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Regulation of Human Red Cell Volume by Linked Cation Fluxes

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Summary. Osmotic volume perturbations in human red blood cells lead to specific changes in cation fluxes. When the cells are shrunken, influx of both Na and K is increased and efflux of both cations is decreased. Thus, all four fluxes react to the stress in a cooperative sense to cause a net accumulation of cations so that water enters the cell to maintain osmotic equilibrium. Our data show that this process leads to a slow return to normal volume. The linkage observed between the several fluxes cannot be explained on the basis of a simple pump-leak hypothesis, but is consonant with a mechanism of volume regulation mediated by a conformational change in a membrane protein or protein complex.

The volume of red cells is maintained essentially constant *in vivo*, yet the detailed physical process responsible for this regulation has remained obscure. Since the red cell membrane is easily distensible [11] the maintenance of cell volume cannot be attributed to the rigidity of the wall and other explanations must be sought. The first demonstration that red cell volume is related to cation permeability was given by Davson [1] who found that cat erythrocytes responded to changes in cell volume by a sharp alteration in the permeability of the cell to Na and K. Similar results were obtained by Ørskov [8] in nucleated pigeon red cells and by Parker and Hoffman [9] in dog red cells. Tosteson and Hoffman [17] showed that the pump and leak for Na and K were adjusted in sheep red cells so that volume remained constant both for high K cells and the genetically different low K strain. This established the relation between cell volume and cation flux but did not reveal either the nature of the sensor or the physical mechanism responsible.

There are numerous examples of volume regulation in other systems that have been ascribed to a control mechanism regulating cell solute content, as for example, crab muscle fibers (Shaw [14]; Lang and Gainer [7]),

flounder red cells (Fugelli [2]) and lobster nerve axons (Grundfest [4]). Rosenberg, Shank and Gregg [13] have studied the mechanism of volume regulation in a large number of mammalian cells and implicated the Na pump. However, no unidirectional fluxes were measured in any of these systems.

In red cells, cation fluxes have been measured both into and out of the cells. In the nucleated red cells of the duck, Kregenow [5, 6] has shown that sudden changes in cell volume lead to an alteration in Na and K fluxes resulting in a return to initial cell volume with a half time of about 1 hr. Simultaneously and independently, Poznansky and Solomon [10] demonstrated that K influx into human red cells is a function of cell volume, an observation which had hitherto been confined to the low K cells of cat and dog, among nonnucleated mammalian red cells. They suggested that the volume-dependent flux changes were caused by a conformational change in the transport mechanism and pointed out that this process could represent a mechanism for regulating cell volume. The present paper is concerned with the dependence of the influx and efflux of both Na and K on human red cell volume. Following a small perturbation in human red cell volume, all four fluxes are changed in a direction which leads to return to normal volume. There is a close linkage between flux changes in the same spatial direction, independent of the direction of the electrochemical potential gradient. This close relationship between fluxes in the same direction is not consistent with the operation of a pump-leak system in the usual sense, but rather fits the concept of a conformation-dependent mechanism which regulates ion transport in both directions across the membrane.

Materials and Methods

Blood was drawn from healthy young adults (heparin 10,000 units/liter blood), plasma and buffy coat were removed following centrifugation, and the cells were suspended in a buffer of the following composition (in mM): $MgCl_2$ 1.2, $CaCl_2$ 2.4, Na_2HPO_4 1.7, NaH_2PO_4 4.2, KCl 5.0, Na_2CO_3 13.5, NaCl 117.8, glucose 10.0. A mixture of 5% CO_2 and 95% air was bubbled into the solution to bring it to pH 7.4. Osmolality was changed either by the addition of sucrose or NaCl or the removal of NaCl. Tracer experiments were carried out using either ^{42}K or ^{24}Na (Isoserve, Inc., Princeton, N.J.) according to the technique described by Poznansky and Solomon [10]. Radioactivity was measured in an automatic gamma counter (Model 4222, Nuclear-Chicago, Des Plaines, Ill.), and all counting was carried out to an accuracy of 0.5%. Na and K were measured in a flame photometer (Model 143, Instrumentation Lab., Lexington, Mass.) to an accuracy of 2% and cell concentrations were computed by difference between plasma and whole blood cation content. The hematocrits were measured by centrifugation at $3{,}000 \times g$ for 5 min and corrections for trapped plasma were made with ^{131}I-labeled albumin. The reproducibility was better than 1%. Changes in cell cation concentrations

were small, amounting to no more than 1 to 2% in 6 hr for K and 15% in 6 hr for Na. These small changes in net cation concentration account for the fact that Rosenberg *et al.* [13] did not find any volume effect on cation content in human red cells over their 2-hr period of observation. Since our measurements took 2 hr, steady-state equations may be used with reasonable accuracy, as discussed by Solomon [15].

Results and Discussion

The reversible nature of the volume perturbation was confirmed in three experiments on K influx. In these experiments, the cells were incubated in hyperosmolar buffer for 2 hr; the osmolarity of the supernatant was then returned to its original value and ^{42}K influx was measured. Control influxes were determined in the hyperosmolar cells and in normal cells exposed to isosmolar buffers. The flux increases caused by cell shrinking were found to be entirely reversible.

The cell membrane must alter its shape to induce the observed shifts in cation flux in the red cells of dog and duck as shown by Romualdez, Sha'afi, Lange and Solomon [12] and Parker and Hoffman [9] for the dog and by Kregenow [5, 6] for the duck. In these species, the volume effect cannot be ascribed either to an altered osmotic environment or to responses to the specific solute used to produce the osmotic change. The volume effect in human red cells as well is independent of solute as shown by Poznansky and Solomon [10] (*see also* footnote to Table 1).

Fig. 1 shows the dependence of Na and K influx on cell volume in one experiment typical of the six experiments whose results are given in the top

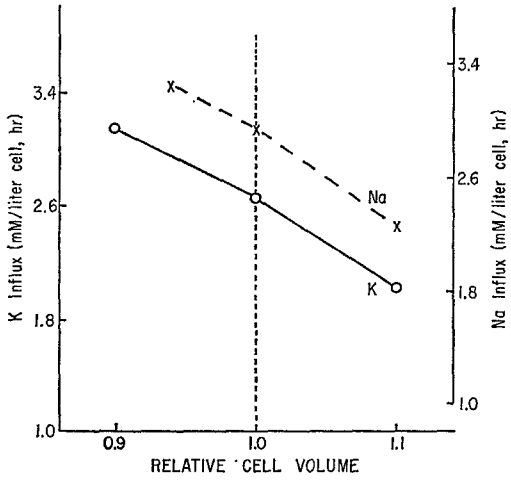

Fig. 1. Relation of K influx and Na influx in mM/liter cell, hr to the relative cell volume

Table 1. Effect of cell volume on cation fluxes

	+50 mOsm NaCl[a]	Control[b]	−50 mOsm NaCl
Approx relative cell volume	0.90	1.0	1.10
K influx[c] (mM/liter cell, hr)	3.18 (2.80−3.27)	2.52±0.12	2.03 (1.90−2.26)
Na influx (mM/liter cell, hr)	3.05 (3.00−3.20)	2.74±0.14	2.10 (2.05−2.25)
K efflux (mM/liter cell, hr)	1.84 (1.80−2.00)	2.38±0.17	2.60 (2.55−2.70)
Na efflux (mM/liter cell, hr)	1.69 (1.60−1.90)	2.40±0.18	2.68 (2.60−2.82)

[a] Cells shrunken with 50 mOsm sucrose in place of 50 mOsm NaCl exhibit essentially identical flux changes.
[b] Control fluxes are ±SD. The fluxes in swollen and shrunken cells are averages with ranges given in brackets.
[c] Fluxes are given in terms of original cell volume.

Table 2. Linkage between cation fluxes following volume perturbation[a]

Flux	Shrink approx 10%	Swell approx 10%
Na influx	+17%	−23%
K influx	+18%	−24%
Na efflux	−30%	+11%
K efflux	−25%	+9%

[a] Data from experiments illustrated in Figs. 1 and 2.

two rows of Table 1. As Table 2 shows, when cell volume is increased by about 10%, Na and K influxes are both decreased by about 23%; conversely, when the cell volume is decreased by about 10% the influxes of both cations are increased by about 17%. Thus, the water movement consequent to the change in cation influx is in the right direction to return the cells to their original volume, independent of any consideration about cation efflux.

However, changes in cell volume also produce an effect on cation efflux, as shown in Fig. 2 for one experiment typical of the three whose results are given in the bottom two rows of Table 1. Table 2 shows that an increase in cell volume by about 10% increases the efflux of both Na and K by about 10%; whereas a 10% volume decrease causes the efflux of both cations to decrease by about 27%. These efflux changes are also in the direction to restore the cell volume to its original state.

The effects are additive. When cell volume shrinks by 10%, equivalent to a 15% decrease in cell water content, the cumulative effect is an increase

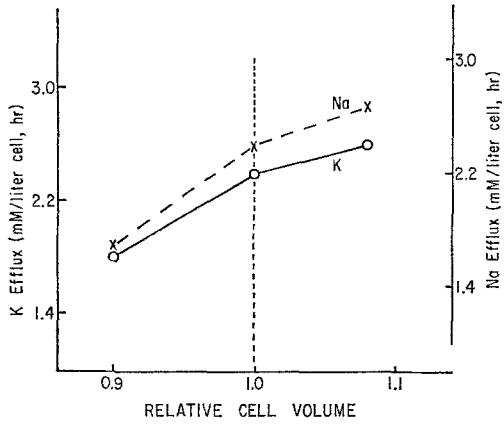

Fig. 2. Relation of K efflux and Na efflux in mM/liter cell, hr to the relative cell volume

of 43% in net K influx plus a 47% increase in net Na influx. Conversely, when the cell swells by 10%, net K influx decreases by 33% and net Na influx by 34%. Net cell cation content is 155 mM/liter cells and the sum of Na and K unidirectional fluxes in normal cells is 5.2 mM/liter cell per hr leading to an average half time exchange of 21 hr. A 45% increase in net flux, though large when looked at in terms of flux, is small from the viewpoint of changes in cation content since it would only alter the half time of cation exchange from 21 hr to 16 hr. This may be contrasted to the situation in the nucleated red cells of the duck in which the fluxes are so high that the half time of cation exchange in the cells used by Kregenow [5, 6] is 6 hr. Furthermore, comparable alterations in duck red cell volume produce larger changes in cation fluxes.

Even though the volume-induced change in cation fluxes in human red cells is small, it is possible to measure the return of human red cell volume towards its initial value following an osmotically induced volume change. We have found it difficult to maintain constant volume in control human red cells for periods much longer than 5 to 8 hr, even in the presence of bacteriostatic agents. Consequently, we have not been able to demonstrate that the cells can return all the way to their original volume. However, Fig. 3 which presents the results of one experiment, typical of the four experiments performed, shows that the volumes of both swollen and shrunken human red cells move unidirectionally towards the initial volume. Volume restoration is half complete in about 10 hr, which agrees satisfactorily with the calculated figure of 9 hr determined from an analysis of total ionic flux changes. These experiments demonstrate that changes in human red cell volume induce cation flux shifts that lead towards restoration

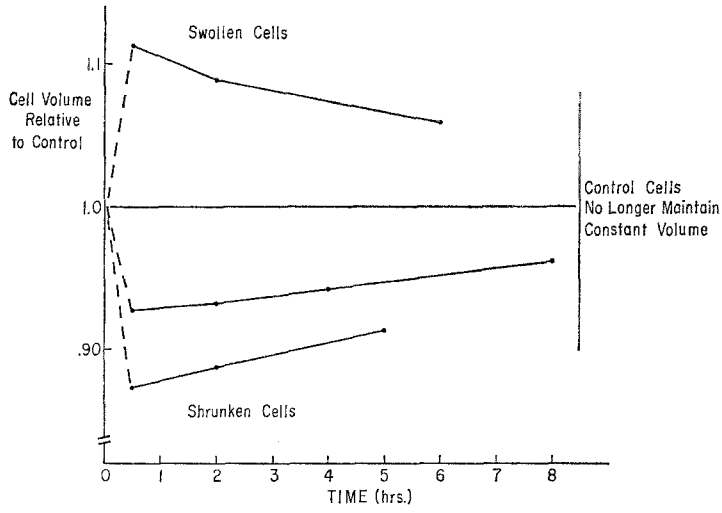

Fig. 3. Change in relative cell volume with time after initial osmotic perturbation. The cells in the upper curve were swollen by removal of 50 mOsm NaCl from the suspending medium. In the lower curves the cells were shrunken with 40 and 75 mOsm NaCl, respectively

of original cell volume in accordance with the suggestion of Poznansky and Solomon [10].

Kregenow [5, 6] has suggested that the operative mechanism is caused by changes in membrane elasticity which cause localized changes in cation permeability, whereas Poznansky and Solomon [10] have suggested that the mechanism may be ascribed to a conformational change in a membrane protein which controls cation transport. The relation with the downhill fluxes may be used to discriminate between these explanations. Localized changes in cation permeability should be reflected by similar actions on the two fluxes that go down electrochemical potential gradients. That is, increased cation permeability ought to cause an increase in both Na influx and K efflux, and a decreased cation permeability should cause a converse effect. Yet as shown in Table 2 for human red cells, shrinking which causes an *in*crease in Na influx causes a *de*crease in K efflux. There is a similar sign difference in the response of these fluxes to cell swelling. These observations argue against a mechanism dependent only on localized permeability changes. Furthermore, they are not consistent with the independent downhill leak which is an integral part of the conventional pump-leak hypothesis.

Instead, all the evidence presented in Table 2 argues for a close linkage between the fluxes of a very different sort. Each condition which causes an

increase in Na *in*flux causes a numerically similar increase in K *in*flux. Volume-induced decreases in influx are similarly linked and an exactly equivalent statement can be made about the linkage between Na and K effluxes. It is important to emphasize that this linkage is not dependent upon concentration gradients which are opposite for the two cations. Rather, it depends upon the direction of the flux. Any volume-induced increase in the inward flux of Na is accompanied by a similar increase in K influx. Such a close linkage in fluxes is consonant with regulation by a protein, or protein complex, whose conformation controls cation transport in each direction across the membrane.

This linkage between Na and K fluxes in the same direction does not fit the simple pump-leak hypothesis. According to this hypothesis, the influx of K into human red cells, an energy-requiring process, is driven by a pump whereas K efflux, primarily a downhill process, is considered to be a leak entirely independent of the pump. Cardiac glycosides have often been used to characterize the pump because they are especially effective in inhibition of the uphill K influx simultaneously with inhibition of Na efflux which is also an energy-requiring process [16], and a linkage between these two fluxes is evident in many conditions (*see* Garrahan [3]). The effects of small reversible perturbations in human red cell volume offer a striking contrast because shrinking the cells increases uphill K influx but depresses uphill Na efflux, whereas swelling has the opposite effect. These observations are not compatible with conclusions drawn from the actions of the cardiac glycoside sensitive cation pump. We cannot, however, exclude the possibility that cell volume changes induce alterations of the characteristics of the pump because Poznansky and Solomon [10] have already shown that changes in cell volume alter the dose-response curve relating K influx to cardiac glycoside concentration. However, it would seem unlikely that these small volume changes alter the pump characteristics radically. It is more likely that the gentle nature of the reversible volume perturbation reveals linkages which have hitherto not been apparent.

The present observations argue against any simple view of a pump and leak mechanism since the several processes are connected in a very subtle way. However, such subleties of behavior are entirely consistent with control by a protein, or a protein complex, as frequently exhibited in other biological systems.

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