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Furosemide-sensitive Na^+ and K^+ transport and human erythrocyte volume

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The relationship between cation transport and cell volume in human erythrocytes was investigated by measuring ouabain-sensitive K^+ influx, ouabain-resistant, furosemide-sensitive K^+ influx, and ouabain + furosemide-resistant K^+ influx, and maximal ouabain binding in microcytic, normocytic and macrocytic red cells. A significant correlation was found between the mean corpuscular volume and furosemide-sensitive K^+ influx normalized either to cell number ($r = 0.636$, $P < 0.001$) or to cell volume ($r = 0.488$, $P < 0.001$). No relationship was seen between mean corpuscular volume and ouabain-sensitive K^+ influx, and the number of ouabain-binding sites per cell was only weakly correlated with mean corpuscular volume ($r = 0.337$, $P < 0.05$). A slight, negative relationship existed between mean corpuscular volume and ouabain + furosemide-resistant K^+ influx expressed per volume of cells ($r = -0.359$, $P < 0.01$), and an apparent relationship between furosemide-sensitive K^+ influx and mean corpuscular hemoglobin concentration ($r = 0.446$, $P < 0.01$) disappeared when microcytic samples were excluded from analysis. Furosemide-sensitive transport, including Na^+ influx and K^+ and Na^+ efflux, was completely absent in microcytic cells from one patient with α-thalassemia minor. In addition, these cells exhibited a furosemide-resistant, Cl^--dependent K^+ influx. Exposure of normal erythrocytes to hypotonic conditions (196 mosM) increased furosemide-sensitive K^+ influx by a mean of 45% ($P < 0.05$), while exposure to hypertonic conditions (386 mosM) had no significant effect. The results indicate that furosemide-sensitive transport and cell volume are interrelated in human erythrocytes. However, the inability to fully recreate this relationship with in vitro manipulation of cell volume suggests that this relationship is established prior to red cell maturation.

Introduction

Involvement of the different monovalent cation transport processes in the regulation of cell volume varies widely among different tissues and is not completely understood in most of them. Ouabain-sensitive (Na/K pump-mediated) fluxes [1], ouabain-resistant, furosemide-sensitive fluxes [2–5], and other, ouabain- and furosemide-resistant fluxes [6,7] have all been implicated in a variety of cells. Particular attention has been focused on furosemide-sensitive fluxes, which represent Cl^--dependent K^+ and Na^+ transport [8,9]. Because these fluxes are commonly termed Na/K/Cl and K/Cl cotransport, these terms will be used here, although cotransport is not proven in human red cells and is therefore hypothetical. Studies with avian [2], dog [4] and rat [5] erythrocytes have demonstrated an important relationship between cotransport and cell volume. In these studies, erythrocytes exhibited marked changes in cotransport activity when cell volume was altered,

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and, in avian erythrocytes, volume increased when cotransport activity was stimulated by adrenergic agents [10].

Similar relationships are not as apparent in human red cells. Duhm and Gobel [11] found a marked increase in furosemide-resistant, but not furosemide-sensitive, K^+ influx with cell swelling and no change in either flux with cell shrinkage. Adragna and Tosteson [12] demonstrated a reduction in furosemide-sensitive Na^+ efflux and no change in furosemide-sensitive K^+ efflux with isosmotic, but not osmotic, cell swelling, and an increase in furosemide-sensitive Na^+ efflux with isosmotic shrinkage. Lauf et al. [13] have reported no change in basal, furosemide-sensitive K^+ flux with volume change but did demonstrate a direct relationship between volume change and an *N*-ethylmaleimide-stimulated K/Cl flux which is relatively resistant to furosemide. A similarly volume-stimulated, bumetanide-resistant K/Cl transport has been recently shown in hemoglobin S and hemoglobin C erythrocytes [14]. Slight increases in volume have been observed in red cells treated with furosemide [15].

In an attempt to clarify the relationships between cation transport and human erythrocyte volume in vivo, we have measured ouabain-sensitive, furosemide-sensitive, and ouabain + furosemide-resistant K^+ influx in microcytic, normocytic and macrocytic human erythrocytes. The relationship between cotransport and cell volume was further investigated by measuring transport in cells exposed to hypotonic and hypertonic media. The results demonstrate that cotransport and cell volume are interrelated in human erythrocytes. An additional finding was the complete absence of Na/K cotransport and the presence of a furosemide-resistant Cl^--dependent K^+ influx in one sample of microcytic cells.

Methods

Erythrocyte samples. Blood samples from 58 patients were obtained at random from the Clinical Pathology and Hematology Laboratories of the New England Medical Center. Mean corpuscular volume and mean corpuscular hemoglobin concentration were determined in these laboratories with either a Coulter S +4 or a Coulter S 880 cell analyzer. The mean corpuscular volume was measured directly in these analyzers and the mean corpuscular hemoglobin concentration was derived from the mean corpuscular volume and the hemoglobin concentration. Samples were stored at room temperature and transport studies were performed within 6 h of venipuncture, a period of time that was shown in initial experiments not to affect K^+ fluxes or intracellular Na or K content.

K^+ influx. ^{86}Rb, a K analog, was used to measure K^+ influx. Cells were centrifuged, washed twice in phosphate-buffered saline (145 mM Na, 4.2 mM K, 142 mM Cl, 5.0 mM glucose and 9.5 mM phosphate, pH 7.4) to remove the buffy coat and plasma, and resuspended in phosphate-buffered saline to a hematocrit of 50%, all performed at room temperature. Aliquots of the cell suspension (50 μl) were preincubated in the appropriate buffer for 15 min at 37°C in plastic microfuge tubes, after which 0.1 μCi of $^{86}RbCl$ (New England Nuclear) was added. The final incubation volume was 0.25 ml and the final concentrations of the various constituents were as stated above for phosphate-buffered saline with the addition of 1 mM $MgCl_2$ and 1 mM $CaCl_2$. At the end of the incubation (usually 1 h), 1.0 ml of ice-cold phosphate-buffered saline was added and the samples were centrifuged for 10 s in a Beckman microfuge, followed by two more rinses in ice-cold phosphate-buffered saline. In this assay, all components of K^+ influx were linear for over 1 h.

The pellets were resuspended in 0.3 ml of 5% trichloroacetic acid, centrifuged, and the supernatants were removed for counting by liquid scintillation. K^+ influx was determined by dividing the counts extracted from the pellet by the total counts added to the incubation, and multiplying by the total amount of K^+ in the incubation medium. Ouabain-sensitive influx was the difference between influx in the presence and that in the absence of 0.1 mM ouabain. Furosemide-sensitive influx was that influx in the presence of 0.1 mM ouabain which was inhibited by 1.0 mM furosemide.

In experiments with hypotonic and hypertonic media, the concentrations of the various constituents were: Na 97 mM, K 4.2 mM, Mg 1.0 mM, Ca 1.0 mM, Cl 97 mM, phosphate (pH 7.4)

6.3 mM, glucose 3.3 mM, and sucrose 0, 90 or 180 mM. Digitoxin, 0.02 mM, was used in place of ouabain. At this concentration, digitoxin inhibits the Na/K pump instantaneously, eliminating the need for preincubation of the cells prior to the addition of the ^{86}Rb [16].

Na^+ influx. Na^+ influx was measured with ^{22}Na (New England Nuclear) using the procedure described above for K^+ influx, except that the final hematocrit during incubation was 25% and the cells were washed with ice-cold 110 mM $MgCl_2$ to terminate the uptake. $MgCl_2$ was used to prevent efflux of ^{22}Na through either the Na/K pump or Na/Na countertransport.

Na^+ and K^+ efflux. The intracellular concentrations of Na^+ and K^+ were adjusted to 70 mM using nystatin [17]. One ml of red cells was incubated at 4°C for 20 min in 5 ml of 70 mM NaCl, 70 mM KCl, 50 mM sucrose, 56 μg/ml nystatin (Squibb), and 10 mM Tris (pH 7.4 at 4°C). After centrifugation, fresh solution was added to the cells for an additional 10 min incubation. The cells were then washed four times at 37°C in the same solution containing, in addition, 10 mM glucose, 0.1% fatty acid-free albumin, and 1 mM potassium phosphate buffer (pH 7.4). This was followed by four washes at 4°C in 149 mM choline chloride, 1 mM $mgCl_2$, and 10 mM Tris (pH 7.4 at 4°C). The cells were then suspended, at a hematocrit of 4%, in 149 mM choline chloride, 1 mM $MgCl_2$, 10 mM glucose, 0.1 mM ouabain and 10 mM Tris (pH 7.4 at 37°C), and the efflux of Na^+ and K^+ at 37°C was measured by the appearance of Na and K in the medium, using atomic absorption spectrophotometry.

Intracellular Na^+ and K^+. Cells were washed three times in ice-cold 110 mM $MgCl_2$, lysed in deionized water, dried, and resuspended in deionized water. Na^+ and K^+ concentrations were determined by atomic absorption spectrophotometry.

Ouabain binding. The method of Schmalzing et al. [18] was employed, using 25 μl of cells, 225 μl of Tris-buffered saline, 100 nM [^{3}H]ouabain (18.0–20.9 Ci/mmol, New England Nuclear), and a 4-h incubation at 37°C.

All data are expressed as the means of triplicate measurements. Best line fits and correlation coefficients were derived by the method of least squares and significance was determined by Student's *t* test.

Results

K^+ transport, ouabain binding and intracellular electrolyte concentrations were measured in a total of 58 samples. In these samples, the mean corpuscular volume ranged from 60.0 to 126 fl, with 19 samples below 80 fl and 17 samples above 100 fl. The microcytic samples included 4 patients with thalassemia minor, 3 with hemoglobin H disease, 2 with iron deficiency, 4 with polycythemia vera, and 6 in whom the diagnosis was either unknown or unavailable. Diagnoses were not available for many of the macrocytic samples. Macrocytosis was the result of hemolytic anemia in two patients and the result of antineoplastic drug therapy in seven others.

A strong correlation was found between mean corpuscular volume and furosemide-sensitive K^+ influx ($r = 0.636$, $P < 0.001$), which is shown in Fig. 1. The best line fit for this correlation has an *x*-intercept that is significantly greater than zero (49.7 fl, 95% confidence limits: 29.9–59.8), and the correlation persisted when influx was expressed per volume, rather than number, of cells ($r = 0.488$, $P < 0.001$), indicating that this relationship be-

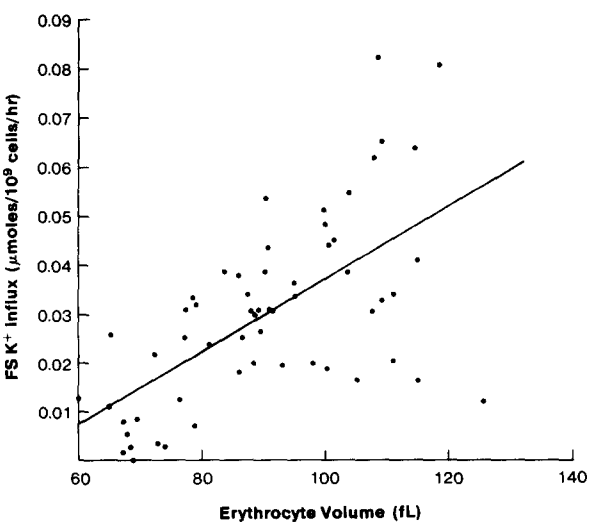

Fig. 1. Furosemide-sensitive (FS) K^+ influx as a function of cell volume in vivo in human erythrocytes. Influx was measured as ^{86}Rb uptake in isotonic, phosphate-buffered saline containing 4.2 mM K^+.

tween cell volume and furosemide-sensitive K^+ influx is not merely the result of a relationship between cell volume and surface area. Weaker correlations existed between mean corpuscular volume and other transport modes. A relationship between ouabain binding, a measure of the number of Na/K pump units per cell (expressed as pmol/10^9 cells), and mean corpuscular volume (slope = 0.0056, x-intercept = 16 fl; $r = 0.337$, $P < 0.05$) disappeared when ouabain binding was expressed per volume rather than number of cells, and no correlation was found between mean corpuscular volume and ouabain-sensitive K^+ influx. A weak, negative correlation was seen between mean corpuscular volume and ouabain + furosemide-resistant K^+ influx (slope = $6.7 \cdot 10^{-4}$, y-intercept = 0.142 μmols/h per ml; $r = -0.359$, $P < 0.01$), but only when influx was expressed per volume of cells. No significant relationship existed between mean corpuscular volume and intracellular Na^+ ($r = -0.27$, $P > 0.1$) or K^+ ($r = 0.01$, $P > 0.5$) concentrations, expressed as mmol/l cells. Furosemide-sensitive K^+ influx was also positively correlated with mean corpuscular hemoglobin concentration (slope = 0.052, x-intercept = 26.5 g/l; $r = 0.446$, $P < 0.01$), but this correlation was weak and no longer significant after microcytic samples were omitted ($r = 0.257$, $P < 0.1$), which suggests that this relationship was merely the result of the hypochromia that was present in many of the microcytic samples.

Microcytic cells from one patient with α-thalassemia minor consistently exhibited no furosemide-sensitive K^+ or Na^+ influx. To determine whether this was due to absence of cotransport activity or to resistance to furosemide, we measured K^+ influx in the absence of Na^+ or Cl^-. For these assays choline was substituted for Na^+, and Cl^- was replaced with NO_3^-. As shown in Table I, normal cells exhibit both Na^+-dependent and Na^+-independent, Cl^--dependent K^+ influx which is inhibited by furosemide. On the other hand, the patient cells exhibited no sodium-dependent K^+ influx and a small furosemide-resistant Cl^--dependent K^+ influx, indicating both an absence of Na/K/Cl cotransport and the presence of a furosemide-resistant K/Cl cotransport. Efflux of Na^+ and K^+ was also measured in these cells, after the intracellular concentration of each cation had been adjusted to 70 mM, using nystatin, in order to maximize cotransport-mediated efflux [19]. Furosemide had no effect on the efflux of either cation (Fig. 2), demonstrating that furosemide-sensitive transport in either direction across the cell was absent in these cells. Despite the absence of cotransport, these cells had normal morphology, other than their microcytosis, and there was no clinical indication of red cell dysfunction. Ouabain-sensitive and ouabain + furosemide-resistant K^+ influx, and ouabain binding were all normal in these cells.

The correlation between cell volume and furosemide-sensitive K^+ influx was investigated further by measuring K^+ influx in normal red cells in hypotonic and hypertonic conditions (Fig. 3). Sucrose was used to vary tonicity so that the concentrations of Na^+, K^+ and Cl^- remained the same. To ensure that early effects of volume changes were not missed, K^+ influx was measured over a 30-min, rather than 60-min, period. Ex-

TABLE I

Na^+ AND Cl^- DEPENDENCE OF K^+ INFLUX IN RED CELLS FROM AN INDIVIDUAL WITH ABSENT FUROSEMIDE-SENSITIVE TRANSPORT AND FROM A NORMAL SUBJECT

Values are the means from triplicate determinations ± S.D. Na was replaced with choline in the no Na assays and Cl was replaced with NO_3 in the no Cl assays. Furosemide, when present, was at a concentration of 1 mM.

Sample	Furosemide	K^+ influx (μmol/l per h)		
		Na/K/Cl	no Na	no Cl
Normal	−	343 ±16.5	149 ±14.8	67.2±2.7
	+	48.6± 0.9	55.9± 2.1	47.8±2.8
Patient	−	84.3± 5.5	82.6± 3.4	55.1±0.5
	+	85.1± 5.1	82.6± 4.9	55.9±1.5

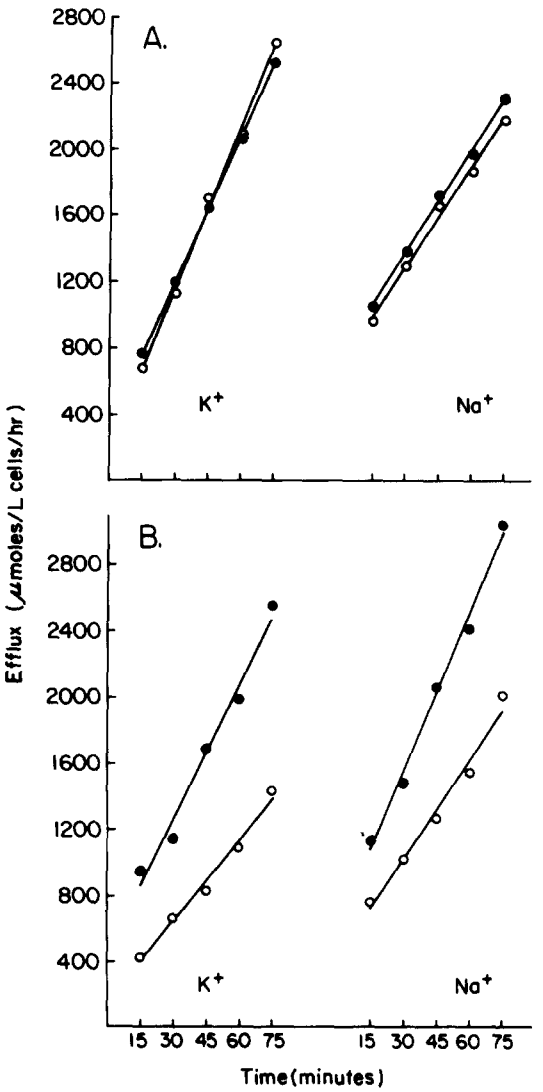

Fig. 2. Sodium and potassium efflux from normal and patient erythrocytes as a function of time. Solid circles are in the absence, and open circles are in the presence, of furosemide. A, patient; B, normal individual.

posure to a hypotonic medium increased furosemide-sensitive K^+ influx in all six samples, with a mean increase of 45% ($P < 0.05$). In the hypertonic medium, five samples showed a slight increase and one sample showed a more marked increase in furosemide-sensitive K^+ influx which, overall, was not significant ($P < 0.1$). Ouabain + furosemide-resistant K^+ also increased in the hypotonic medium (171 ± 66%, data not shown) and was unchanged in the hypertonic medium.

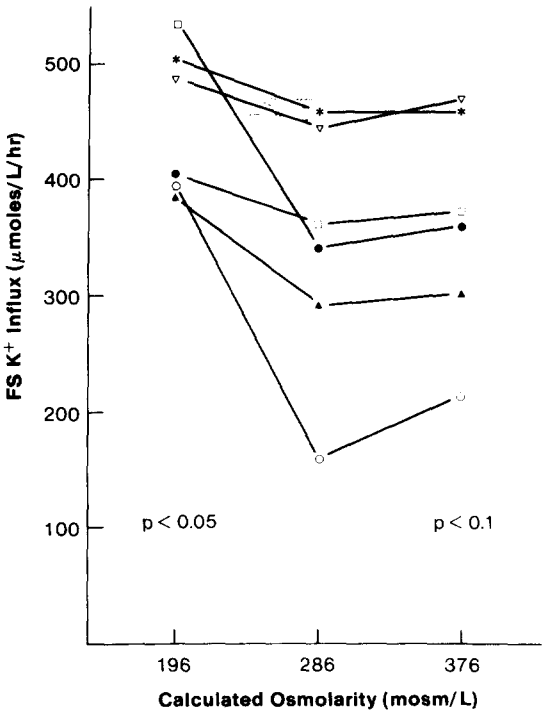

Fig. 3. Furosemide-sensitive (FS) K^+ influx in normal human erythrocytes exposed to varied osmotic conditions. Influx is expressed per original volume of cells (in isotonic medium). Symbols indicate different individuals.

Discussion

The highly significant correlation which exists between furosemide-sensitive K^+ influx and the in vivo cell volume in human erythrocytes suggests that a direct relationship exists between cotransport and erythrocyte volume. This relationship appears to be independent of other parameters which could influence cotransport activity and which could vary with cell volume, such as cell surface area and intracellular electrolyte concentrations. As there is theoretically a direct relationship between the quantity of plasma membrane and the cell volume, any process which is associated with the plasma membrane might be expected to vary with cell volume. Any correlation on this basis would extrapolate to a zero value at a volume close to zero and would disappear if transport were expressed per volume, rather than number, of cells, as is the case for the relationship between the number of Na/K pump units per cell

and cell volume. The correlation between furosemide-sensitive K^+ influx and cell volume, however, extrapolates to zero transport at a volume of 50 fl, and the correlation persists when influx is expressed per volume of cells, indicating that the relationship between cotransport activity and cell volume is independent of surface area.

The intracellular concentrations of Na^+ and K^+ can also influence cotransport activity [19], but these did not vary with mean corpuscular volume and were not significantly correlated with furosemide-sensitive K^+ influx, and cannot account for the correlation between cotransport activity and cell volume. Furosemide-sensitive K^+ influx was also related to mean corpuscular hemoglobin concentration in our samples, a finding that has been reported by Duhm and Gobel [15]. However, the correlation with mean corpuscular hemoglobin concentration is weaker than that with mean corpuscular volume, and it is no longer significant when microcytic samples are omitted suggesting that it is due to the low mean corpuscular hemoglobin concentration in many of the microcytic samples. Cotransport activity may also vary with erythrocyte age, being higher in younger, less dense cells [14]. Although reticulocyte counts were not performed in our samples, we believe that differences in cell age among the samples cannot explain our results. The microcytic samples included disorders with high (hemoglobin H disease) and low (iron deficiency) reticulocyte counts, and the macrocytic samples likewise included disorders with high (hemolytic anemia) and low (antineoplastic drug therapy) counts.

Various abnormalities in cation transport have been described in red-cell disorders [20–22] but they are not consistent with a relationship between furosemide-sensitive transport and cell volume. In addition, significant heterogeneity may exist in the circulating red cells in these disorders, possibly influencing our results. However, because our samples encompassed a wide spectrum of red-cell disorders, we believe that the relationship between cotransport and cell volume is not the result of the underlying disorders and cell heterogeneity. In fact, it is likely that these factors have other, volume-independent, effects on cotransport which may be obscuring the correlation between cotransport and cell volume.

Because cotransport can mediate a net flux of NaCl or KCl across the cell, attention has been focused on its potential role in regulating cell volume. In our studies furosemide-sensitive K^+ influx increased significantly in each of six samples of normal red cells exposed to a hypotonic medium, demonstrating that cotransport activity can be influenced by changes in cell volume. Shrinkage of the cells in hypertonic medium produced a slight increase in furosemide-sensitive K^+ influx which was not significant. These findings are consistent with the greater furosemide-sensitive K^+ influx which we have found in macrocytic cells but do not explain the reduced cotransport activity in microcytic cells. Duhm and Gobel [11] performed similar experiments and found no change in furosemide-sensitive K^+ influx with either cell shrinkage or swelling, although furosemide-resistant K^+ influx increased markedly with cell swelling. Lauf et al. [13] also found no change in cotransport with volume change, but did find a direct relationship between volume change and *N*-ethylmaleimide-stimulated, bumetanide-resistant K/Cl transport. A volume-stimulated, bumetanide-resistant K/Cl cotransport has recently been described in hemoglobin S and hemoglobin C erythrocytes [14]. Cell swelling has also been reported to decrease furosemide-sensitive Na^+ and K^+ efflux in human red cells [12].

The relationship between cell volume and cotransport has also been explored by measuring erythrocyte volume and net cation fluxes after inhibition of cotransport with furosemide. Slight increases in red cell volume after incubation with furosemide have been reported [15], while other investigators have found no changes in net Na^+ fluxes with furosemide treatment [23,24]. Cotransport therefore does not appear to control volume in human red cells and cannot explain the correlation between furosemide-sensitive K^+ influx and mean corpuscular volume which we have found.

In one of our samples, from a patient with α-thalassemia minor, furosemide-sensitive K^+ influx was absent and further investigation revealed that there was a complete absence of furosemide-sensitive Na^+ or K^+ transport in these cells. The lack of Na^+-dependent K^+ influx suggests an absence of Na/K/Cl cotransport, which has not been documented previously in human red cells.

Although several reports [25,26] contain samples which appear to lack furosemide-sensitive Na^+ or K^+ efflux, there is no indication that both fluxes were absent in the same sample and that furosemide-sensitive influx was also absent; furthermore, flux measurements in the absence of Na^+ or Cl^- were not performed. In addition to lacking Na/K cotransport, the erythrocytes from our patient also exhibited a furosemide-resistant, Cl^--dependent K^+ influx similar to that described in hemoglobin C and hemoglobin S red cells [13]. Whether this is a property of hemoglobinopathic cells in general needs to be explored. It is of interest that the cells from this patient, other than being microcytic, exhibited no other obvious structural or functional abnormalities, which suggests that Na/K/Cl cotransport has no critical function in circulating human erythrocytes.

The basis of the relationship between cotransport activity and cell volume remains to be determined. Specifically, it is not clear whether cotransport activity regulates cell volume or whether cell volume determines cotransport activity. The former is unlikely due to the fact that furosemide has little effect on red cell volume [15]. The latter is suggested by the fact that swelling of erythrocytes in hypotonic medium increases furosemide-sensitive K^+ influx. However, cell shrinkage does not reduce furosemide-sensitive K^+ influx, so that these experiments cannot fully explain the relationship between cell volume and furosemide-sensitive K^+ influx. The inability to fully recreate the correlation between furosemide-sensitive K^+ influx and cell volume with in vitro manipulations of cell volume in normal, circulating erythrocytes, and the lack of a significant effect of furosemide on the volume or cation content of normal, circulating erythrocytes [15,23,24] suggest that the cotransport-volume relationship is established during red cell development and that active coupling of these two parameters disappears upon maturation.

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