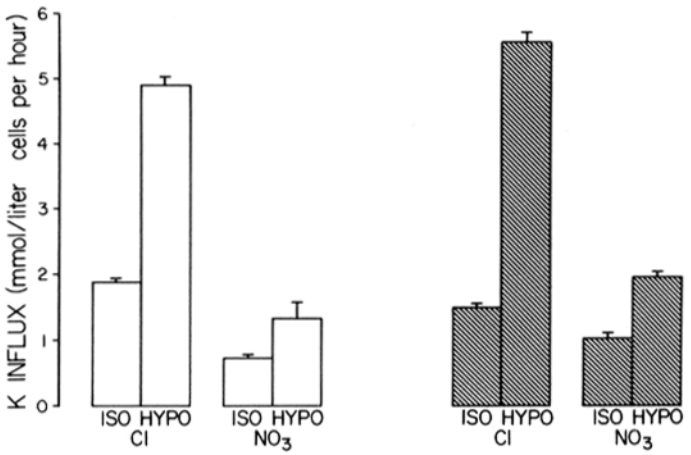

FIGURE 7. Effect of extracellular Na on OR K influx in swollen and nonswollen human erythrocytes. Erythrocytes were anion-equilibrated and incubated in isotonic (labeled "ISO") or hypotonic media (labeled "HYPO") with Cl or NO_3. The compositions of the hypotonic Na media were the same as those described in Figs. 5 and 6. The isotonic media contained 120 mM Na*X* and 30 mM K*X*, where *X* is Cl or NO_3. All media contained 0.3 mM ouabain, 10 mM glucose, 10 mM Tris·MOPS, pH 7.4 at 37°C. In media without Na (hatched bars), Na was replaced by *N*-methylglucamine ($n = 4$).

Effect of Replacement of Intracellular Na

The failure to observe inhibition of the volume-sensitive K influx upon the removal of external Na cannot be construed as definitive evidence that (Na + K + 2Cl) cotransport was not responsible for mediating the volume-sensitive K influx observed here. Both human (Canessa et al., 1986) and avian (Haas et al., 1982) erythrocytes exhibit a furosemide-sensitive K influx in the absence of external Na, which is believed to represent a partial reaction (K/K exchange mode) of the (Na + K + 2Cl) cotransport pathway. This pathway is abolished upon removal of intracellular Na (Canessa et al., 1986). Therefore, additional studies were performed in swollen cells to evaluate the effect of removal of intracellular Na on volume-sensitive K influx (Fig. 8). In this group of experiments, the nystatin method was used to swell the cells and to alter cell electrolytes,

as needed. In swollen cells without cell Na (cell K_i = 140 mmol/liter cell water, hatched bars, Fig. 8), K influx was higher than that in swollen cells with 10 mM cell Na (open bars, $p < 0.01$). The finding that volume-activated K influx was independent of both extracellular and intracellular Na strongly suggests that this transport process was not mediated by the (Na + K + 2Cl) cotransport pathway.

Effect of Transport Inhibitors

Both SITS (0.1 mM) and DIDS (0.1 mM) had only a small effect on reducing K influx in swollen human erythrocytes (Fig. 9). In contrast, DIDS has been shown to be effective in suppressing volume-sensitive K transport in toadfish erythrocytes (Lauf, 1985).

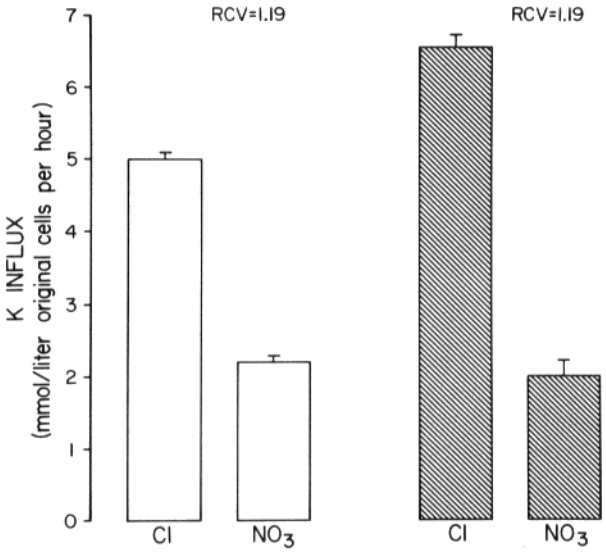

FIGURE 8. Effect of removal of intracellular Na on OR K influx in isosmotically swollen human erythrocytes. Erythrocytes were treated with the nystatin method to contain 10 mM NaCl plus either 130 mM KCl or 140 mM KCl. Swelling was achieved by varying the sucrose concentration during exposure to nystatin. After nystatin was washed off, cells were anion-equilibrated with Cl or NO_3. The incubation media for ^{86}Rb influx contained 120 mM *N*-methylglucamine Cl or NO_3 and 30 mM KCl or KNO_3. All media also contained 0.5 mM ouabain, 10 mM glucose, and 10 mM Tris·MOPS, pH 7.4 at 37°C (n = 6).

Amiloride, a known inhibitor of Na transport pathways, has recently been shown (Cala, 1983) to inhibit K/H exchange in *Amphiuma* erythrocytes, where an interconversion of Na/H and K/H exchange pathways has been proposed. Amiloride (0.1 mM) had no effect on K influx, whether it was added after swelling the cells in hypotonic media or added to shrunken cells that were later swollen in the continued presence of amiloride.

Quinine, an inhibitor of the Gardos channel (Ferreira and Lew, 1977) and of the *N*-ethylmaleimide–stimulated K transport in human erythrocytes (Ellory and Dunham, 1980), had little or no effect on K influx in swollen cells.

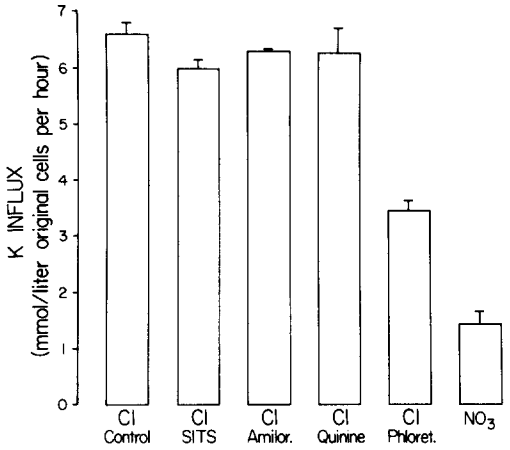

FIGURE 9. Effect of various transport inhibitors on OR K influx in swollen human erythrocytes. Cells were incubated in a hypotonic medium with a composition similar to that described in the legend to Fig. 5. Transport inhibitors were added at least 15 min before the addition of isotope and were present throughout the period of incubation (n = 3).

Phloretin, a nonspecific inhibitor of many facilitated diffusion pathways, inhibited 30% of the OR K influx in swollen cells.

Effect of Inhibitors of the Ca-activated Channel

Table II shows that various inhibitors of the Gardos channel (Ferreira and Lew, 1977) were ineffective in suppressing the OR K influx in swollen human erythrocytes. This finding makes it unlikely that the Ca-activated K channel is responsible for mediating the volume-activated K influx.

Effect of Swelling on K Efflux

The constancy of the erythrocyte volume in anisotonic media (Brugnara et al., 1985), taken together with the increase in unidirectional K influx in swollen

TABLE II

Effect of Inhibitors of the Ca-activated K Channel

Inhibitor	K influx
	mmol/liter original cells·h
None (control)	6.58±0.24
Quinine (0.5 mM)	7.21±0.24
Trifluoperazine (10^{-5} M)	7.70±0.19
Chlorpromazine (10^{-5} M)	6.70±0.95
3,3′-Dipropylthiadicarbocyanine iodide (10^{-5} M)	7.27±0.32
3,4,5-Trimethoxybenzoic acid octyl-ester (0.2 mM)	8.08±0.46

Erythrocytes were washed and incubated in the standard hypotonic flux medium described in the legend to Fig. 5. Inhibitors were added to prewarmed erythrocyte suspensions at least 15 min before the addition of ^{86}Rb. The relative cell volume for the experiment was 1.24 (n = 3).

cells, suggested the possibility that swelling may activate a Cl-dependent but tightly coupled K/K exchange process that does not mediate net K transport. The next set of experiments was therefore designed to examine the augmentation of K efflux with cell swelling in the presence and absence of external K.

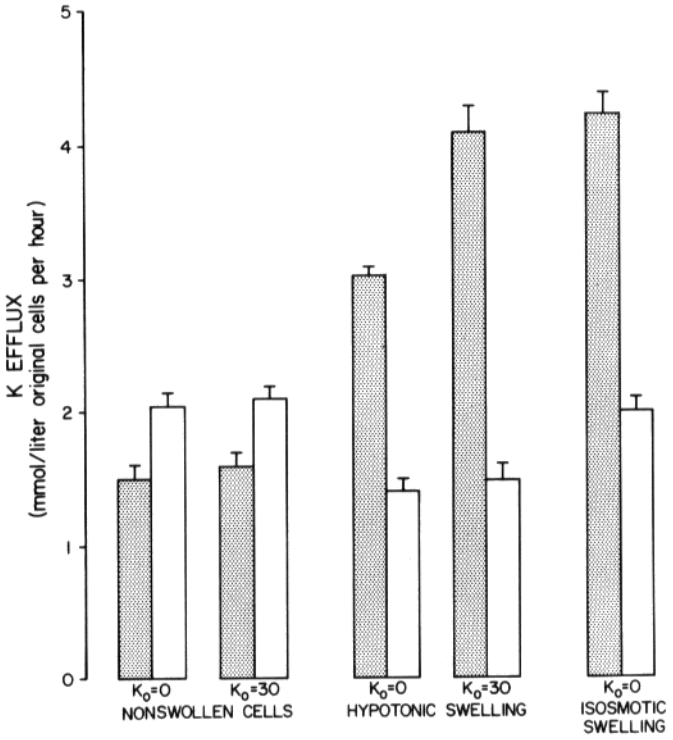

FIGURE 10. The effect of hypotonic and isosmotic cell swelling on K efflux. Cells were loaded with ^{86}Rb, swollen hypotonically or isosmotically as described in the Methods, and anion-equilibrated in Cl or NO_3 media. The nonswollen cells (left panel) were incubated in isotonic media containing 50 mM NaCl or $NaNO_3$, 0 or 30 mM KCl or KNO_3, and 100 or 70 *N*-methylglucamine Cl or NO_3, osmolality 298 ± 3 mosmol/kg. Hypotonically swollen cells (middle panel) were incubated in media containing 50 mM NaCl or $NaNO_3$, 0 or 30 mM KCl or KNO_3, and 50 or 20 *N*-methylglucamine Cl or NO_3, osmolality 200 ± 2 mosmol/kg. Isosmotically swollen cells (right panel) were incubated in media containing 50 mM NaCl or $NaNO_3$ and 100 mM *N*-methylglucamine Cl or NO_3, osmolality 300 ± 3 mosmol/kg. All media contained 0.3 mM ouabain, 6.7 mM glucose, and 6.7 mM Tris·MOPS, pH 7.4 at 37°C (n = 3).

In isotonic K-free media, K efflux was slightly higher in NO_3 as compared with Cl media (nonswollen cells, Fig. 10). The absence of a Cl-dependent K efflux from nonswollen cells in the absence of external K has also been described recently by others (Lauf et al., 1984; Haas and Schmidt, 1985). In hypotonic Cl media, K efflux was consistently enhanced even in the absence of external K.

This finding suggests that the volume-activated K transport process is capable of net efflux under zero-*trans* conditions. Whereas nonswollen cells failed to show a Cl-dependent K efflux, an appreciable Cl-dependent K efflux was observed in swollen cells. In hypotonic Cl media containing 30 mM K, a further increase in K efflux was observed as compared with the efflux in K-free media. This increment in K efflux upon addition of external K is probably due to a Cl-dependent K/K exchange. Taken together, these results suggest that the swelling-activated, Cl-dependent K transport pathway mediates both net K transport and K/K exchange.

Isosmotic cell swelling (Fig. 10) also stimulated K efflux in K-free media. The increase in K efflux was also observed in 150 mM *N*-methylglucamine Cl media (not shown) and was thus independent of external Na. Like K influx, K efflux was higher in isosmotically swollen cells (Fig. 10) than in hypotonically swollen cells, probably because of the higher cell Cl and K concentrations. Furthermore, swollen cells loaded with KCl alone without Na (nystatin method) showed an activation of Cl-dependent K efflux in media without Na or K (150 mM *N*-methylglucamine Cl alone; not shown). Thus, the properties of swelling-activated K efflux were the same as those of swelling-activated K influx.

TABLE III

OR Cl-dependent K Fluxes with Hypotonic Swelling

	K efflux	K influx	Net K efflux
	mmol/liter original cells·h		
Donor 2	2.479	1.197	1.282
Donor 3	2.065	1.196	0.869

Erythrocytes were divided into two aliquots—one for K efflux and the other for K influx. K efflux and influx were measured in hypotonically swollen cells as described in the Methods. For the composition of the medium used for K influx and efflux, see the legend to Table I (0 sucrose). All fluxes are expressed as OR Cl-dependent K fluxes. Net K efflux was calculated by subtracting K influx from K efflux. The relative cell volume was 1.22 for cells from donor 2 and 1.19 for cells from donor 3. Each value represents a mean of duplicate measurements from a single experiment.

Comparison of Swelling-activated K Influx and K Efflux

In interpreting the relationship between K influx and K efflux, it is important to stress that the experiments with K influx shown in Figs. 1–9 and those with K efflux shown in Fig. 10 were performed on erythrocytes from different donors. To compare the relative magnitudes of K/K exchange and net K flux at physiological K concentrations, OR Cl-dependent K fluxes were measured at 5 mM external K simultaneously in erythrocytes from two normal subjects. Table III shows that the OR Cl-dependent K efflux exceeded the OR Cl-dependent K influx in both donors by a small amount. Thus, the swelling-activated K transport pathway in the human erythrocytes appears to be capable of causing a small net K efflux at physiological K concentrations. However, the rate of the net K efflux is so slow ($\simeq$1 mmol/liter original cells·h) that it may not be possible to detect volume regulation by this mechanism in acute (4–6 h) studies.

DISCUSSION

The present studies demonstrate that swelling of normal human erythrocytes produces a significant increase in K influx. Earlier workers, investigating the effect of swelling on K influx, have reported either a decrease (Poznansky and Solomon, 1972) or no change in K influx (Dunham and Benjamin, 1984; Lauf et al., 1985). A combination of factors, such as lesser degrees of cell swelling (Dunham and Benjamin, 1984; Lauf et al., 1985), a lower Rb concentration (Lauf et al., 1985), and perhaps a lower activity of Cl-dependent K influx in their subjects, may have contributed to this discrepancy. In addition, one of the earlier reports (Dunham and Benjamin, 1984), showing no appreciable change in the Na-stimulated and furosemide-sensitive K influx with 12% cell swelling, is not necessarily at odds with the present findings, because the volume-sensitive K influx reported here was not stimulated by Na and was only partially inhibited by furosemide. It is noteworthy that the effect of swelling on K influx observed here was unambiguous; it was observed consistently in every subject tested and was constant in magnitude in a given subject over several months.

The sensor responsible for the rise in K transport with cell swelling has not been identified. By causing a dilution of net negative intracellular changes, hypotonic swelling may result in alterations in the Cl ratio, cell pH, and membrane potential, any of which could trigger the rise in K transport. However, the finding of a comparable alteration of K influx with the addition of NaCl and sucrose to hypotonic media (which produce directionally opposite changes in the Cl ratio [Freedman and Hoffman, 1979; Dunham and Ellory, 1981]) makes it unlikely that changes in membrane potential (and therefore cell pH), which may occur with hypotonic swelling, serve as sensors to trigger the rise in K transport. Furthermore, the volume dependence of K transport cannot be attributed to changes in the intracellular Na or K concentrations, since these parameters remained virtually unchanged during isosmotic cell swelling. Some other signal associated with the change in cell volume, such as a conformational change in the membrane or a change in the intracellular divalent cation concentration, may be responsible for the volume dependence of K transport in human erythrocytes.

The swelling of mammalian cells activates a variety of specific but different K transport pathways. The mechanism of the swelling-activated K transport in human erythrocytes was therefore explored by measuring its susceptibility to various transport inhibitors and to the effects of anion and cation substitution. The swelling of the human lymphocyte activates a conductive K transport pathway with inhibition characteristics similar to those of the Ca-activated K channel (Sarkadi et al., 1985). In contrast, swelling-activated K transport in human erythrocytes was found to be insensitive to many known inhibitors of the Gardos channel (Table II). Therefore, it is unlikely that K transport in swollen human erythrocytes is mediated by the Ca-activated K channel (Gardos, 1958).

The finding of a specific Cl requirement raised the possibility that the swelling-activated K transport process may be mediated by the (Na + K + 2Cl) cotransport pathway. However, swelling-activated K transport was relatively insensitive to low concentrations of loop diuretics (Fig. 5) and persisted in the total absence of

extracellular and intracellular Na (Figs. 7 and 8). These findings virtually exclude the possibility that the (Na + K + 2Cl) cotransport pathway was responsible for mediating volume-activated K transport. Indeed, the failure to observe an Na-augmented K influx in swollen cells suggests that swelling may inhibit the (Na + K + 2Cl) cotransport pathway.

Cell swelling has been reported to activate (K + Cl) cotransport in avian erythrocytes (Kregenow, 1974; Kregenow and Caryk, 1979; Haas and McManus, 1985). The swelling-activated, Cl-dependent K transport in sheep erythrocytes (Ellory and Dunham, 1980; Dunham and Ellory, 1981) is also postulated to be (K + Cl) cotransport (Lauf, 1985). The properties of the volume-sensitive K transport pathway, such as the specific requirement for Cl or Br, the persistence of this transport in the absence of both external and intracellular Na, and the relatively low sensitivity to bumetanide, are all consistent with the proposal that the volume-sensitive K transport seen in swollen human erythrocytes is mediated by (K + Cl) cotransport. Evidence for (K + Cl) cotransport would be stronger if Cl transport via the volume-sensitive pathway could be measured accurately. However, because of the enormous Cl fluxes via the Cl/HCO_3 exchanger, Cl transport via the volume-sensitive pathway cannot be measured. Therefore, it is not possible to draw definitive conclusions regarding the precise nature of the Cl-dependent K transport activated by cell swelling.

Swelling also activated the unidirectional efflux of K from swollen cells (Fig. 10). The increment in K efflux with isosmotic cell swelling (Fig. 10) confirms a recent report (Adragna and Tosteson, 1984) and extends it by describing the properties of the swelling-activated K efflux. Like K influx, swelling-activated K efflux is Cl dependent and independent of both external and intracellular Na, and is observed with both hypotonic and isosmotic swelling (Fig. 10). Thus, the properties of swelling-activated K efflux are similar to those of swelling-activated K influx.

The finding that unidirectional K efflux was enhanced by external K in the presence of hypotonic swelling (Fig. 10) suggests that the swelling-activated K transport pathway mediates a K/K exchange in the presence of external K. A comparison of Cl-dependent K efflux and K influx (Table III) suggests that at least 50% of the rate of unidirectional K fluxes at physiological K concentrations may represent K/K exchange.

In addition to K/K exchange, the swelling-induced K transport pathway also appears to be capable of mediating a net K efflux from swollen cells. Both the observation of Cl-dependent K efflux in the absence of external K (Fig. 10) and the finding that Cl-dependent K efflux exceeded K influx at 5 mM K (Table III) support this conclusion. The capacity of the swelling-induced K transport pathway to mediate net K efflux from swollen cells suggests that this pathway may contribute to volume regulation. The apparent inability of the human erythrocyte to regulate volume in short (4–6 h) experiments (Brugnara et al., 1985) may be due to the predominance of K/K exchange, the relatively slow rate of net K efflux at physiological K concentrations (Table III), the counteracting influence of swelling on other Na or K transport pathways, or a combination of these factors.

In summary, hypotonic or isotonic swelling of normal human erythrocytes results in a consistent and striking increase in K influx, whereas shrinking the cell produces a small but statistically significant decrease in K influx. Both swelling-activated unidirectional K efflux and K influx exhibit similar properties, including a specific requirement for Cl or Br, independence from extracellular and intracellular Na, and activation by hypotonic and isosmotic swelling. The Cl-dependent K transport pathway is capable of mediating both net cation transport and K/K exchange and appears to mediate a small net K efflux at physiological K concentrations.

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