Coordinated Regulation of Na/H Exchange and [K-Cl] Cotransport in Dog Red Cells

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ABSTRACT Swelling-activated [K-Cl] cotransport and shrinkage-activated Na/H exchange were studied in dog red cells with altered internal Mg or Li content. The two pathways responded in a coordinated fashion. When cells were depleted of Mg, [K-Cl] cotransport was stimulated and Na/H exchange was inhibited. Raising internal Mg had the opposite effect: [K-Cl] cotransport was inhibited and Na/H exchange was stimulated. Li loading, previously shown to stimulate Na/H exchange, inhibited [K-Cl] cotransport. From these reciprocal effects and from other evidence, we surmise that the regulation of Na/H exchange and [K-Cl] cotransport is conducted and coordinated by a discrete mechanism that responds to changes in cell volume and is sensitive to cytoplasmic Mg and Li concentrations.

INTRODUCTION

Dog red cells respond to perturbations of cell volume by activating transport systems that function minimally or not at all at normal cell volume. Cell shrinkage triggers Na/H exchange and cell swelling triggers both [K-Cl] cotransport and Na/Ca exchange. Na/H exchange and Na/Ca exchange are volume regulatory in that they promote net ion fluxes that correct the volume perturbation. Though activated by cell swelling, [K-Cl] cotransport is not volume regulatory in dog red cells, since the K concentration is so low that little net loss of KCl can occur via this pathway (Parker et al., 1975; Parker, 1983*a*, *b*, *c*; Parker and Castranova, 1984).

Volume-sensitive transporters have been described in many other cell types, but the mechanisms by which ion transport pathways become activated or deactivated in response to alterations of cell water content are not understood (Chamberlin and Strange, 1989). Work in duck red cells has suggested that a shrinkage-activated pathway ([Na-K-2Cl] cotransport) and a swelling-activated pathway ([K-Cl] cotransport) are closely coordinated (Haas and McManus, 1985; Starke and McManus, 1990), but whether the interplay between the two pathways is mediated by an

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alteration in transporter specificity or a separate mechanism has been difficult to assess. In the present study we show that coordinated shifts of volume-activated transport are also seen in dog red cells, where the transport pathways—[K-Cl] cotransport and Na/H exchange—carry no ions in common. Coordination between the shrinkage-activated transporter and the swelling-activated transporter must therefore involve a common regulatory mechanism.

METHODS

Venous blood from healthy mongrel dogs was drawn into heparinized vacuum tubes within 15 min of the start of each experiment. After centrifugation the plasma and buffy coat were discarded and the cells were washed twice with isotonic, buffered NaCl (Cell wash, Table I).

Media

Table I lists preincubation, incubation, and wash media.

TABLE I

Wash and Incubation Media (Concentrations in Millimolar)

	NaCl	KCl	$MgSO_4$	EDTA	NMDGCl*
			mM		
Cell wash	140	—	—	—	—
Preincubation and albumin wash	110–180	—	0–3	0–0.5	—
Flux	90–210	4	—	—	0–120

All solutions contained 5 mM glucose and were buffered with 10 mM HEPES adjusted to pH 7.4 at 37°C by titration with Tris base. Before the flux determination, the cells used in the experiments depicted in Fig. 7 were preincubated with 200 mM LiCl that had been buffered to pH 7.4 with HEPES.
*N-Methyl-*d*-glucamine hydrochloride.

Alteration of Cytosolic Mg

Cell Mg was altered by a modification of the procedures of Flatman and Lew (1980) and Flatman (1988). Cells, washed twice in preincubation media containing either 0.5 mM EDTA or appropriate concentrations of $MgSO_4$, were resuspended at a cell/medium ratio of 0.03. A stock solution of 0.5 mM A23187 in dimethylsulfoxide was added to yield a final ionophore concentration of 2.5 μM. After incubation for 30 min at 37°C the suspension was centrifuged at 4°C and washed free of A23187 with ice-cold solutions identical to the preincubation media except for the presence of 0.5% bovine serum albumin and the absence of ionophore. Preliminary studies confirmed that the ionophore was effectively removed by washing the cells four times in 30 vol of cold albumin solution.

Flux Measurements

^{22}Na and ^{86}Rb influx were determined as previously described (Parker, 1983*a*, *b*, *c*; Parker, 1986; Parker et al., 1989). Briefly, isotope (1–2 μCi/ml) was added to a 37°C suspension in which the cell/medium ratio was 1:20. Between 0 and 30 min portions of the suspension were centrifuged at 4°C, and the cells were washed twice with cold, isotope-free flux solution (Table I) before packing at 40,000 *g* in special lucite well tubes. The cell button was weighed and extracted with perchloric acid, and the radioactivity was determined by liquid scintillation

(^{86}Rb) or gamma (^{22}Na) counting. Influx was calculated as the initial rate of isotope entry divided by the specific activity of the isotope in the bathing medium. Rb efflux was performed by the same method previously described for Na efflux (Parker, 1978): cells, loaded with ^{86}Rb by using external ATP as a reversible permeabilizing agent, were washed and incubated in isotope-free media. At 0, 20, and 40 min samples of the red cell suspension were centrifuged. The rate of appearance of radioactivity in the supernate was used to calculate a first-order efflux rate constant.

Mg and Cell pH Determinations

Total cell magnesium was assayed in red cell extracts by atomic absorption as previously described (Parker et al., 1989). Values for free cell Mg were calculated from the chloride ratio and the external Mg by the equation $[Mg]_i/[Mg]_o = ([H]_i/[H]_o)^2 = ([Cl]_o/[Cl]_i)^2$, where $[Mg]_i$, $[H]_i$, and $[Cl]_i$ refer to the intracellular concentrations (millimoles per kilogram cell water) of free Mg, Cl, and H ions, and $[Mg]_o$, $[H]_o$ and $[Cl]_o$ refer to the corresponding extracellular concentrations in millimoles per kilogram medium water (Flatman and Lew, 1980). Intracellular pH was estimated from the chloride ratio by the same equation.

Cell Water Content

Cell suspensions were placed in special lucite tubes with a well at the bottom and centrifuged at 40,000 *g* for 5 min with no brake in Sorvall RC2B centrifuges. The supernate was removed and the pellet was dried for 30 min at 90°C and 10 min in a microwave oven. A 1.2% correction was made for trapped medium (Parker, 1971).

RESULTS

Freshly drawn dog red cells suspended in their own plasma have a cell water content of 63–64% (Parker, 1973). Fig. 1 shows time courses for Na and K influx (measured with ^{22}Na and ^{86}Rb) in swollen and shrunken dog red cells, and justifies the sampling period of 30 min used to measure initial rates. Na influx is higher in shrunken cells, whereas K influx is higher in swollen cells.

Swelling-induced K flux was previously described in dog red cells (Parker and Hoffman, 1965, 1976). Its anion dependence and sensitivity to furosemide were reported in studies of net K efflux from K-loaded cells (Parker, 1983*a*). Characterization of this pathway with ^{86}Rb influx measurements demonstrated its similarity to the [K-Cl] cotransport systems found in human (Ellory and Hall, 1988), sheep (Fujise and Lauf, 1987), rabbit (Al Rohil and Jennings, 1989), and duck (Haas and McManus, 1985) red cells. Above a cell water level of 65–66% wet weight, K influx was stimulated by cell swelling (Fig. 2 *A*). Replacement of chloride by nitrate (Fig. 2 *A*), sulfamate, or methanesulfonate (not shown) inhibits swelling-induced K influx. The pathway was partially inhibited by 1 mM furosemide, an effect enhanced by increasing external K (Fig. 2 *B*), as has been previously reported for the red cells of sheep (Lauf, 1984) and duck (Lytle and McManus, 1987). We found no stimulation of Rb efflux by external K, a result that is at some variance with that of Kaji (1989) in human red cells.

Characteristics of the Na/H exchanger in dog red cells have been previously reported (Parker, 1983*c*, 1986; Parker and Castranova, 1984). Like [K-Cl] cotransport, this pathway is volume sensitive, but is stimulated by shrinkage and inhibited by

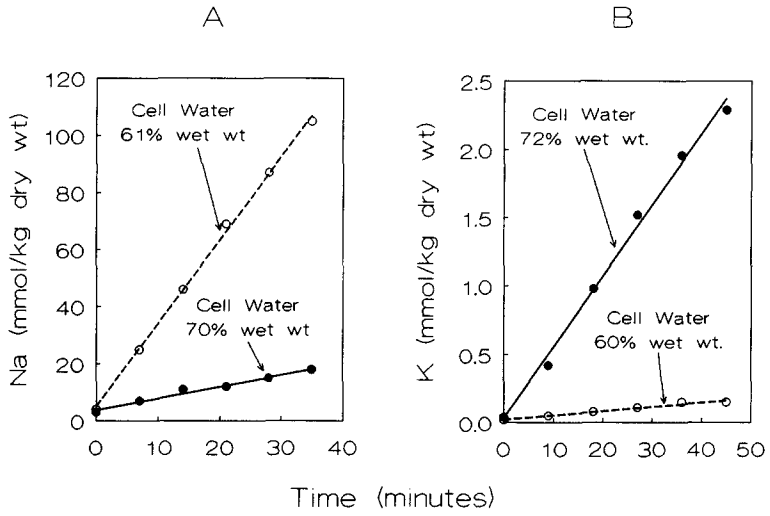

FIGURE 1. Time course of Na and K influx as measured with ^{22}Na (*A*) and ^{86}Rb (*B*) in swollen (*open circles, dashed lines*) and shrunken (*solid circles, solid lines*) cells.

swelling. Activity of Na/H exchange is minimal at cell water contents >66–67% (Parker, 1986).

Figs. 3 and 4 show data obtained from untreated cells and from cells preincubated with A23187 in media containing either EDTA or various concentrations of Mg. The external Mg concentration at which exposure to A23187 causes neither gain nor loss

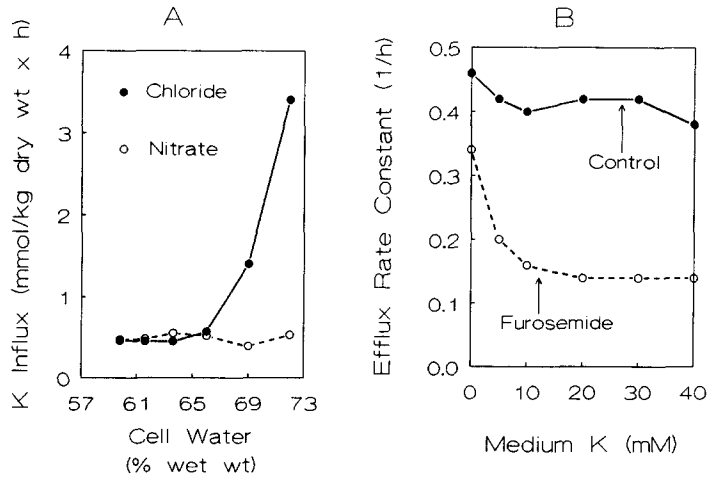

FIGURE 2. Unidirectional K flux (measured with ^{86}Rb) as a function of percent cell water, Cl replacement, and furosemide. (*A*) Effect of replacing chloride (*solid circles, solid line*) with nitrate (*open circles, dashed line*) on swelling-induced K influx. (*B*) ^{86}Rb efflux rate constant as a function of medium K concentration in the absence (*solid circles, solid line*) and presence (*open circles, dashed line*) of 1 mM furosemide. Each study is representative of at least three replicate experiments.

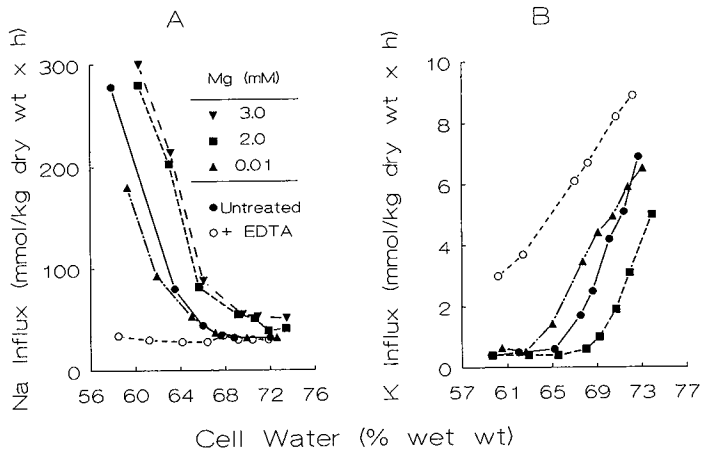

FIGURE 3. Influx of Na (*A*) and K (*B*) as a function of initial percent cell water. Solid circles and solid lines are data from fresh cells that were not preincubated. Open circles are data from cells depleted of Mg by preincubation in A23187 plus 0.5 mM EDTA. Other symbols indicate preincubation of cells with A23187 plus 0.01–3 mM Mg as noted in the figure.

of Mg from dog red cells is ~0.3 mM (Parker et al., 1989). Before measuring ion flux ionophore was eluted from the cells by washing them in ice-cold solutions containing albumin.

The experiments depicted in Fig. 3 show values for Na and K influx as a function of cell volume (percent cell water). As previously reported, preincubation of the cells with EDTA and ionophore prevented activation of Na influx by cell shrinkage

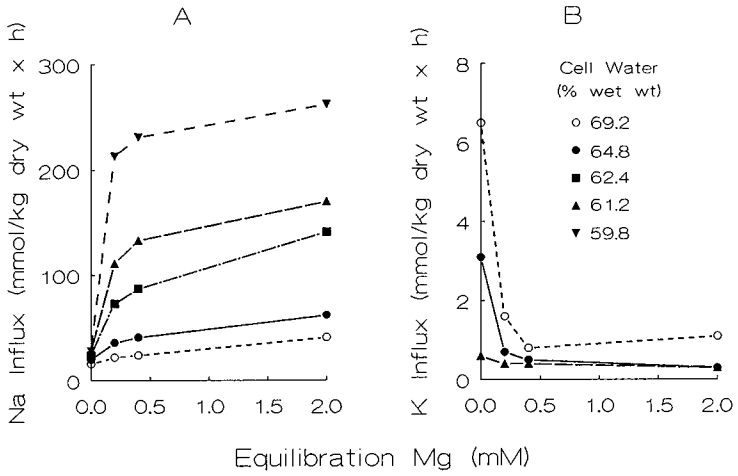

FIGURE 4. Influx of Na (*A*) and K (*B*) as a function of Mg concentration in the preincubation medium. Preincubation with magnesium/A23187, removal of ionophore, and the subsequent flux determinations were carried out in media of various tonicities, yielding cells with percent waters noted on the figure. Magnesium concentrations of the preincubation media are plotted on the abscissae. In these studies, zero Mg represents a Mg-free preincubation solution containing 0.5 mM EDTA.

(Parker et al., 1989). In all other instances, Na and K transport remained volume sensitive in cells with altered Mg content, although the relationship between cell volume and transport was changed. Na influx was diminished in shrunken cells when their Mg content was reduced by preincubation. On the other hand, raising cytosolic Mg stimulated Na influx at all but the highest cell volumes (Fig. 3 *A*). We have previously reported that the Mg-induced increment in Na influx is completely amiloride sensitive.

K influx was stimulated at all cell volumes in cells depleted of Mg by preincubation with EDTA and ionophore; raising cytosolic Mg inhibited K influx (Fig. 3 *B*). The effect of varying cell Mg on [K-Cl] cotransport was first reported by Lauf in sheep red cells (1985) and confirmed in human sickle cells by Brugnara and Tosteson (1987). Persistence of volume-sensitive [K-Cl] cotransport in Mg-depleted duck red cells was emphasized by Starke and McManus (1990).

Fig. 4 shows the effects of varying cytosolic Mg on Na and K influx in cells with normal volume, swollen in hypotonic media, or shrunken in hypertonic media. The zero Mg points in all cases represent cells preincubated with A23187 and 0.5 mM EDTA in a Mg-free medium. Na influx was greatest in shrunken cells loaded with Mg. At all volumes, increasing cell Mg stimulated Na influx, an effect that diminished as cell volume was increased (Fig. 4 *A*). K influx, on the other hand, was greatest in Mg-depleted, swollen cells. Loading cells with Mg inhibited K influx (Fig. 4 *B*).

The results in Fig. 4 could be interpreted as indicating that the degree of Mg loading was affected by the volume of the cells during preincubation with ionophore. Evidence against this view is shown in Fig. 5. Cells treated according to the procedures used in the experiments described in Fig. 4 had, after removal of the ionophore, levels of free and total Mg that were little influenced by the volume at which the Mg equilibration was carried out (Fig. 5, *A* and *B*). The water content of shrunken and swollen cells was constant over the range of Mg concentrations used in the preincubation medium (Fig. 5 *C*). The intracellular pH was slightly higher in swollen cells than shrunken ones, as might be expected from the dilution of impermeant intracellular charged solutes, but was not greatly affected by altering cell Mg (Fig. 5 *D*). Thus, in Fig 4 cell volume and Mg content can be regarded as independent variables: the effects of changing volume are not due to different degrees of Mg loading at different volumes.

The dramatic effect of including EDTA in the preincubation with ionophore (Figs. 3 and 4) raised the possibility that calcium depletion, rather than Mg depletion, might have been responsible for the large changes in transport. Against that interpretation are the data in Fig. 6. Cells were preincubated with ionophore and 0.5 mM Mg, plus graded concentrations of either EDTA or EGTA. At EDTA concentrations equal to or greater than 0.5 mM the Na flux of shrunken dog red cells was decreased, whereas EGTA had no effect (Fig. 6 *A*). Similarly, the K flux in swollen dog red cells increased with the addition of EDTA, but not EGTA to the preincubation medium (Fig. 6 *B*). Under physiological conditions at 37°C and pH 7.4 the two chelators have similar Ca affinities ($pK'Ca_{EDTA} = 7.4$, $pK'Ca_{EGTA} = 8.0$), whereas Mg is more tightly bound by EDTA ($pK'Mg_{EDTA} = 6.4$) than by EGTA ($pK'Mg_{EGTA} = 1.9$) (Durham, 1983). At 0.5 mM Mg and 1 mM EGTA, free Mg would be 0.46 mM, but in the presence of 1 mM EDTA, free Mg would be <1 μM.

Therefore, the effects on transport of treating cells with ionophore plus EDTA are due to removal of Mg, not Ca.

The observation that Mg loading brought about a concerted shift in the volume–response curves for [K-Cl] cotransport and Na/H exchange (Fig. 3) suggested that a similar effect might be seen with Li loading, previously shown to stimulate Na/H exchange at all cell volumes (Parker, 1986). Fig. 7 shows that Li-loaded cells had a

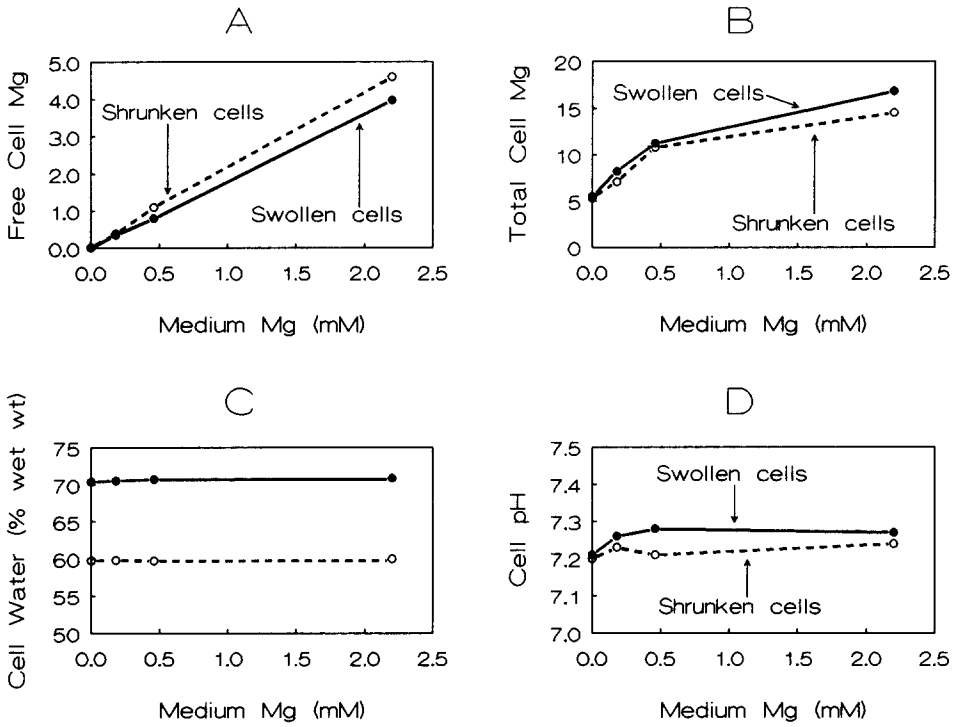

FIGURE 5. Effect of the level of Mg added to the A23187 preincubation medium on the final concentration—after washing to remove ionophore—of free (*A*) and total (*B*) Mg, percent cell water (*C*), and cell pH (*D*) in cells that were swollen (*solid circles, solid lines*) or shrunken (*open circles, dashed lines*). Free Mg in *A* is expressed in millimoles per kilogram cell water. Total Mg in *B* is expressed as millimoles per kilogram dry weight. Preincubation and cell washings were carried out at a tonicity designed to yield cells of the appropriate volume. The zero Mg point was obtained by preincubating cells in the absence of Mg, but in this case without addition of EDTA. Results are representative of four replicate experiments.

decreased K influx, although the volume sensitivity was preserved. Thus, Li loading, like Mg loading, promotes a coordinated activation of Na/H exchange and inhibition of [K-Cl] cotransport.

DISCUSSION

These experiments demonstrate a reciprocal pattern in the responses of the Na/H exchanger and the [K-Cl] cotransporter: whenever one was stimulated, the other was

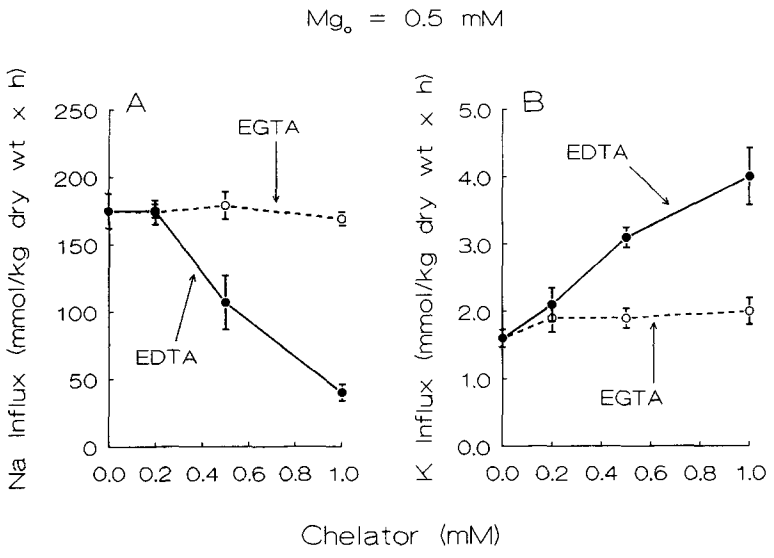

FIGURE 6. Effects of adding EDTA (*solid symbols, solid lines*) or EGTA (*open symbols, dashed lines*) to preincubation media containing 0.5 mM Mg on Na influx in shrunken cells (59.8% water) (*A*) and on K influx in swollen cells (69.0% water) (*B*). Mean ± SEM for three studies.

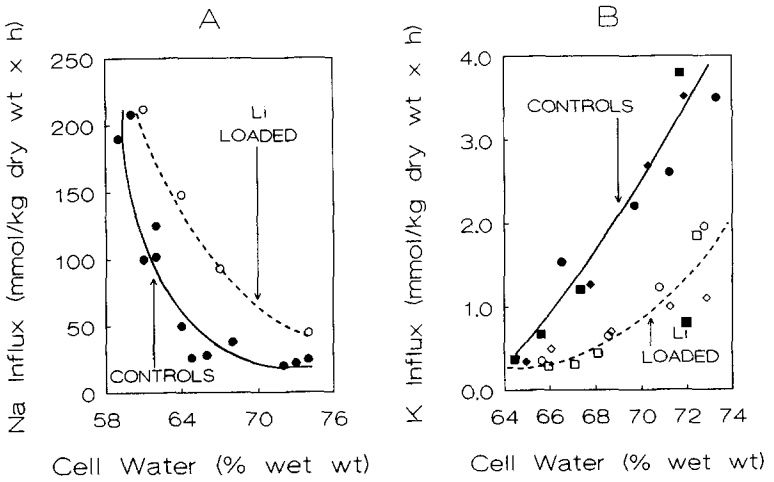

FIGURE 7. *A* is a replot of data from a previously published figure (Parker, 1986). Results for Li-loaded cells are shown in open circles and a dashed line, while controls are indicated by solid circles and a solid line. *B* shows potassium influx as a function of cell water content in control (*solid symbols*) and Li-loaded (*open symbols*) cells. Li loading was accomplished by preincubation cells for 60 min at 37°C in 200 mM LiCl (Parker, 1986) (see Table I). Controls consisted of fresh cells (*squares*), cells preincubated 60 min in 140 mM NaCl (*diamonds*), or cells preincubated for 60 min in 200 mM NaCl (*circles*). Three separate studies are plotted together. Curves were drawn by eye.

inhibited. Shrinking the cells, or raising their Mg or Li content, stimulated Na/H exchange and inhibited [K-Cl] cotransport. Swelling them, or reducing their Mg content, inhibited Na/H exchange and stimulated [K-Cl] cotransport. Assuming that Na/H exchange and [K-Cl] cotransport are carried out by different membrane proteins, do these divergent responses to volume perturbation, or alteration in Mg or Li, represent independent effects on the two transport systems? Alternatively, does the pattern of responses signify that the two transport pathways are under a common influence? Is there, for example, a separate mechanism in the cell via which the influences of cell volume, or cytosolic Mg or Li, are exerted in a coordinated fashion, so that the two transporters respond in a reciprocal manner?

There is evidence that Mg does not act on the transport process directly: Cells almost totally depleted of Mg showed no shrinkage-induced activation of Na/H exchange (Fig. 3 *A*). Nevertheless, the transporter itself is still functional in these cells, because it can be stimulated by internal acidification (Parker et al., 1989). Presumably Mg affects the activation or expression of Na/H exchange. Li also appears to act indirectly, rather than on the transport process itself (Parker, 1986). Although the stimulatory effect of Li on Na/H exchange was originally assumed to be an allosteric one, similar to the action of protons (Aronson, 1985), subsequent work has led us to reject that interpretation. Resealed ghosts made from dog red cells show no increase in Na/H exchange when either shrunken or Li loaded (unpublished observations), even though they do show activation of the pathway after internal acidification (Grinstein et al., 1987). Presumably the mechanism that responds to cell shrinkage or to increased Li is disabled by hemolysis and resealing of the ghosts, but the Na/H exchanger itself is still functional.

Anion replacement experiments offer additional evidence for a reciprocal relationship between the functional expression of shrinkage-activated and swelling-activated pathways. Replacement of chloride with nitrate or thiocyanate in dog red cells inhibits shrinkage-activated Na/H exchange and stimulates swelling-activated Ca/Na exchange (Parker, 1983*b*, *c*). Lauf (1990) has recently reported that partial replacement of chloride by nitrate or thiocyanate in low-K sheep red cells stimulates swelling-activated [K-Cl] cotransport.

Evidence that these anion replacement studies are not due to direct action on the transport process itself comes from studies of "transporter fixation." It is possible in dog red cells to "lock" Na/H exchange in an activated state by exposing shrunken cells to small concentrations of a fixative, such as glutaraldehyde or *N*-phenylmaleimide. Afterwards, if the cells are swollen they continue to behave as though shrunken. In cells fixed in this way, replacement of chloride by thiocyanate has no effect on Na/H exchange, perhaps because the anion affects an activator rather than the transport process itself (Parker, 1984; Parker and Glosson, 1987).

Volume-sensitive transport in duck red cells also shows reciprocal behavior. The pathways stimulated by shrinkage ([Na-K-2Cl] cotransport) are regulated so that as one turns off, the other turns on, whether in response to catecholamines, changes in cell volume, or manipulation of cytosolic Mg (Starke and McManus, 1990). It is possible in relation to the duck red cell system that the coordination of these responses is not due to a separate, common volume-sensitive regulator, but rather to the ability of a single, volume-sensitive pathway to carry out either [K-Cl] cotransport

or [Na-K-2Cl] cotransport, but not both at once. The experiments reported in this paper suggest that such a model is highly unlikely, at least in dog red cells, since the shrinkage-activated pathway (Na/H exchange) and the swelling-activated pathway ([K-Cl] cotransport) carry no ions in common.

Table II summarizes observations from this paper, as well as from the literature, on stimuli that exert reciprocal effects on ion transport in red cells of dogs, sheep, humans, and ducks. The various manipulations (Mg, Li, SCN, resealing, and catecholamines) appear to follow a pattern. Na/H exchange and [Na + K + 2Cl] cotransport, the two shrinkage-activated pathways, show similar responses to all the agents listed; these same agents exert precisely the opposite effects on the two swelling-induced pathways, Na/Ca exchange and [K-Cl] cotransport. These findings suggest that the responses of the shrinkage- and swelling-activated transport pathways are coordinated by a cellular system that senses cell volume.

The means by which cytoplasmic Mg and Li cause these coordinated shifts in

TABLE II

Stimuli That Elicit Coordinated, Volume-sensitive Transport Responses in Red Cells

	Shrinkage-activated transport pathways		Swelling-activated transport pathways	
	Na/H exchange	[Na + K + 2Cl] cotransport (a)	Na/Ca exchange (b)	[K-Cl] cotransport
Mg	A	A (c)	—	I
Li	A (d)	—	—	I
SCN	I (e)	—	A (f)	A (g)
Reseal	I (h)	—	A (i)	A (j)
Catecholamines	—	A (a)	—	I (a)

A, activation. *I*, inactivation. "Reseal" refers to the technique of hemolysis and resealing the red cell ghost. *a*, (duck) Haas and McManus, 1985; *b*, (dog) Parker, 1978; *c*, (duck) Starke and McManus, 1990; *d*, (dog) Parker, 1986; *e*, (dog) Parker, 1983*c*; *f*, (dog) Parker, 1983*b*; *g*, (sheep) Lauf, 1990; *h*, (dog) Parker, unpublished observations; *i*, (dog) Parker, 1989; *j*, (human) Dunham and Logue, 1986.

volume-sensitive transport are not clear. It has been suggested in relation to [K-Cl] cotransport that phosphorylation is required for volume activation, and/or that a phosphatase may also be involved (Sachs, 1988; Kracke and Dunham, 1989; Jennings and Al-Rohil, 1990), both of which might require the presence of Mg ions (Ballou and Fischer, 1986). On the other hand, Mg is not required for volume activation of [K-Cl] cotransport (Figs. 3 *B* and 4 *B*). Li is known to interact with several steps of the phosphoinositide pathway (Vickers et al., 1984). Modulation of the volume response might involve these mechanisms, but the question of how cells detect perturbations in their volume in the first place remains unresolved.

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