Swelling Activation of K-Cl Cotransport in LK Sheep Erythrocytes: a Three-State Process

PHILIP B. DUNHAM, JADWIGA KLIMCZAK, and PAUL J. LOGUE

From the Department of Biology, Syracuse University, Syracuse, New York 13244

ABSTRACT K-Cl cotransport in LK sheep erythrocytes is activated by osmotic swelling and inhibited by shrinkage. The mechanism by which changes in cell volume are transduced into changes in transport was investigated by measuring time courses of changes in transport after osmotic challenges in cells with normal and reduced Mg concentrations. When cells of normal volume and normal Mg are swollen, there is a delay of 10 min or more before the final steady-state flux is achieved, as there is for swelling activation of K-Cl cotransport in erythrocytes of other species. The delay was shown to be independent of the extent of swelling. There was also a delay after shrinkage inactivation of cotransport. Reducing cellular Mg concentration activates cotransport. Swelling of low-Mg cells activates cotransport further, but with no measurable delay. In contrast, there is a delay in shrinkage inactivation of cotransport in low-Mg cells. The results are interpreted in terms of a three-state model:

$$A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} B \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} C$$

in which A state, B state, and C state transporters have relatively slow, intermediate, and fast transport rates, respectively. Most transporters in shrunken cells with normal Mg are in the A state. Swelling converts transporters to the B state in the rate-limiting process, followed by rapid conversion to the C state. Reducing cell Mg also promotes the A → B conversion. Swelling of low-Mg cells activates transport rapidly because of the initial predominance of B state transporters. The results support the following conclusions about the rate constants of the three-state model: k_{21} is the rate constant for a Mg-promoted process that is inhibited by swelling; k_{12} is not volume sensitive. Both k_{23} and k_{32} are increased by swelling and reduced by shrinkage; they are rate constants for a single process, whereas k_{12} and k_{21} are rate constants for separate processes. Finally, the A → B conversion entails an increase in J_{max} of the transporters, and the B → C conversion entails an increase in the affinity of the transporters for K.

Address reprint requests to Dr. P. B. Dunham, Department of Biology, Syracuse University, 130 College Pl., Syracuse, NY 13244-1220.

Jadwiga Klimczak's present address is Bristol-Myers Squibb Co., Syracuse, NY.

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INTRODUCTION

Activation of K-Cl cotransport can play a role in regulation of cell volume by promoting KCl efflux and an osmotically obliged efflux of water. This has been observed in red cells of birds (Kregenow, 1971, 1981), of teleosts (Lauf, 1982), in immature mammalian red cells (Brugnara, Kopin, Bunn, and Tosteson, 1985; Lauf and Bauer, 1987; O'Neill, 1989*a*), in human red cells with abnormal hemoglobins (Berkowitz and Orringer, 1987), and in Ehrlich ascites tumor cells (Thornhill and Laris, 1984). K-Cl cotransport has also been reported in some types of epithelial cells where it plays a role in transcellular transport of salt and in some instances in water transport as well (Corcia and Armstrong, 1983; Greger and Schlatter, 1983; Reuss, 1983; Larson and Spring, 1984; Guggino, 1986; Sasaki, Ishibashi, Yoshiyama, and Shiigai, 1988; Zeuthen, 1991). K-Cl cotransport was proposed early in red cells from sheep of the LK (low $[K]_c$) phenotype, where it was observed as a Cl-dependent K flux that was particularly sensitive to osmotically induced increases in cell volume (Ellory and Dunham, 1980; Dunham and Ellory, 1981; Lauf, 1983). Cl-dependent K transport appeared to be electrically neutral (Dunham and Ellory, 1981). Brugnara, Van Ha, and Tosteson (1989) presented the first direct evidence for coupling between Cl and K fluxes in sheep and human red cells. Recently Kaji (1993) demonstrated unequivocally that K-Cl cotransport in human red cells is electroneutral.

K-Cl cotransport is not measurable in normal, mature human red cells (Duhm, 1987), but it can be induced by cell swelling (Kaji, 1986), and is present in immature human red cells (Hall and Ellory, 1986; Canessa, Fabry, Blumenfeld, and Nagel, 1987). These observations indicate that K-Cl cotransport contributes to the decrease in cell volume during the maturation of reticulocytes, and further that it is responsible in part for the abnormally low volume of mature red cells with mutant hemoglobins, HbS and HbC, cells in which K-Cl cotransport continues to function (Brugnara et al., 1985; Brugnara, Bunn, and Tosteson, 1986; Berkowitz and Orringer, 1987; Brugnara and Tosteson, 1987; Canessa et al., 1987; Olivieri, Vitoux, Galacteros, Bachir, Bloquit, Beuzard, and Brugnara, 1992). Important, unresolved questions about the K-Cl cotransporter are the nature of the signal of volume changes, the associated sensor of the signals, and the mechanism of transduction of this signal from the sensor to the transporter. For reviews on K-Cl cotransport in red cells, see Parker and Dunham (1989), Dunham (1990), and Lauf, Bauer, Adragna, Fujise, Zade-Oppen, Ryu, and Delpire (1992).

We have reported measurements of steady-state kinetics of Cl-dependent K influx in LK sheep red cells of normal and swollen volumes (Bergh, Kelley, and Dunham, 1990). We found that both the maximum velocity of transport (J_{max}) and the apparent affinity for external K, K_o ($K_{1/2}$), were increased by swelling. These two effects on kinetic constants were separable in that changes in J_{max} and $K_{1/2}$ for K_o could be induced independently. Reducing cellular Mg, Mg_c, caused an increase in J_{max} without affecting $K_{1/2}$ and swelling of low-Mg_c cells reduced $K_{1/2}$ without further effect on J_{max}. Mg appears to be an endogenous inhibitor of K-Cl cotransport since reducing intracellular Mg activity activates cotransport in both human and sheep red cells (Lauf, 1985; Brugnara and Tosteson, 1987; Sachs, 1988; Bergh et al., 1990;

Dunham, 1990, 1992; Delpire and Lauf, 1991). (Mg_c also stimulates cotransport at low concentrations in human red cells [Brugnara and Tosteson, 1987; Sachs, 1988].) Swelling may activate the J_{max} of cotransport by relieving the inhibition by Mg_c, either by simply diluting $[Mg]_c$ or by otherwise modifying binding of Mg to something.

A recent, productive approach to studying the regulation of K-Cl cotransport is the analysis of the time courses of changes in transport after volume changes. Kregenow (1971) observed a delay of 1–2 min after osmotic swelling in the increase in ^{42}K efflux (presumably K-Cl cotransport) from duck red cells. Lytle, C., and T. J. McManus (personal communication) confirmed this observation. Similar observations on K-Cl cotransport have been made in red cells from LK sheep (Lauf, 1988; Dunham, 1990, 1992), pigs (Kim, Sergeant, Forte, Sohn, and Im, 1989), humans (Kaji and Tsukitani, 1991), and dogs (Parker, Colclasure, and McManus, 1991). Delays in the activation of a transporter by volume changes are not confined to K-Cl cotransport. Delays of minutes have also been reported for swelling activation of Cl channels in T lymphocytes (Cahalan and Lewis, 1988) and for two instances of shrinkage activation of a transporter, of the Na/H exchanger in *Amphiuma* red cells (Siebens and Kregenow, 1985) and of the Na-K-Cl cotransporter in squid giant axon (Breitwieser, Altamirano, and Russell, 1990). Finally, there is a delay in the swelling inactivation of the Na/H exchanger in dog red cells (Parker et al., 1991).

A physiological significance of these delays has not been established, but they have proven useful in studying the control of K-Cl cotransport. In the first systematic study of these phenomena, Jennings and Al-Rohil (1990) measured time courses in rabbit red cells of activation of K influx with swelling and inactivation with shrinkage. There was a delay in the increase in transport after swelling, as observed by others. However, there was little or no delay in inactivation after shrinkage of swollen cells. These results were interpreted in terms of a two-state model in which transporters are in either a state with low (or zero) transport rate, A, or an activated state, B, permitting a higher flux:

$$A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} B \tag{1}$$

In cells of physiological volume, most transporters are in the A state, and activation by swelling causes an increase in the ratio B/A and in the ratio of the unimolecular rate constants, k_{12}/k_{21}. A specific protein phosphatase inhibitor, okadaic acid, slowed the rate of activation of transport after swelling (Jennings and Schulz, 1991), suggesting that k_{12} is the rate constant for dephosphorylation of a protein. (The same observation has been reported for human red cells [Kaji and Tsukitani, 1991].) Compelling arguments were presented that swelling increases k_{12}/k_{21} in rabbit cells by reducing k_{21}, not by increasing k_{12} (Jennings and Al-Rohil, 1990). It follows that the volume-sensitive process is a protein kinase for which k_{21} is the rate constant, and not the phosphatase. It was not possible to assign this kinase to any of the known classes of protein kinases (Jennings and Schulz, 1991).

In this study, we measured the time course of activation of K-Cl cotransport in red cells from sheep of the LK phenotype. The presence of a delay was confirmed. We showed further that in cells with reduced $[Mg]_c$ (in which cotransport is elevated), swelling activates transport further, but with no measurable delay. Therefore it

appears that swelling activates cotransport in two steps, a slow, Mg-dependent step followed by a rapid, Mg-independent process. These findings are consistent with our earlier results on the separable swelling-induced changes in J_{max} and $K_{1/2}$ of cotransport (Bergh et al., 1990). These results on the time courses of volume-sensitive changes in cotransport cannot be explained by a simple two-state model; a three-state model is presented that can explain them.

Preliminary reports of some of these results have been published (Dunham, 1990, 1992).

MATERIALS AND METHODS

Cells

Blood was drawn from the jugular vein into heparinized containers from Suffolk breed sheep of the LK phenotype. The sheep are maintained at the Vinzant Family Farms in Borodino, NY. The red cells were washed free of plasma and white cells by three successive, brief centrifugations and resuspension in an isotonic medium (290 mosmol/kg, measured using a vapor pressure osmometer; Wescor Inc., Logan, UT) containing: 140 mM NaCl, 10 mM KCl, 10 mM Tris-HCl, and 5 mM glucose, with pH adjusted to 7.4 at 37°C.

Influxes

Unidirectional influxes of K were measured using ^{86}Rb as a tracer (Rb is a good alternate substrate for K in K-Cl cotransport in sheep erythrocytes [Dunham and Ellory, 1981]). All fluxes were measured in media containing 10 mM K, 0.1 mM ouabain, 5 mM glucose, and 10 mM Tris-HCl, pH 7.4. The media also contained sufficient choline chloride to achieve the desired osmolality, 190–290 mosmol/kg. Osmolalities >290 were achieved by adding crystalline sucrose to a medium of 290 mosmol/kg. A few experiments were carried out in Cl-free medium with either choline methyl sulfate and Tris-methyl sulfuric acid or choline nitrate and Tris-HNO_3 substituted for choline chloride and Tris-HCl. The methods for measuring and calculating the influxes were slight modifications of earlier methods (Sachs, Ellory, Kropp, Dunham, and Hoffman, 1974).

In experiments in which cells were equilibrated in media of various osmolalities to bring the volume-sensitive fluxes to a constant rate, influxes are expressed in millimoles of K per original liter of cells per hour (i.e., the volume of cells in medium of physiological osmolality, 290 mosmol/kg, calculated from hemoglobin concentrations of lysates) (Figs. 1 and 11). In most experiments K influx varied as a function of time in some aliquots of cells as a consequence of osmotically induced changes in cell volumes at time zero. In these experiments, influxes are referred to as "K uptake," are expressed as millimoles of K per original liter of cells, and are plotted against time (Figs. 2–9 and 12). In one experiment in which all cell aliquots are at physiological volume (Fig. 10), fluxes are expressed simply as millimoles per liter cells per hour.

Though K-Cl cotransport under physiological conditions generally promotes a net KCl efflux, unidirectional influxes rather than effluxes were used as a measure of the activity of the transporter. Cell swelling stimulates both effluxes and influxes of Cl-dependent K transport. There is some asymmetry in the transporter, and it does not mediate obligatory exchanges (Dunham, 1990). Jennings and Al-Rohil (1990) pointed out the difficulty of using efflux measurements to study the early events of the time course of swelling-stimulated transport (though it has been accomplished with human cells [Kaji and Tsukitani, 1991]). A small extent of cell lysis would contribute large errors to early estimates of efflux, but trivial errors to early estimates of influx.

Cell Volume Changes

These were provoked by altering extracellular osmolality. Two assumptions were made about cell volume changes. First, the change to a new steady volume is complete 2 s after a step change in external osmolality as shown for human red cells (Moronne, Melhorn, Miller, Ackerson, and Macey, 1990). Second, changes in the volume of cell water are proportional to the ratio of the osmolalities of the original and the new media. Red cells are not perfect osmometers, but in the range of cell volumes used in this study their behavior is close to ideal.

The effects of altered cell volume on the time course of unidirectional K influx were determined as follows. Cells equilibrated in media of the desired compositions were packed into pellets by centrifugation, and the supernatant solution was removed by aspiration. The cells were resuspended at 10% hematocrit in a medium of a different osmolality (or the same osmolality for controls) containing an appropriate amount of ^{86}Rb and the same $[K]_o$, 10 mM. Samples of the cell suspension removed at desired intervals were rapidly chilled by addition of 5 ml of ice-cold isotonic medium, and then were washed free of extracellular tracer by two more centrifugations and resuspensions in the cold, isotonic medium. Samples of the cell lysates were taken for determinations of radioactivities and hemoglobin concentrations.

It is important for the interpretation of the results that the cell volumes remain constant after volume changes. If swelling caused a net KCl efflux, cell volume could be expected to decline. The experiments were designed to minimize this. $[K]_o$ was 10 mM in all experiments, and physiological $[K]_c$ in LK sheep cells was ~15 mM (Dunham and Hoffman, 1971). In experiments in which swelling of 35% was induced, $[K]_c$ would be reduced to 11 mM. With the Na/K pump inhibited, the forces on K would be outward through K-Cl cotransport, which could cause a slight shrinkage, and the membrane potential, inside negative, which would promote a slow K influx through K channels. In swollen rabbit red cells with K-Cl cotransport an order of magnitude greater than that in swollen sheep cells, with a much higher $[K]_c$ and with 5 mM $[K]_o$, cells shrank ~10% in 2 h (Jennings and Al-Rohil, 1990). Therefore, cell volume in swollen LK sheep cells in 10 mM $[K]_o$ is unlikely to change significantly during the experiments reported here, the longest of which was 3 h.

Reducing Cellular [Mg]

This was accomplished as described before (Bergh et al., 1990), a modification of the method of Flatman (1982). Cells were preincubated for 20 min in an isotonic medium with the ionophore for divalent cations, A23187, at 10 μM, and then washed free of the ionophore by three successive centrifugations and resuspensions. Control experiments were carried out to determine if the effect of this pretreatment, stimulation of Cl-dependent K influx, was due to A23187 and not to reduced cellular divalent cation concentration. In the same control experiments, attempts were made to attribute changes in flux to changes in cellular [Mg], as has been shown for ferret red cells (Flatman, 1987). Cells were preincubated with A23187 without Mg, and with Mg at various concentrations from 0 to 1 mM. As will be shown below, transport was stimulated at [Mg] in the medium of 0.1 mM and lower. At higher Mg concentrations there was inhibition. We conclude that stimulation of Cl-dependent K influx by preincubation with A23187 in a nominally Mg-free medium is due to reduced cellular [Mg], probably to the micromolar range, consistent with earlier conclusions (Lauf, 1985; Flatman, 1987).

A23187 reduces $[Mg]_c$ in exchange for protons. Since reducing intracellular pH (pH_c) stimulated K-Cl cotransport in LK sheep cells (Zade-Oppen and Lauf, 1990), it is important to estimate the effect of Mg depletion on pH_c. Total $[Mg]_c$ in sheep red cells is ~1.2 mmol/liter cells (Delpire and Lauf, 1991). If all of this Mg is removed by A23187 and replaced by 2.4 mmol/liter of protons, the reduction of pH_c, estimated from the buffer capacity of human red

cells (Freedman and Hoffman, 1979), is ~0.05 pH units, which would cause a trivial increase in cotransport (Zade-Oppen and Lauf, 1990). Furthermore, whatever pH gradient is generated will be dissipated by HCO_3-Cl exchange through the anion exchanger.

Analysis of Data

Changes in time course of K influxes were analyzed using an equation for the two-state model derived by Jennings and Al-Rohil (1990) using the principles of chemical relaxation kinetics. The equation for the relaxation from the initial to the final steady-state flux is:

$$C_t = J_1 t + \tau(J_1 - J_0)[\exp(-t/\tau) - 1] \quad (2)$$

where C_t is the ^{86}Rb content of the cells in millimoles per original liter of cells at time t; J_0 is the initial steady-state influx before volume change; J_1 is the final steady-state influx; τ is the time constant for the relaxation from the initial to the final steady state. (Jennings and Al-Rohil [1990] refer to the time constant as the time lag; we refer qualitatively to large time constants as delays.) In the two-state model (Eq. 1), τ is equal to $(k_{12} + k_{21})^{-1}$, the reciprocal of the rate of relaxation to the new steady state, where k_{12} and k_{21} are the forward and backward rate constants, evaluated in the final steady state, for the conversion of A to B. J_0 was determined by linear regression analysis of the values for C_t in cells at constant volume. In all experiments J_0 was measured simultaneously with a change in time course of influx provoked by a cell volume change in order that changes in time course unrelated to volume changes could be detected.

Two parameter fits to Eq. 2 by a nonlinear least-squares iterative procedure (Marquardt algorithm [Marquardt, 1963]) using "A Programming Language" (APL) yielded estimates of J_1 and τ. (J_0 was an experimentally determined constant and C_t and t were known dependent and independent variables, respectively.) Asymptotic standard errors were obtained (standard errors of nonlinear functions are useful informal measures of goodness of fit, but generally underestimate true uncertainty [Motulsky and Ransnas, 1987]).

Materials and Abbreviations

Choline chloride was obtained from Syntex Agribusiness (Springfield, MO), and was further purified by precipitation from hot ethanol. Choline nitrate and choline methyl sulfate were prepared using anion exchange columns as described earlier (Wiater and Dunham, 1983). A23187 was from Calbiochem-Novabiochem Corp. (La Jolla, CA). ^{86}Rb was from New England Nuclear Corp. (Boston, MA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

LK refers to the phenotype of sheep in which the red cells have low K concentrations; LK is also used to refer to the low-K red cells. $[K]_c$ and $[K]_o$ are cellular and external concentrations of K, respectively. K_c and K_o are cellular and external K without respect to concentration. The same notation is used for Cl, Mg, and H (protons); a^c_{Mg} and a^o_{Mg} are cellular and external Mg activities, respectively. pH_c is intracellular pH. IOVs are inside-out membrane vesicles.

RESULTS

Steady-State Relationship of K-Cl Cotransport to Cell Volume

Fig. 1 shows unidirectional K influxes into LK sheep red cells equilibrated in media with a range of osmolalities from 410 to 190 mosmol/kg (all with 10 mM $[K]_o$). The cells had been equilibrated in these media for 90 min, which will be shown below to be sufficient to allow a steady-state flux to be reached. Fluxes were measured in both Cl and Cl-free media (with methyl sulfate the substitute anion), and are plotted

against (osmol/kg)$^{-1}$, which is proportional to the volume of cell water. Physiological, normal medium, 3.45 (osmol/kg)$^{-1}$ (290 mosmol/kg), is marked on the abscissa of the figure. There was no effect on K influx of swelling or shrinking in the absence of Cl, as shown earlier (Dunham and Ellory, 1981). Swelling of cells caused a marked increase in Cl-dependent K influx (K-Cl cotransport), and shrinkage caused a substantial inhibition. The time courses of the conversions of fluxes by volume changes between these steady-state fluxes are the subject of the rest of this study.

Delay in Swelling-induced Increase in K-Cl Cotransport

The time course of K uptake after swelling is shown in Fig. 2. At time zero the medium of aliquots of cells (open triangles and circles) was diluted to 240 mosmol/kg, causing swelling of ~20% ($[K]_o$ was kept constant at 10 mM). ^{86}Rb was added

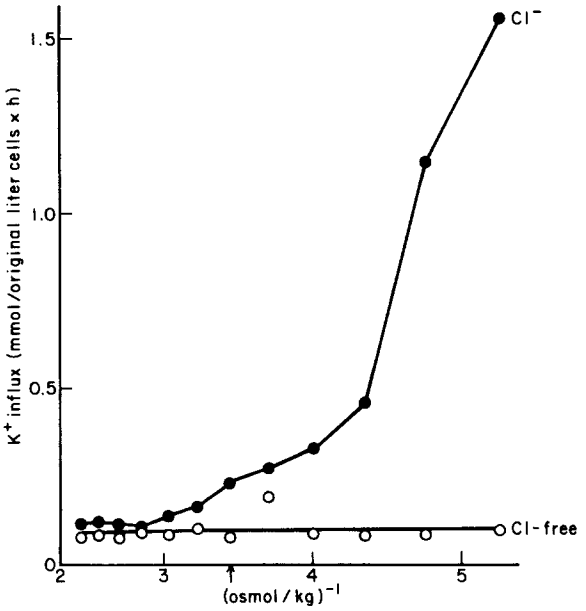

FIGURE 1. Unidirectional K influxes into LK sheep red cells swollen or shrunken in media with osmolalities above and below physiological (290 mosmol/kg, or 3.45 [osmol/kg]$^{-1}$, marked by the arrow on the abscissa) and then equilibrated 90 min to bring the volume-sensitive fluxes to a new steady state. The media contained either Cl (●; total influx) or methyl sulfate (○; Cl-independent flux) as the anion. $[K]_o$ in all media was 10 mM. Fluxes are expressed as millimoles per hour per original liter of cells (i.e., the volume at physiological osmolality). The lines were fitted by eye.

simultaneously with the hypotonic challenge. Control cell volumes (filled symbols) were kept constant. The K uptakes were measured in both Cl and Cl-free media with methyl sulfate as the substitute anion. In cells at constant volume, there was a linear increase in ^{86}Rb content. The flux in Cl medium was several-fold higher than in Cl-free medium, as expected. In cells swollen at time zero in Cl-free medium, the uptake was about the same as in cells of unaltered cell volume in Cl-free medium, also as expected (Dunham and Ellory, 1981). Swelling in Cl-medium caused an increase in K influx. However, there was a delay in the increase; the time courses of K influx during the first 3 min are about the same in cells of normal and swollen volumes, and diverge thereafter. Osmotically induced changes in volumes of red cells are complete in 2 s (Moronne et al., 1990), so the delay has some other explanation.

Varying the Extent of Swelling

In terms of the two-state model (Eq. 1), activation by swelling increases the ratio of rate constants, k_{12}/k_{21}, due to an increase in k_{12} or a decrease in k_{21} (Jennings and Al-Rohil, 1990). These possibilities can be distinguished by determining the relationship between the extent of swelling and τ, the time constant for relaxation to the new steady-state flux. The ratio k_{12}/k_{21} is a direct function of extent of swelling. If k_{12} increases, $(k_{12} + k_{21})^{-1}$, τ, must decrease with greater swelling. If k_{21} decreases, τ must increase with greater swelling. (It is also possible that there are changes in both rate constants: an increase in k_{12} and a decrease in k_{21}, or an increase in both rate constants, with a greater increase in k_{12}.)

In an attempt to determine which rate constant is volume sensitive, we determined τ for cells swollen 20 and 50%. At time zero, solutions of three different osmolalities containing ^{86}Rb were added to three aliquots of packed cells. All solutions contained

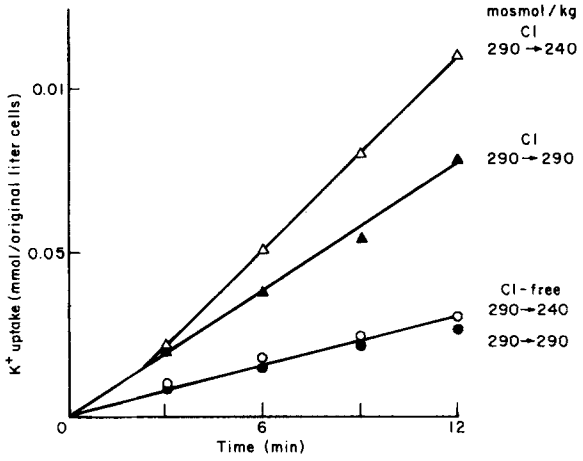

FIGURE 2. Time courses of K uptake into LK sheep cells of constant volume (▲,●) kept in media of normal, physiological osmolality, 290 mosmol/kg (290 → 290) or swollen ~20% at time zero (△,○) by transfer to hypotonic media of 240 mosmol/kg (290 → 240) simultaneously with addition of ^{86}Rb. Uptakes were measured in cells in Cl (▲,△) or Cl-free media (●,○; methyl sulfate the substitute anion), and are expressed in millimoles/original liter of cells (i.e., the volume at physiological osmolality). The lines were fitted by eye.

10 mM KCl and sufficient choline Cl to make the three final osmolalities, 290 mosmol/kg for cells at constant volume, 240 mosmol/kg for 20% swelling, and 190 mosmol/kg for 50% swelling. Fig. 3 shows the time courses of unidirectional K uptake determined for 90 min. It was constant for cells of constant volume, and increased with obvious delays for the swollen cells to final steady-state fluxes, which were greater for the more swollen cells. It is important to note that the data in Fig. 3 (and of all other time course experiments shown where there are obvious delays) fit reasonably well to Eq. 2, which is a single exponential. The estimates of τ for 20 and 50% swelling were nearly the same, 21 ± 7 and 24 ± 4 min, respectively, and therefore there appeared to be no effect of the extent of swelling on τ. But the question is of fundamental importance, so the experiment in Fig. 3 was repeated a number of times.

Table I summarizes mean steady-state fluxes and time constants from the results of the experiment in Fig. 3 and from seven other experiments like it. (Results of 11

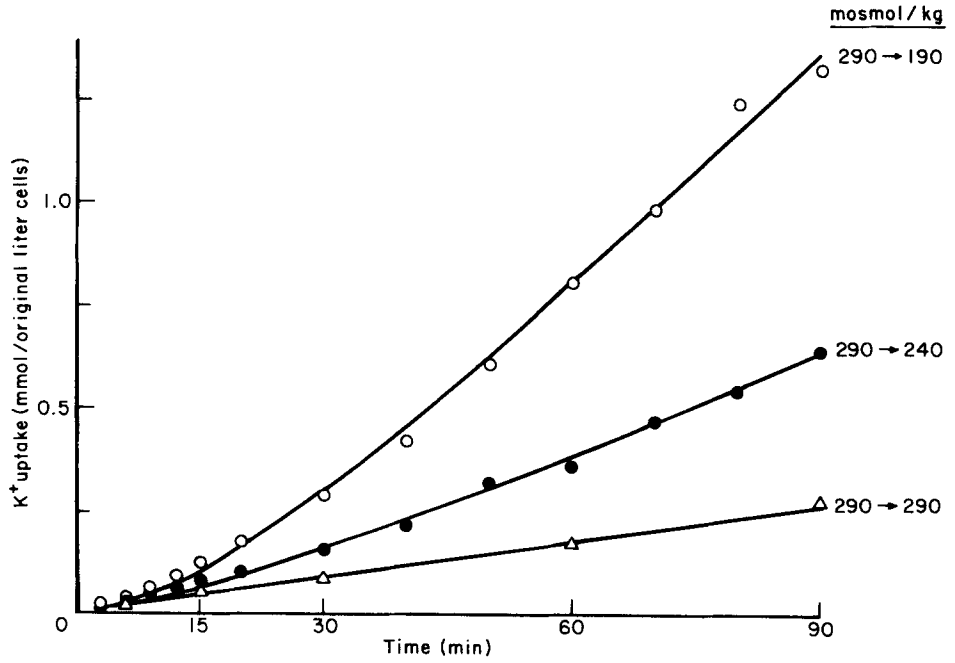

FIGURE 3. Time courses of K uptake into cells swollen ~20% (●) or ~50% (○) at time zero by transfer from medium of normal osmolality to hypotonic media of 240 (290 → 240) or 190 (290 → 190) mosmol/kg. ^{86}Rb was added simultaneously. An aliquot of cells was kept at constant volume in a medium of normal osmolality (△, 290 → 290). The flux into these cells permitted estimation of J_0, the initial steady-state influx. Shown below are J_0 and estimates of the J_1's, the final steady-state influxes after swelling, and the τ's, the time constants of the relaxation to the final steady states. These estimates were obtained by a two-parameter nonlinear least-squares fit of the results to a two-state model as described in Materials and Methods. The lines in this and all subsequent figures (unless otherwise indicated) are from these computer fits. Results of this experiment and seven others like it are summarized in Table I.

	Estimated parameters	
	Steady-state influxes	τ
	mmol/(original liter per h)	*min*
J_0, constant volume (290 → 290)	0.17 (r = 0.998)	—
J_1, swollen 20% (290 → 240)	0.49 ± 0.03	21 ± 7
J_1, swollen 50% (290 → 190)	1.21 ± 0.07	24 ± 5

other experiments of the same design were excluded on the basis of following not very stringent constraints: either the time course of influx into cells of constant volume did not remain constant [$r < 0.99$ for linear regression] or the standard errors for the estimates of either of the parameters, J_1 or τ, exceeded the estimates.) The final steady-state influxes were significantly different for the two extents of swelling, but the time constants were not. There were large differences in the estimates of time constants among experiments, ranging from 6 to 75 min for cells swollen 20%, and from 5 to 36 min for cells swollen 50%. The time constant was

greater for 20% swollen cells in five of the eight experiments, but the mean time constants were nearly the same for the two extents of swelling. We conclude that both k_{12} and k_{21} are volume sensitive, with k_{12} increasing and k_{21} decreasing upon swelling. The rate constants may change to varying extents in different experiments, but both are nevertheless variable. Of course there may be conditions, for example with very slight swelling, where only one rate constant changes, but this has not been tested, and it would be difficult to do so.

Time Course of Shrinkage-inactivated K Uptake

If just one rate constant in the two-state model is volume sensitive, there will be an asymmetric response to shrinking and swelling in the following sense. If the delay in the response to swelling is due to a decrease in the rate constant for the reverse reaction, there will be a much smaller time constant in the relaxation process after shrinkage of swollen cells because this response will be due to an increase in this rate

TABLE I

Analysis of Time Courses of Swelling-Activated K Influxes at Two Swollen Volumes in Eight Experiments

Influx	Steady-state influxes	Time constant (τ)
	mmol/(original liter per h)	*min*
Initial (J_0)	0.26 ± 0.03	—
Final (J_1), 20% swelling	0.96 ± 0.17	27 ± 8
Final (J_1), 50% swelling	1.62 ± 0.23	20 ± 4

Initial steady-state influxes were measured. Final steady-state influxes and time constants were calculated using Eq. 2 (see Materials and Methods). Cells were swollen by transfer to media of (mosmol/kg) 240 (20% swelling) or 190 (50% swelling). Shown are means ± SEMs (n = 8) from the experiment in Fig. 3 and seven others of the same design. The differences between the three steady-state fluxes were all statistically significant ($P < 0.05$) as determined by a one-way analysis of variance and a multiple comparison test (Fisher protected least significant difference). The two time constants were not significantly different ($P = 0.27$; paired t test, two-tailed).

constant (which is now the rate constant for a forward reaction). If both rate constants are volume sensitive, then a significant delay is expected in the time course of shrinkage inactivation of K influx as well as for swelling activation. To test this, we compared time courses of swelling-activated and shrinkage-inactivated fluxes in the same experiment. Fig. 4 shows the results of an experiment in which normal volume cells were swollen and swollen cells were shrunken. The time constants for the two relaxation processes were nearly the same, ~10 min. Therefore, the two relaxation processes can be symmetrical, consistent with the view that both rate constants in the two-state model are volume sensitive.

Time Courses in Rabbit Red Cells

As stated above, Jennings and Al-Rohil (1990) obtained results on rabbit red cells which supported the view that only k_{21} is volume sensitive. The time constant after

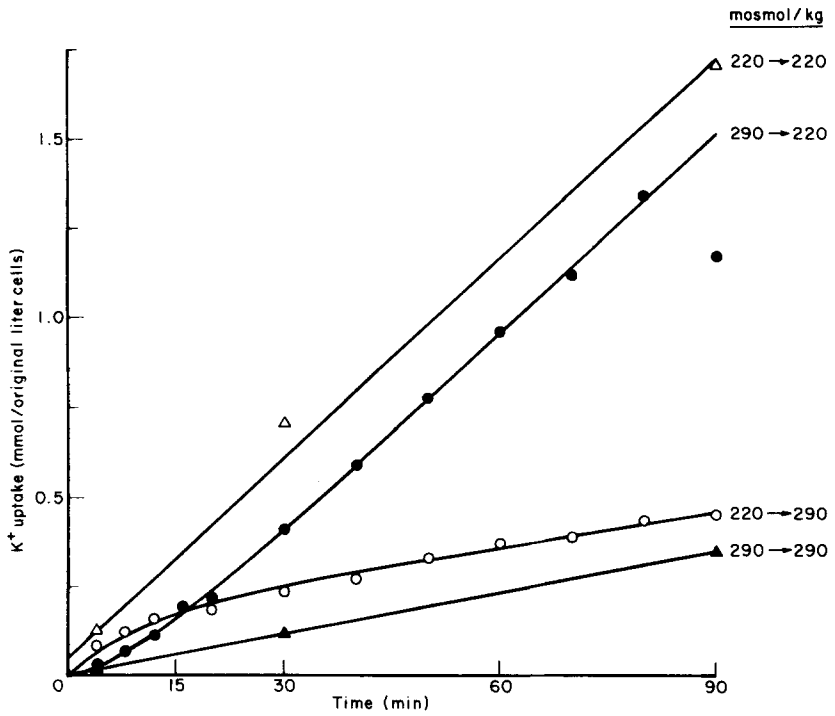

FIGURE 4. Time courses of K uptake into cells either in normal medium (290 mosmol/kg) and swollen at time zero (●, 290 → 220), or swollen by equilibration for 90 min in hypotonic medium (220 mosmol/kg) and then shrunken at time zero by transfer to normal medium (○, 220 → 290). Aliquots of cells were kept at constant volume, swollen (△, 220 → 220) or normal (▲, 290 → 290), for estimates of J_0's. Shown below are estimates from these results of initial and final steady-state influxes (J_0 and J_1, respectively) and the time constants (τ) of relaxation to the final steady-state fluxes. J_0 for swollen cells (220 → 220) and J_1 for initially normal volume cells (290 → 220) were identical. Similar results were obtained in one other experiment of the same design.

Initial cell volume	Estimated parameters	
	Steady-state influxes	τ
	mmol/(original liter per h)	*min*
Normal		
J_0 (290 → 290)	0.23 (r = 0.999)	—
J_1 (290 → 220)	1.12 ± 0.02	10.6 ± 1.1
Swollen		
J_0 (220 → 220)	1.12 (r = 0.997)	—
J_1 (220 → 290)	0.20 ± 0.02	10.2 ± 0.9

swelling was larger with increased extents of swelling, and there was little delay in the reduction of influx after shrinkage of swollen cells. To determine if the differences in results between rabbits and sheep were attributable to a species difference or to differences in techniques, we repeated the experiment in Fig. 4 on rabbit red cells. We used unseparated red cells rather than young erythrocytes after fractionation on a

density gradient as Jennings and Al-Rohil (1990) did. Our results on rabbit red cells are shown in Fig. 5. Gratifyingly, we obtained the same results that Jennings and Al-Rohil (1990) did: a significant delay (time constant of ~7 min) in the relaxation process after swelling (filled circles), but no discernible delay in relaxation after shrinkage of swollen cells (open circles). Therefore, there is a species difference.

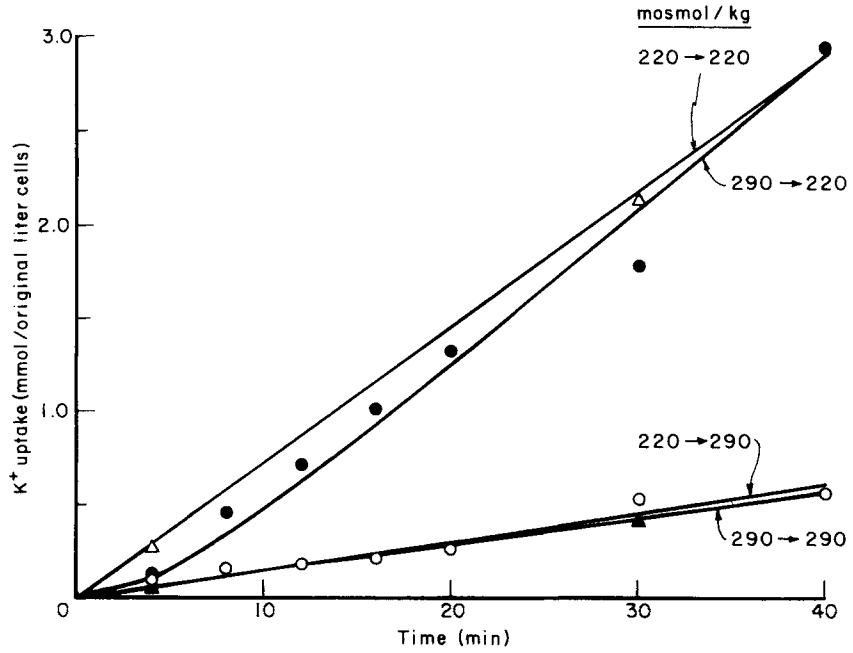

FIGURE 5. Time courses of K uptake into rabbit red cells either in normal medium (290 mosmol/kg) and swollen at time zero (●, 290 → 220), or swollen by equilibration 90 min in hypotonic medium (220 mosmol/kg) and then shrunken at time zero by transfer to normal medium (○, 220 → 290) (the design of this experiment was the same as the one in Fig. 4). Aliquots of cells were kept at constant volume, swollen (△, 220 → 220) or normal (▲, 290 → 290), for estimates of J_0's. Values are shown to 40 min; these and four values from 50 to 90 min were used to obtain the estimates given below of final steady-state influxes (J_1) and the time constants (τ) for relaxation to the final steady-state fluxes. There was no discernible delay in the relaxation to J_1 after shrinkage of swollen cells (220 → 290).

Initial cell volume	Estimated parameters	
	Steady-state influxes	τ
	mmol/(original liter per h)	*min*
Normal		
J_0 (290 → 290)	0.91 (r = 0.999)	—
J_1 (290 → 220)	5.07 ± 0.22	6.8 ± 2.7
Swollen		
J_0 (220 → 220)	4.35 (r = 0.999)	—
J_1 (220 → 290)	0.84 (r = 0.994)	—

Effect of Reduced $[Mg]_c$ on Time Course of Swelling-activated K Influx

Our results on steady-state kinetics lead to the expectation that there may be a more complex time course than the two-state model provides. Swelling causes changes in both J_{max} and $K_{1/2}$, and the changes can be separated by manipulating $[Mg]_c$ (Bergh et al., 1990). Therefore, we tested the effect of reduced $[Mg]_c$ on the time course of K influx in swollen cells to determine if the overall time course of swelling activation

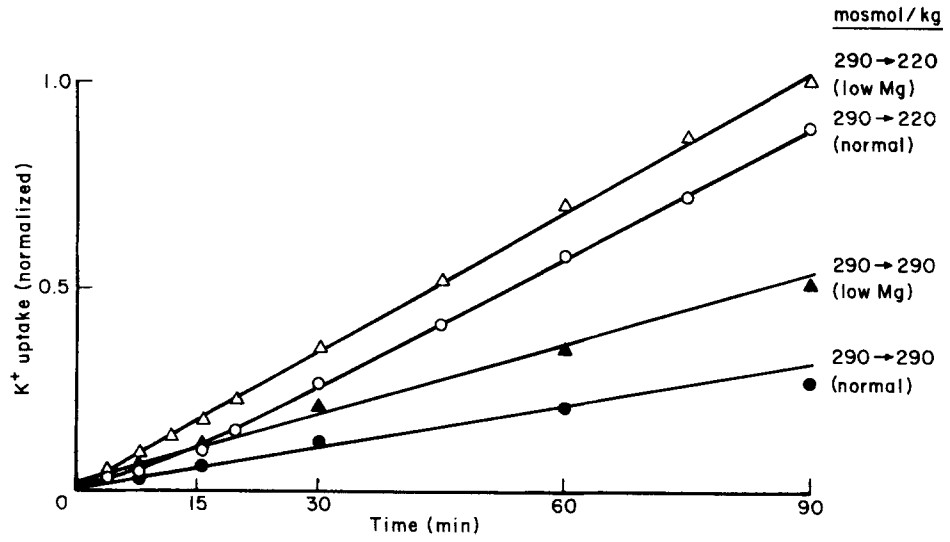

FIGURE 6. Time course of K uptake into cells of normal Mg_c (●,○) or low-Mg_c cells (▲,△) (reduced by pretreatment with A23187 as described in Materials and Methods). Aliquots of normal Mg_c (○) and low-Mg_c cells (△) were swollen ~30% at time zero by transfer to hypotonic medium (220 mosmol/kg) (290 → 220). Other aliquots were kept at normal volume (●,▲, 290 → 290) for estimates of J_0's, the initial steady-state fluxes. Results are shown from two experiments on cells from two different sheep. The fluxes differed by ~1.5-fold between the two experiments. Therefore, the results were normalized by setting the highest flux in each experiment at 1.0 and then calculating all other fluxes as the appropriate fractions. Shown are the means of these normalized fluxes. The estimates of J_0's and the final steady fluxes, J_1's, and τ's, the time constant for relaxation to J_1, are shown below. There was no discernible delay in the relaxation to J_1 in low-Mg_c cells; the values for this influx were well fitted by a straight line. Similar results were obtained in three other experiments of the same design.

Cells	Estimated parameters	
	Steady-state influxes	τ
	normalized fractions per 100	*min*
Normal Mg_c		
J_0 (290 → 290)	3.2 (r = 0.989)	—
J_1 (290 → 220)	10.6 ± 0.02	8.1 ± 1.3
Low Mg_c		
J_0 (290 → 290)	6.0 (r = 0.995)	—
J_1 (290 → 220)	11.4 (r = 0.999)	—

could be resolved into two phases. The results in Fig. 6 suggest that it can. These are the results of two experiments on cells from two different sheep, both control cells (with unaltered $[Mg]_c$) and cells with reduced $[Mg]_c$. The absolute influxes in the two experiments differed by a factor of ~1.5, so the results of the two experiments were normalized as described in the figure legend. After swelling of control cells (open circles), there was a typical delay in activation of influx (τ = ~8 min). As expected, K influx is stimulated in low-Mg_c cells of constant volume (filled triangles) compared with control cells of constant volume (filled circles) (Lauf, 1985), presumably due to an increase in J_{max} (Bergh et al., 1990). Upon swelling, K influx in low-Mg_c cells increased further (open triangles), presumably due to a decrease in $K_{1/2}$ (from ~80

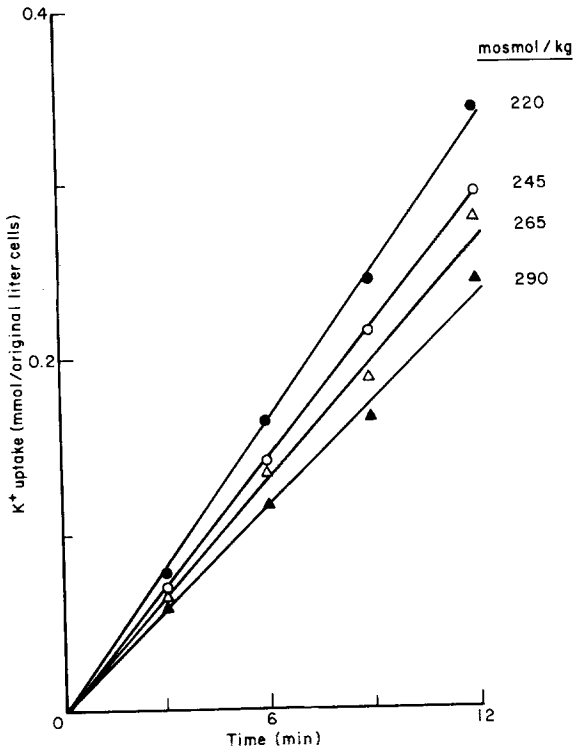

FIGURE 7. Time courses of K uptake into low-Mg_c cells swollen at time zero to varying extents (~9, 14, or 32%) by transfer to media of 265 (△), 245 (○), or 220 (●) mosmol/kg, respectively. Cells were also kept at constant volume (290 mosmol/kg, ▲). The lines were fitted by linear regression; all correlation coefficients were 0.997 or greater.

to ~30 mM $[K]_o$ [Bergh et al., 1990]), but with the striking difference from control cells that no delay in the time course of activation was discernible. In these experiments, the final steady-state influx in cells swollen 50% is about the same in cells with normal and reduced $[Mg]_c$, demonstrating the important point that the effects of swelling and reducing $[Mg]_c$ are not additive.

To verify that the stimulation of influx by A23187 treatment and apparent elimination of the delay are due to reduced $[Mg]_c$, the following control experiments were carried out. In cells treated with A23187 + Mg_o at 0.1 mM, the influx was not stimulated, as will be shown below, and the usual delay was observed after swelling (results not shown). In another experiment, cells were incubated in a nominally

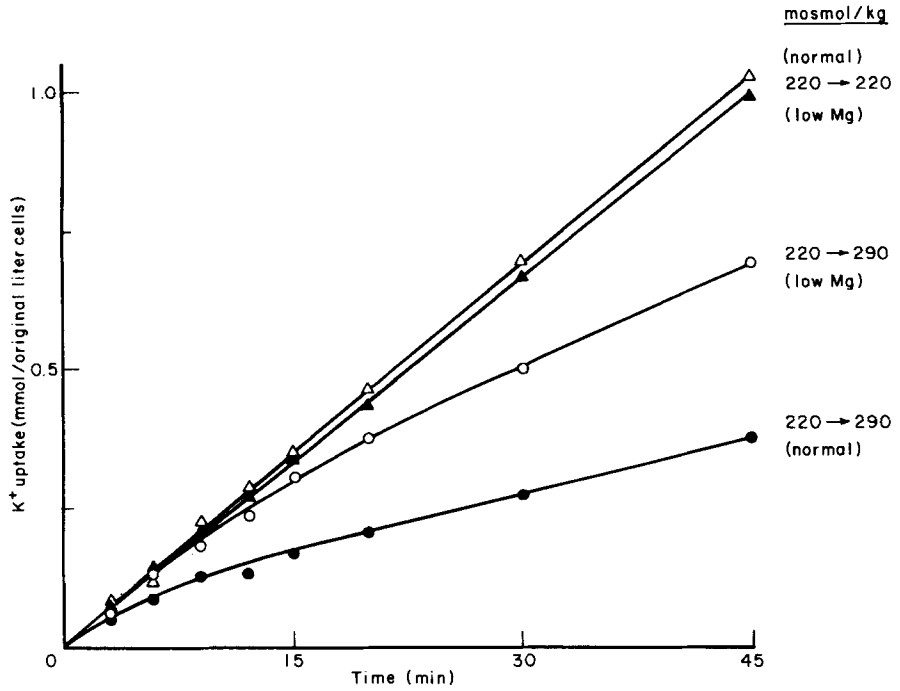

FIGURE 8. Time courses of K uptake into swollen cells, normal Mg_c or low Mg_c, shrunken at time zero. An aliquot of cells had been treated with A23187 to reduce $[Mg]_c$, and then all cells were swollen by equilibration with hypotonic medium (220 mosmol/kg) for 90 min. At time zero, aliquots of the two types of cells were shrunken by transfer to 290 mosmol/kg medium (○, low Mg_c; ●, normal Mg_c, 220 → 290) containing ^{86}Rb. Other aliquots of cells were kept swollen (△, normal Mg_c; ▲, low Mg_c, 220 → 220), from which J_0's were estimated. Values are shown to 45 min; estimates of J_0's, J_1's, and τ's (shown below) were made using these and additional values obtained at 60, 75, and 90 min. Similar results were obtained in two other experiments of the same design.

Cells	Estimated parameters	
	Steady-state influxes	τ
	mmol/(original liter per h)	*min*
Normal Mg_c		
J_0 (220 → 220)	1.48 (r = 0.999)	—
J_1 (220 → 290)	0.40 ± 0.005	4.0 ± 0.2
Low Mg_c		
J_0 (220 → 220)	1.47 (r = 0.999)	—
J_1 (220 → 290)	0.90 ± 0.06	14 ± 4

Mg-free medium with A23187 for 10 min, and then Mg was added (to 0.1 mM). In these cells also there was normal K uptake and the usual delay after swelling. These results show that the elimination of the delay is reversible, and confirm that changes in $[Mg]_c$ are responsible.

Fig. 7 shows time courses of K uptake into low-Mg_c cells swollen to four extents, from 9 to 32%, less than the 50% swelling in the experiments in Fig. 6. There were no

discernible delays, showing that, within the limit of the method to resolve delays, the elimination of the delay in low-Mg_c cells was independent of the extent of swelling.

Time Course of Shrinkage-inhibited Influx in Low-Mg_c Cells

It was important to determine the time course of shrinkage inactivation of K uptake in low-Mg_c cells. Fig. 8 shows influxes into swollen cells, both normal and low Mg_c,

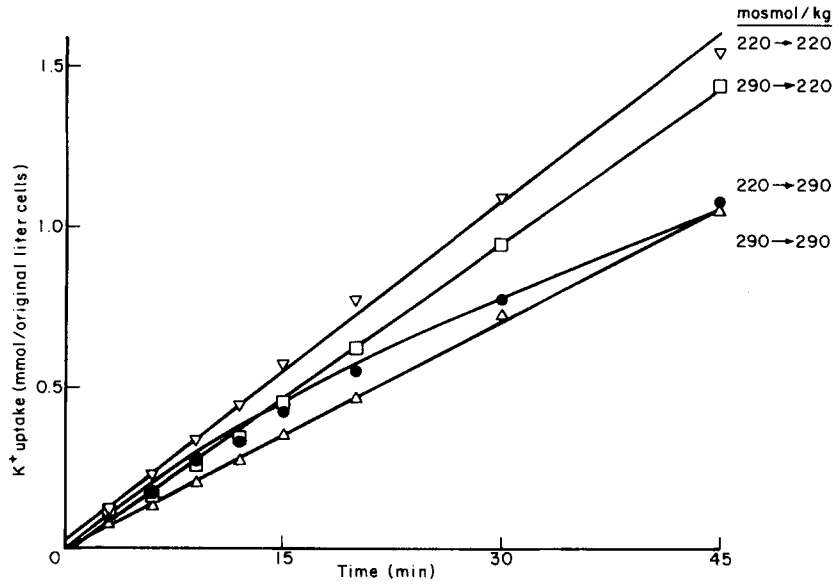

FIGURE 9. Time courses of K uptake in low-Mg_c cells either in normal medium and swollen at time zero (□, 290 → 220) or swollen by equilibration 90 min in hypotonic medium (220 mosmol/kg) and then shrunken at time zero (●, 220 → 290). Aliquots of cells were kept at constant volume, swollen (▽, 220 → 220) or normal (△, 290 → 290), for estimates of J_0's. Values are shown to 45 min; estimations of parameters used these plus values obtained at 60 and 75 min. Shown below are estimates of final steady-state fluxes, J_1's, and τ, the time constant for 220 → 290. There was no discernible delay for 290 → 220, and the values for this uptake were well fitted by a straight line.

Initial cell volume	Estimated parameters	
	Steady-state influxes	τ
	mmol/(original liter per h)	*min*
Swollen		
J_0 (220 → 220)	2.30 (r = 0.999)	—
J_1 (220 → 290)	0.97 ± 0.005	15.6 ± 2.8
Normal		
J_0 (290 → 290)	1.40 (r = 0.999)	—
J_1 (290 → 220)	1.83 (r = 0.997)	—

kept at constant (swollen) volume, or shrunken at time zero. The delay with control cells (filled circles) was slightly less than that usually observed (see Fig. 5). A delay of a magnitude similar to that in most control cells ($\tau = \sim 14$ min) was observed with low-Mg_c cells (open circles). These results confirm the asymmetric behavior of

low-Mg_c sheep cells: no delay in the time course in one direction of volume change, and delay in the other.

To confirm the qualitative differences between time courses of volume-sensitive fluxes in normal and low-Mg_c cells, the experiment in which aliquots of cells were simultaneously swollen or shrunken (Fig. 4) was repeated on low-Mg_c cells. The results (Fig. 9) confirm the absence of a delay in swelling activation (open squares) and the presence of a delay during shrinkage inactivation (filled circles) in low-Mg_c cells.

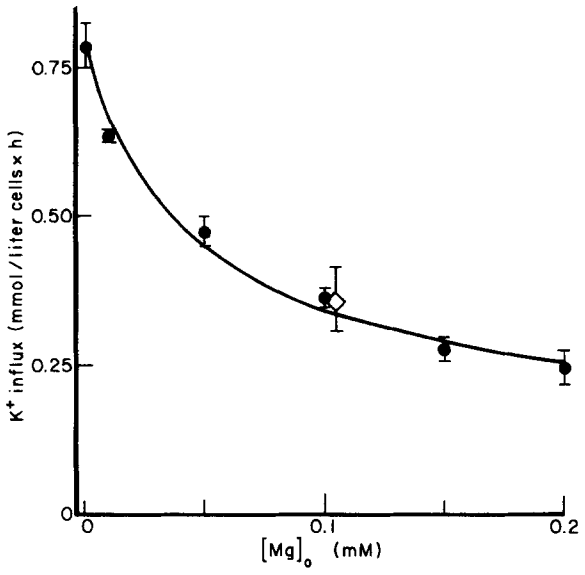

FIGURE 10. K influxes in LK sheep red cells in steady state with intracellular Mg varied by preequilibration in media with various Mg concentrations ($[Mg]_o$) + A23187 (10 μM; see Materials and Methods). At zero $[Mg]_o$, the medium also contained 1 mM EDTA. The influxes were measured after removal of the A23187, and were also measured on a control aliquot of cells that had not been preincubated with A23187 (◇). Fluxes are expressed in millimoles per liter per hour. Error bars indicate SDs (n = 3). The curve was fitted to the data using the equation: $J = 0.787 - \{J_\infty/[1 + (K_i/[Mg]_o)]\}$, where J = measured influxes in A23187-treated cells, $[Mg]_o$ = extracellular [Mg] during A23187 incubation, $0.787 = J$ at 0 $[Mg]_o$, $J_\infty = 0.787$ − the asymptotic, minimum influx, and $K_i = [Mg]_o$ at half-maximal inhibition. Estimates of the constants were: $J_\infty = 0.657 \pm 0.057$ mmol/liter per h and $K_i = 0.048 \pm 0.014$ mM (±asymptotic standard errors). The minimum flux was 0.787 − 0.657 = 0.130 mmol/liter per h. Similar results were obtained in two other experiments of the same design (see text).

Relation between Cellular Mg Activity and Cotransport

The relationship between cellular Mg activity, a^c_{Mg}, and cotransport recently worked out by Delpire and Lauf (1991) provides a basis for determining if dilution of a^c_{Mg} due to swelling can be the activating signal of cell swelling. (Reduction of a^c_{Mg} is unlikely to be the only signal since swelling of low-Mg_c cells activates cotransport further, but it may nevertheless contribute.) We calculated from the results of Delpire and Lauf (1991) that a twofold reduction of a^c_{Mg} caused at most a 31% activation of cotransport. This reduction of a^c_{Mg} would require swelling by at least twofold, which would be lytic. (The swelling required will, in fact, be greater because of buffering of changes in a^c_{Mg}.) Much smaller extents of swelling cause much larger increases in cotransport (Fig. 1). So, while Mg_c may play a role in regulation of cotransport, a reduction in its

activity by swelling does not appear to be the mechanism of the relief of inhibition of cotransport by Mg_c.

This point was sufficiently important that we repeated the experiment of Delpire and Lauf (1991). Fig. 10 shows K uptakes measured in cells that had been preequilibrated in media with A23187 and a range of Mg concentrations from 0 to 0.2 mM. The influx in untreated cells (open diamond) was near the middle of the range of rates of influx. Assuming that a^c_{Mg} in untreated cells is the same as in treated cells with the same flux, a^c_{Mg} is in equilibrium with an external [Mg] of 0.102 mM. Reducing a^c_{Mg} twofold (cells in equilibrium with $[Mg]_o$ = 0.05 mM) would have caused only a 27% increase in K influx (estimated from the curve in Fig. 10), nearly the same

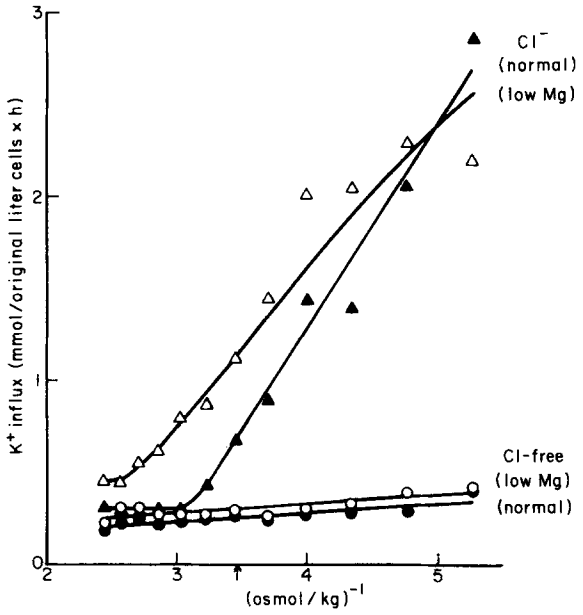

FIGURE 11. Steady-state K influxes into LK sheep red cells with both normal Mg_c (▲,●) and low Mg_c (△,○) swollen or shrunken in media of osmolalities above and below physiological (3.45 $[osmol/kg]^{-1}$; arrow on the abscissa) and then equilibrated 60 min to bring the volume-sensitive fluxes to steady state. The media contained either Cl (▲,△; total influx) or NO_3 (●,○; Cl-independent influx) as the anion. $[K]_o$ in all media was 10 mM. Fluxes are expressed as millimoles per original liter of cells per hour (i.e., the volume at physiological osmolality). The lines for total influxes were fitted by eye except for normal Mg_c cells below 3.2 $(osmol/kg)^{-1}$; this line and the lines for the Cl-independent influxes were fitted by linear regression.

as the 31% increase estimated from the results of Delpire and Lauf (1991). In another experiment of the same design, a 17% increase in influx was estimated for a twofold decrease in a^c_{Mg}. These results support the conclusion that dilution of a^c_{Mg} by swelling is not a major contributor to the signal of swelling.

The results in Fig. 10 can be used to estimate a^c_{Mg} in untreated cells. Assuming that a_{Mg} in the medium, a^o_{Mg}, is the same as $[Mg]_o$ (with Mg in the medium, no chelator of divalent cations was added), a^c_{Mg} can be estimated from the $[Mg]_o$ at which untreated cells appeared in equilibrium, 0.102 mM, and from the proton gradient (A23187 promotes the exchange of divalent cations with protons). At steady state, the proton gradient, $[H]_c/[H]_o$, is equal to $[Cl]_o/[Cl]_c$, and $a^c_{Mg}/[Mg]_o = \{[Cl]_o/[Cl]_c\}^2$. Using

$[[Cl]_o/[Cl]_c]^2 = 2.06$ as reported for LK sheep red cells (Delpire and Lauf, 1991), $a^c_{Mg} = 0.21$ mM. In two other experiments of the same design, estimates for a^c_{Mg} were 0.35 and 0.28 mM; the mean is 0.28 ± 0.05 mM. Delpire and Lauf (1991) presented somewhat higher estimates for a^c_{Mg} in LK sheep red cells, 0.40–0.45 mM.

Steady-State Relation of Cotransport to Cell Volume in Low-Mg_c Cells

The experiment in Fig. 1 was repeated on low-Mg_c cells: K influxes in the presence and absence of Cl in low-Mg_c cells equilibrated in media with a range of osmolalities, and plotted against $(osmol/kg)^{-1}$; the results are shown in Fig. 11 (open symbols). (Similar results were obtained on the relationship between K-Cl cotransporter and cell Mg in dog red cells; Parker, McManus, Starke, and Gitelman, 1990.) Measure-

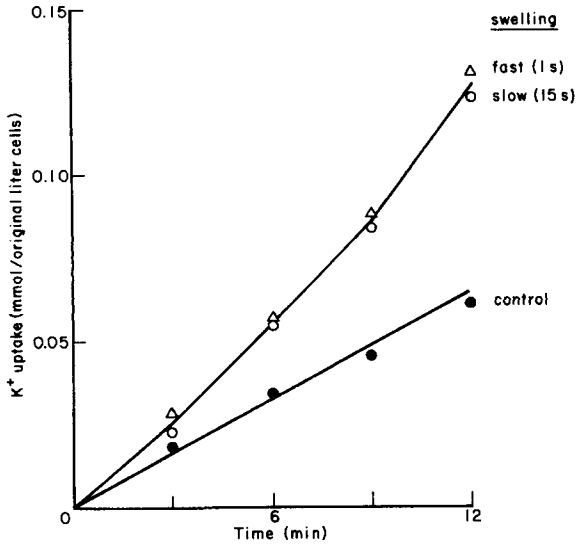

FIGURE 12. Time courses of K uptake into cells swollen ~20% either rapidly (△, ~1 s) or slowly (○, ~15 s). Swelling was achieved by appropriate additions of hypotonic medium to suspensions of cells in medium of normal osmolality, 290 mosmol/kg. The hypotonic medium contained 10 mM K and 10 mM Tris-HCl, pH 7.4. For rapid swelling, a sufficient volume of the hypotonic medium was added to a cell suspension (with rapid mixing) to reduce osmolality to 240 mosmol/kg. For slow swelling, 5 vol of the hypotonic medium was added at 3-s intervals so that the final osmolality was also 240 mosmol/kg. One aliquot of cells was kept at constant volume in normal medium (●). The lines were fitted by eye. Similar results were obtained in three other experiments of the same design.

ments on control cells (normal Mg_c) were determined in the same experiment (filled symbols). The results with control cells were similar (but not identical) to those in Fig. 1: constant Cl-dependent flux of ~0.08 mmol/liter per h in the most shrunken cells, at <3 $(osmol/kg)^{-1}$. (This is threefold higher than the minimum Cl-dependent flux in Fig. 1. The substitute anion was $MeSO_4$ in Fig. 1 and NO_3 in Fig. 11. The difference in Cl-dependent fluxes could mean either that NO_3 is an inhibitor or that $MeSO_4$ is a substrate; on the other hand, different sheep were used as donors in the two experiments.) For low-Mg_c cells, the flux in Cl-free media was also independent of cell volume and was only slightly higher than in control cells (0.27 vs. 0.23 mmol/liter per h). At shrunken volumes, the Cl-dependent K influx in low-Mg_c cells was greater than in control cells. Cl-dependent K influx in severely shrunken low-Mg_c cells appeared to reach a constant minimum of ~0.22 mmol/liter per h. In low-Mg_c

cells of physiological volume and swollen somewhat, K influx was higher than in control cells, but in cells swollen ~50% (5 [osmol/kg]$^{-1}$) the curves converge as expected. Fig. 6 shows that the K influxes are the same in normal Mg_c and low-Mg_c cells ~1 h after 50% swelling and effects of reducing $[Mg]_c$ and swelling are not additive.

Effect of Rate of Swelling on Time Course of Influx

One question arising about the signal for swelling is whether the rate of change in volume is important. For example, if the signal is a consequence of a transient mechanical pressure arising during swelling, or a rapid decrease in the concentration of a signal solute, then the rate of change of cell volume would be critical. The results of several experiments suggest that it is not. The strategy was to induce the same extent of swelling either in a single step reduction in osmolality or in five step reductions 3 s apart to permit volume to reach a new steady-state between each step. In the two procedures the final extents of swelling were the same, but the maximum transient pressure in the second procedure was probably fivefold less than in the first. The time courses of increase in influx were nearly the same (Fig. 12). This experiment was repeated with low-Mg_c cells with the same result (not shown). These results are consistent with those of Lohr and Grantham (1986), who found that very gradual volume changes of kidney tubules could elicit regulation of cell volume.

DISCUSSION

The object of this study was the signal transduction process by which changes in cell volume modulate the activity of the K-Cl cotransporter. The approach was to study the time courses of the swelling- or shrinkage-induced changes in rates of K transport in LK sheep red cells. The goal was to determine the simplest model to which the results could be fitted, and to identify the steps in the model that are sensitive to changes in cell volume. Using this approach, Jennings and Al-Rohil (1990) obtained results in rabbit red cells that could be fitted to a two-state model in which swelling of the cells activates transport by inhibiting a reverse reaction, not by stimulating a forward reaction. As explained above, the evidence was mainly of two types. First, after swelling, the time constant of relaxation to a new steady-state flux increased with greater extents of swelling. Second, there was little delay in relaxation to a new steady-state flux after shrinkage of swollen cells.

Our results with sheep red cells differed from those on rabbit cells in two important respects. First, the magnitude of the time constant for relaxation after swelling did not vary significantly with the extent of swelling (Fig. 3; Table I). Second, there were similar time constants for reaching new steady-state fluxes after swelling of normal cells and after shrinkage of swollen cells (Fig. 4). In a two-state model, these results require that both forward and reverse reactions are volume sensitive since the time constant, $(k_{12} + k_{21})^{-1}$, is constant and k_{12}/k_{21} increases with increased swelling. The rate constant for the forward reaction must increase with swelling and the rate constant for the reverse reaction must decrease. We obtained some of the same results on rabbit red cells (Fig. 5) as Jennings and Al-Rohil (1990) did, which shows that rabbits and sheep are different, and that the differences in results on cells from the two species are not attributable to techniques.

Three-State Model

The time courses of changes in K influx with low-Mg_c cells (Figs. 6–8) indicate that the simple two-state model, Eq. 1, is inadequate to explain the results on sheep cells. An important test for the two-state model is that the rate of approach to a new steady state should depend only on the final condition and not on the direction of approach (Jennings and Al-Rohil, 1990). Swelling activation of normal Mg_c and low-Mg_c cells proceeds to the same final steady state with very different time courses. The simplest interpretation of these results is that there are three states. Several three-state models can be considered: linear models, A ↔ B ↔ C, in which A cannot go directly to C, and triangular models,

$$\begin{array}{ccc} A & \leftrightarrow & C \\ & \searrow\ \nearrow & \\ & B & \end{array}$$

in which all three states are interconvertible. A linear model has four rate constants and a triangular model has six. In this sense the linear model is simpler, though the rate constants have a degree of interdependence in the triangular model. A triangular model was not considered because a linear three-state model was found to which the results could reasonably be fitted.

It will be assumed that one state predominates in swollen cells with or without Mg_c because the flux is the same in swollen cells ± Mg_c. Another state is assumed to predominate in cells of normal volume and normal Mg_c, and a third in cells of normal volume and low Mg_c. It is unlikely that the state predominating in swollen cells is the state in the middle (B) because direct conversion would be precluded between the two states predominating in cells of normal volume. It is also unlikely for two reasons that the state predominating in normal volume, normal Mg_c cells is in the middle. First, if the B → C conversion were Mg dependent (which it would be if normal Mg_c cells were the middle state), then the flux could not be activated in cells of normal, constant volume by reducing Mg_c, and it can be. Second, it was shown that reducing cell Mg_c activates the flux and swelling low-Mg_c cells activates it further. However, lowering the Mg_c of swollen cells does not activate the flux further. Therefore, normal volume, low-Mg_c cells must be intermediate between normal volume, normal Mg_c cells and swollen cells, and the middle, B state predominates in these cells. The three-state model is:

$$A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} B \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} C \tag{3}$$

with A state transporters having a low flux rate (or perhaps zero flux) and predominating in cells of normal volume and normal Mg_c. Reduction of Mg_c increases the fraction of transporters in the B state, which have a higher flux. The conversion to the C state, with the highest flux, is caused by swelling of cells of normal Mg_c (A → B → C) or swelling of low-Mg_c cells (B → C). Swelling activates by inducing the same effect as reducing Mg_c, but has an additional effect since swelling can activate the flux in low-Mg_c cells. The swelling-induced activation of A → B is rate limiting. Two relaxation processes might be resolvable, but arguments will be made

that B → C and B → A are rapid ($\tau \leq 1$ min), so the time courses of K uptake after swelling of normal Mg_c cells and shrinkage of swollen cells appear as single relaxation processes with time constants of 10–20 min.

These proposals are consistent with our earlier findings from steady-state kinetics, which showed that the swelling-induced changes in J_{max} and $K_{1/2}$ for K_o are separable, and that the increase in J_{max} can be induced by reducing $[Mg]_c$ (Bergh et al., 1990). Thus the two swelling-induced changes in kinetic constants would be in sequence, first a slow, rate-limiting increase in J_{max} dependent on Mg_c (A → B), followed by a rapid decrease in $K_{1/2}$ (B → C).

An alternative to a three-state model is a two-state model in which the rate constants can change slowly. With this type of model, the time courses of changes in transport would not be single exponentials because there would be an exponent of an exponent in Eq. 2. All the slow time courses shown above were fitted by single exponentials, indicating that the time constants for the change in the rate constants are too small to be resolved. Therefore, this type of model does not appear to apply.

In the following sections, the data will be analyzed in terms of the model in more detail in the hope of gaining further insight into the signal transduction process. Considerable reliance will be placed on assumptions and guesses. First, estimates will be made of the four rate constants in the three-state model. From these, time constants will be calculated and compared with measured time constants for swelling activation and shrinkage inactivation of K influx. Next, estimates will be made of the fluxes through the transporter in each of its three proposed states and of the kinetic constants, J_{max} and $K_{1/2}$, for each of the states. The rate constants will be used to calculate the equilibrium distribution of the states in cells at normal volume ± Mg_c and at swollen volume. From the distribution of states and the flux through the transporter in each state, fluxes will be calculated for cells in each of these three conditions. Similarity of calculated fluxes to measured fluxes will contribute to validation of the model and of the various assumptions and guesses used to generate the calculated fluxes.

Rate Constants

k_{23} and k_{32}. The time courses of swelling activation and shrinkage inactivation for low-Mg_c cells have been determined (Figs. 6–9). In the terms of the three-state model, these changes are associated with the conversions

$$B \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} C$$

For convenience, the swelling activation and shrinkage inactivation will be denoted B → C and C → B, respectively, even though there are C state transporters in cells of normal volume and B state transporters in swollen cells. The following conclusions can be drawn about the rate constants for the changes in low-Mg_c cells, k_{23} and k_{32}. B → C is rapid ($\tau \leq 1$ min), presumably due to an increase in k_{23}. C → B is slow, probably due primarily to a decrease in k_{23}. The results suggest further that with swelling both k_{23} and k_{32} increase. The argument is as follows. At some point along the steep portions of the curves in Figs. 1 and 11, half of the transporters must be in the C state; with > 50% of transporters in the C state, the slope of the curve would

decrease as C, the fraction of transporters in the C state, approaches 1.0 (the slope is beginning to decrease in Fig. 1 near 5 [osmol/kg]$^{-1}$). In Fig. 11 for low-Mg_c cells, the other half of the transporters must be in the B state (reducing $[Mg]_c$ promotes the A → B conversion). It would be possible to estimate the individual rate constants, k_{23} and k_{32}, for B → C if τ for this process were known. There was no discernible delay, but a τ of 1 min will be assumed (near its lower detectable limit). At the volume where B = C, k_{23} and k_{32} are the same and $\tau = 1/(k_{23} + k_{32}) = 1.0$ min. Therefore, $k_{23} = k_{32} = 0.5$ min^{-1} (these are, of course, lower limit estimates of the rate constants).

k_{32} as well as k_{23} must have increased with swelling, though to different extents, for the following reasons. For shrinkage-induced inactivation of low-Mg_c cells (C → B), τ, $(k_{23} + k_{32})^{-1}$, was ~15 min (Figs. 8 and 9), and the sum of the rate constants at the new steady state after shrinkage, $k_{23} + k_{32} = 0.07$ min^{-1} (1/15 min^{-1}), is less than either k_{23} or k_{32}, 0.5 min^{-1}, for swollen cells. Therefore, both rate constants must decrease with shrinkage, and both must increase with swelling. After shrinkage, the ratio k_{32}/k_{23} must be > 1 (because B > C), but k_{32} cannot exceed 0.07 min^{-1}. If, for example, $k_{32}/k_{23} = 4$ in low-Mg_c cells of normal volume, $k_{23} = 0.014$ and $k_{32} = 0.056$. With swelling to the volume at which B = C, k_{23} would increase ~36-fold and k_{32} would increase ~9-fold, each to 0.5 min^{-1}. Only if τ for B → C had exceeded 5 min would it have been possible to argue that k_{32} is volume insensitive, and a delay of 5 min could easily have been detected. (The same general conclusions about k_{23} and k_{32} would be reached if τ for B → C were much less than 1 min, though the specific estimates of the rate constants would be different.) The estimates of k_{23} and k_{32} obtained from the ratio $k_{32}/k_{23} = 4$ will be used in calculations below. Other assumptions about k_{32}/k_{23} gave less satisfactory results.

That k_{23} and k_{32}, the forward and reverse rate constants for B ⇄ C, change in the same direction with volume changes is consistent with the view that they are rate constants for a single reaction (even though they change to different extents).

k_{12} and k_{21}. The time courses of the

$$A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} B$$

conversions in sheep cells have not been determined; they occur during incubation with A23187. However, they can be inferred. Since A → C is slow (τ = ~10 min) and B → C is rapid ($\tau \leq 1$ min), A → B is rate limiting and probably has the same time course as A → C, i.e., a τ of ~10 min. Therefore, $k_{12} + k_{21} = 0.1$ min^{-1} for A → B. It will be argued below that k_{21} is volume sensitive, decreasing with swelling. The time course of B → A can also be inferred. With the same extents of shrinkage, C → B and C → A both have slow time courses of relaxation (Fig. 8). Therefore, C → B is rate limiting and B → A may have a small time constant associated with a shrinkage-induced increase in k_{21}, and $k_{12} + k_{21}$ = at least 1.0 min^{-1} for B → A. Taking $\tau = 10$ min for A → B and 1 min for B → A and assuming that k_{21} decreases 100-fold with swelling and that k_{12} is constant, the following values can be calculated: $k_{12} = 0.091$ min^{-1}, k_{21} decreases from 0.91 to 0.0091 min^{-1} with swelling, and k_{12}/k_{21} increases from 0.1 to 10.

In the time courses of the A ⇄ C conversions (swelling and shrinking of normal Mg_c cells), there are similar delays in the conversions in the forward and reverse

directions (Figs. 3, 4, and 7 and Table I). The rate constants are complex because of the intermediate state (Stein, 1986):

$$A \underset{(k_{21}\cdot k_{32})/(k_{21}+k_{23})}{\overset{(k_{12}\cdot k_{23})/(k_{21}+k_{23})}{\rightleftharpoons}} C \tag{4}$$

This formulation can be used to gain further insight into the process. The A → C conversion can be considered in simpler terms by noting that upon swelling the ratio $(k_{12} \cdot k_{23})/(k_{21} \cdot k_{32})$ increases (the two denominators are the same). Since forward and reverse A ⇄ C conversions can occur with similar rate constants of ~ 10 min (Fig. 4), swelling must cause an increase in the numerator and a decrease in the denominator. It was argued above that both k_{23} and k_{32} increase with swelling. Therefore, k_{21} must decrease with swelling, as was just assumed. The results indicate that k_{21} is the rate constant for a reaction promoted by Mg. There is no evidence that k_{12} is volume sensitive, but as shown above, it needn't be, and it is assumed to be volume insensitive.

TABLE II

Estimates of the Rate Constants in the Three-State Model for Volume-sensitive K-Cl Cotransport in Normal Mg_c Cells

	Rate constants		Time constants (τ)	
	Normal volume	Swollen 50%		
	min^{-1}			
k_{12}	0.091	0.091	Normal → swollen	10 min
k_{21}	0.91	0.0091		
k_{23}	0.014	0.50	Swollen → normal	18 min
k_{32}	0.056	0.50		

Time constants for swelling-activated and shrinkage-inactivated transport were calculated from the rate constants. See text for the procedures for estimating the rate constants. $\tau = (k_{21} + k_{23})/(k_{12}\cdot k_{23} + k_{21}\cdot k_{32})$ (see Eq. 4). τ was calculated for the normal → swollen conversion from the rate constants for swollen cells and for the swollen → normal conversion from the rate constants for normal volume cells.

The estimates of the four rate constants in the preceding paragraphs for cells of normal and swollen volumes are presented in Table II. From these rate constants, the time constants were calculated for the A ⇄ C conversions, swelling activation and shrinkage inactivation of normal Mg_c cells. The time constants were taken to be equal to the reciprocal of the sum of the numerator and denominator in Eq. 4. These time constants, shown in Table II, 10 min for swelling and 18 min for shrinking, are of the same order of magnitude as those observed in all experiments (e.g., Table I and Fig. 4). They differ from one another, but by less than a factor of two, and in some of our experiments the time constants were this different. These calculated time constants are consistent with the conclusion that both a forward and reverse rate constant are volume sensitive. The rate constants in Table II will be used below to calculate the equilibrium distribution of states of the transporter after making estimates of the fluxes through the transporter in the three states.

Fluxes through the Transporter in the Three States

The results in Figs. 1 and 11 on steady-state fluxes over a range of cell volumes were used to make estimates of the fluxes through the transporters in the A, B, and C states of the model at $[K]_o$ = 10 mM, the concentration in all experiments. In Fig. 1, shrinking the cells by increasing the osmolality above normal resulted in a substantial decrease in the Cl-dependent K influx, but not to zero. Between 2.8 and 2.4 $(osmol/kg)^{-1}$, the four lowest levels of $(osmol/kg)^{-1}$ used, the Cl-dependent K influx was constant at 0.023 ± 0.003 mmol/original liter of cells per h, approximately sevenfold lower than the Cl-dependent flux in normal medium (similar results were obtained in Fig. 11). In the three-state model, shrinking increases the fraction of transporters in the A state, but to what extent cannot be specified. The constant minimum flux over a range of hyperosmolalities is taken to mean that all of the transporters are in the A state, and that the flux in this state, J_A, is not zero, but is 0.023 mmol/original liter of cells per h. Implicit in this argument is the assumption that all transporters remain active, but slower, with shrinkage. There is no evidence to support this assumption; if it is wrong, some of the conclusions drawn below will be incorrect quantitatively, but valid qualitatively.

The results in Fig. 11 on shrunken low-Mg_c cells would permit making an estimate of the flux through B state transporters if the ratio B/A were known. This ratio is obviously not known with certainty, but can be argued to be high, say 10, on the following grounds. There is no obvious delay in the swelling activation of low-Mg_c cells, so most of the transporters must be in the B state. A substantially larger fraction remaining in the A state would have resulted in an appreciable delay in activation of K influx by swelling. Therefore, in shrunken, low-Mg_c cells, Cl-dependent K influx should be close to J_B at 10 mM $[K]_o$. It was 0.22 mmol/original liter of cells per h in Fig. 11, ~10-fold higher than the estimate for J_A at 10 mM $[K]_o$, the Cl-dependent influx in shrunken, normal Mg_c cells. We will use 0.22 mmol/original liter of cells per h as an estimate of J_B.

The curve for low-Mg_c cells in Fig. 11 is steep in the region of 4.5 $(osmol/kg)^{-1}$, indicating (as argued above) that a majority of the transporters have not been converted to the high-flux C state (had they been, the curve would display a decreasing slope, which it appears to do in more swollen cells). J_C, the flux through C state transporters, can be calculated if it is assumed that 50% of transporters are in the C state and 50% in the B state at 4.5 $(osmol/kg)^{-1}$ and B + C = 1.0. (It is also assumed that there are no transporters in the A state in swollen low-Mg_c cells.) J_B is 0.22 mmol/liter per h. The total Cl-dependent K influx, J_T, in low-Mg_c cells at 4.5 $(osmol/kg)^{-1}$ is 1.7 mmol/liter per h and $J_C = [J_T - (1 - C) \cdot J_B]/C$ = 3.2 mmol/liter per h. This rough estimate of J_C is $> 100 \times J_A$. The estimate is somewhat sensitive to the assumption of C as follows. For C = 0.3, J_C = 5.5 mmol/liter per h; for C = 0.7, J_C = 2.3 mmol/liter per h. The next step in the analysis is to obtain estimates of the kinetic constants for the transporters in the three states.

Kinetic Constants

The conversion B → C was concluded above to involve a decrease in the $K_{1/2}$ of the cotransporter for K_o. It is possible to make an estimate of the magnitude of this

change. The Michaelis-Menten equations for J_B and J_C are: $J_B = J_{max}/\{1 + (K^B_{1/2}/[K]_o)\}$ and $J_C = J_{max}/\{1 + (K^C_{1/2}/[K]_o)\}$, where $K^B_{1/2}$ and $K^C_{1/2}$ are the $K_{1/2}$'s for K_o of the B state and C state transporters, respectively. J_{max} is assumed to be the same for the B and C states (the increase in J_{max} was concluded above to take place with the A → B conversion). The calculation requires a value for J_C/J_B; it is 14.5 (J_C and J_B were estimated above to be 3.2 and 0.22 mmol/original liter of cells per h, respectively). Solving each Michaelis-Menten expression for J_{max}, equating one expression to the other, and rearranging yields:

$$K^B_{1/2} = \{J_C/J_B[1 + (K^C_{1/2}/[K]_o)] - 1\}[K]_o \quad (5)$$

Neither of the $K_{1/2}$'s is known, but a guess about $K^C_{1/2}$ can be made. The lowest $K_{1/2}$ we observed in swollen cells was 28 mM (Bergh et al., 1990), some combination of the $K_{1/2}$'s of A, B, and C state transporters, but mostly B and C. $K^B_{1/2}$ was calculated from Eq. 5 with $[K]_o$ = 10 mM and assuming, for a start, that $K^C_{1/2}$ = 15 mM. The $K^B_{1/2}$ obtained was 353 mM. If $K^C_{1/2}$ is assumed to be 1 mM (15-fold lower), $K^B_{1/2}$ is 150 mM, only 2.4-fold lower. Therefore, the estimate of $K^B_{1/2}$ is not highly sensitive to the assumption of $K^C_{1/2}$. Is $K^B_{1/2}$ likely to be as high as 353 mM? We have attempted to measure the $K_{1/2}$ of shrunken cells, which it is expected to exceed 80 mM, the $K_{1/2}$ of normal volume cells (Bergh et al., 1990). The curves for flux vs. $[K]_o$ were indistinguishable from straight lines (results not shown); we could probably have detected a $K_{1/2}$ of 150 mM. Therefore, a $K^B_{1/2} > 200$ mM is not unreasonable. With $K^C_{1/2}$ = 15 mM, J_C = 3.2 mmol/liter per h, and $[K]_o$ = 10 mM, J^C_{max} is 8.0 mmol/liter per h, which is reasonable: measured J_{max}'s in swollen cells are in the range of 6–10 mmol/liter per h (Bergh et al., 1990), and we assume that the J_{max}'s are the same for the B and C states. If $K^C_{1/2}$ were assumed to be much lower than 15 mM, then unreasonably low J_{max}'s would be predicted (e.g., for $K^C_{1/2}$ = 5 mM, J^C_{max} = 4.8 mmol/liter per h). Therefore, the assumption of $K^C_{1/2}$ = 15 mM does not appear unreasonable and $K_{1/2}$ appears to decrease 20-fold (from 353 to 15 mM) with the swelling-induced B → C conversion. J^B_{max} was calculated to be 8.0 mmol/liter per h from J_B = 0.22 mmol/liter per h at 10 mM $[K]_o$ and $K^B_{1/2}$ = 353 mM. The identity of J^B_{max} and J^C_{max} is gratifying and may appear to validate the calculations, but is in fact a consequence of them. J^A_{max} was calculated from $K^B_{1/2}$ ($K_{1/2}$ was assumed to be the same for the A and B states) and J_A at 10 mM $[K]_o$, 0.023 mmol/liter per h.

Table III shows the estimates developed above of the fluxes through the transporter in the three states at 10 mM $[K]_o$ and the estimates of the $K_{1/2}$'s and J_{max}'s for the three states. The calculations used the assumptions outlined above that $K_{1/2}$ does not change with the A ⇄ B conversions and J_{max} does not change with the B ⇄ C conversions. There is support for these assumptions in our earlier work (Bergh et al., 1990) and in this study, but they may be oversimplifications. It is also assumed that the number of functioning transporters does not vary with cell volume; i.e., that A + B + C is constant. This means that increasing J_{max} requires an increase in the turnover number of the transporters, not an activation of latent transporters. This may also be incorrect. On the other hand, the analysis has yielded numbers that do not seem totally unreasonable, and which can explain how, with swelling of shrunken cells, $K_{1/2}$ decreases ~20-fold, J_{max} increases ~10-fold, and influx increases >100-fold.

TABLE III

Estimates of J_A, J_B, and J_C, the K Influxes at 10 mM $[K]_0$ through the Three States of the Transporter in the Three-State Model, and of the Kinetic Constants for K Influx for the Three States, J_{max} and $K_{1/2}$

State	J	$K_{1/2}$	J_{max}
A	0.023	353	0.83
B	0.22	353	8.0
C	3.2	15	8.0

J and J_{max} are in millimoles/original liter of cells per hour; $K_{1/2}$ is in millimolar. The assumptions and other bases for the calculations of the values are explained in the text.

If there are two $K_{1/2}$'s for K influx, then the double reciprocal plots of Cl-dependent K influx in Bergh et al. (1990) should not be straight lines, but should bend toward the origin at high $[K]_o$'s. We calculated K influxes for two $K_{1/2}$'s, 15 and 353 mM, with an equal number of transporters at the two $K_{1/2}$'s, and $[K]_o$'s up to 120 mM, the highest $[K]_o$ used in Bergh et al. (1990). In a double reciprocal plot of these calculated fluxes, the flux at 120 mM $[K]_o$ was 96% of the flux at 120 mM estimated from a nonlinear least-squares fit to the calculated values. Therefore, because the highest $[K]_o$ is one-third of the higher $K_{1/2}$, the double reciprocal plot of the calculated fluxes was difficult to distinguish from a straight line; the same would be true for experimentally determined fluxes.

Equilibrium Distributions of States

From the rate constants in Table II, it is possible to calculate the equilibrium distribution of the three states of the transporter using the King-Altman method (Stein, 1986). The relative numbers of transporters (as fractions) for cells of normal volume, both normal and low Mg_c, and cells swollen 50%, are shown in Table IV. For

TABLE IV

Estimated Equilibrium Distribution of Transporter in the Three States (N = Fraction in Each State), and Contributions of Each State to Total Flux (J), in Normal Mg_c and Low-Mg_c Cells of Normal Volume and Swollen 50%

Cells		A state	B state	C state	Total	Measured fluxes
Normal volume, normal Mg_c	N	0.889	0.089	0.022	1.0	—
	J	0.0205	0.0196	0.0704	0.111	0.14
Normal volume, low Mg_c	N	0.074	0.741	0.185	1.0	—
	J	0.002	0.163	0.592	0.757	0.81
Swollen 50%, ± Mg_c	N	0.048	0.476	0.476	1.0	—
	J	0.001	0.105	1.523	1.629	1.7

Measured fluxes for cells of the three conditions are shown for comparison. N, the fraction of transporter in each state, was calculated using the King-Altman method (Stein, 1986) for a linear three-state system using the rate constants from Table II as follows. T = A + B + C, where A, B, and C are the fractions of the transporters in each state and the total, T, = 1.0. $A/T = k_{21} \cdot k_{32}/\Sigma$; $B/T = k_{12} \cdot k_{32}/\Sigma$; $C/T = k_{12} \cdot k_{23}/\Sigma$; $\Sigma = k_{21} \cdot k_{32} + k_{12} \cdot k_{32} + k_{12} \cdot k_{23}$. J, the flux through the transporters at 10 mM $[K]_0$ in each state, were $J_A \cdot A$, $J_B \cdot B$, and $J_C \cdot C$; J_A, J_B, and J_C were from Table III. The fluxes are in millimoles per original liter of cells per hour. The measured fluxes are from Figs. 1 and 11 (see text).

cells of normal volume and normal Mg_c and for swollen cells, the rate constants in the columns labeled "normal volume" and "swollen 50%" in Table II, respectively, were used. For cells of normal volume and low Mg_c, it was assumed that k_{23} and k_{32} were those of normal volume cells, but that the reduction of Mg_c caused the 100-fold reduction of k_{21} to that of swollen cells. The calculated distributions of the transporters among the three states for cells of the three different conditions were as expected: mostly A state in cells of normal volume and Mg_c, mostly B state in low-Mg_c cells, and equal fractions of B and C states in swollen cells.

It is important to compare measured fluxes with fluxes calculated from (*a*) the estimated fluxes through the transporter in each state (Table III) and (*b*) the fractions of the transporters in each state (Table IV). These calculated fluxes are shown in Table IV, along with measured fluxes from Figs. 1 and 11. In cells of normal volume and Mg_c, the calculated flux, 0.11 mmol/liter cells per h, is a bit lower than the Cl-dependent flux at normal volume in Fig. 1 (0.14 mmol/liter cells per h); it is about half of the Cl-dependent flux in cells of normal volume and Mg_c in Fig. 11. In low-Mg_c cells the calculated flux, 0.76 mmol/original liter cells per h, is nearly the same as the measured flux for such cells in Fig. 11 (0.81 mmol/liter cells per h). The calculated flux for cells swollen 50%, 1.6 mmol/original liter cells per h, is nearly the same as the measured flux in cells swollen 50% in Fig. 1 (1.4 mmol/liter cells per h), and is a bit lower than the flux for such cells in Fig. 11 (2.0 mmol/liter cells per h). The similarity of the measured and calculated fluxes for cells in the three conditions helps to validate the model and also the assumptions and approximations made in estimating the rate constants (Table II), the fluxes through each state (Table III), and the kinetic constants (Table III). Of course there are a lot of adjustable parameters; however, in addition to the fluxes, the kinetic constants and equilibrium distribution of states appear reasonable.

Signals of Volume Change

The results on signal transduction may help illuminate the nature of the signal of changes in cell volume. Two classes of signals can be envisioned: a change in concentration of a cytoplasmic solute, and a mechanical change in the membrane. In red cells there is evidence against both of these classes of signals. Jennings and Schulz (1990) presented results suggesting that the signal of swelling in rabbit red cells is not a mechanical change in the membrane. Echinocytes and discocytes, which have drastically altered membrane shapes, have the same rate of K-Cl cotransport as the erythrocytes from which they were formed. In contrast, Sachs (1988), O'Neill (1989*b*), and we (Dunham and Logue, 1986; Kracke and Dunham, 1990) have presented results suggesting that the signal is not a change in concentration of a cytoplasmic solute. Volume-sensitive K-Cl cotransport was observed in resealed human red cell ghosts, which are nearly devoid of cytoplasm, and in IOVs made from LK sheep red cell membranes; IOVs have no cytoplasm. (Brugnara, Van Ha, and Tosteson [1988] found very little effect on K-Cl cotransport of varying the volume of resealed human red cell ghosts. There is no obvious explanation for this different result.)

The disparate results on the nature of the signal are a conundrum that we try to resolve by proposing two different signals in series (Dunham, 1992). We propose that a mechanical signal promotes the B $\rightarrow$ C conversion and that a dilution of a

cytoplasmic solute promotes the A → B conversion. The bases for these suggestions are as follows. Human erythrocytes have the same slow rate of swelling activation as rabbit and sheep red cells (Kaji and Tsukitani, 1991), but swelling activation in human resealed ghosts proceeds without measurable delay (Sachs, 1988). In recent measurements of K influxes into sheep red cell IOVs, there was also no discernible delay in the time course of swelling activation of the influx (which was sevenfold for 30% swelling) (Dunham, 1992; Kelley, S. J., and P. B. Dunham, unpublished results). Therefore, we conclude that the making of ghosts and IOVs somehow induces the A → B conversion, which is consistent with our earlier observation that K-Cl cotransport is considerably higher in resealed ghosts than in intact cells (Dunham and Logue, 1986). In the three-state model, it is from the B state that swelling activation occurs without delay. If only the B → C conversion takes place in ghosts and IOVs, it follows that the signal for the B → C conversion is mechanical. The nature of the mechanical change which is the signal is unclear; it has been claimed not to be tension or a change in radius of curvature of the membrane (Kracke and Dunham, 1990). Fig. 12 shows that the rate of application of the signal is not critical. A role for the cytoskeleton has been proposed in sensing cell volume in cells in general (Mills, 1987; Hoffmann and Simonsen, 1989; Watson, 1991) and in red cells in particular (Garay, Nazaret, Hannaert, and Cragoe, 1988).

If a mechanical signal can activate cotransport in sheep cells, the question arises why Jennings and Schulz (1990) saw no activation in rabbit cells with mechanical distortion of the membrane. If the two-state model applies to rabbit red cells, there is no B → C conversion. Perhaps the A → B conversion in sheep cells, as in rabbit cells, cannot be provoked by a mechanical signal, but by a dilution of a cytoplasmic solute as Jennings and Schulz (1990) have proposed (though a specific solute was not implicated). Our results (Fig. 10) suggest that in sheep cells, dilution of $[Mg]_c$ is not the primary signal (but it could be in rabbit cells). Colclasure and Parker (1992) recently proposed that dilution of total cytoplasmic protein is the signal for swelling activation of K-Cl cotransport in dog red cells. These authors claimed that their results were not comparable to ours on IOVs since much of the volume-dependent K flux in IOVs was Cl independent. In fact the Cl-dependent K flux was increased sixfold by fourfold swelling (Kracke and Dunham, 1990). Obviously the question about the signal(s) of swelling is unsettled; the answer is more complex than originally envisioned if two signals operate in series.

Role of Phosphate Metabolism

Jennings's group (Jennings and Al-Rohil, 1990; Starke and Jennings, 1993) has argued that two separate reactions control the

$$A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} B$$

conversions in rabbit cells, for which a two-state model applies. They presented evidence that k_{12} is the rate constant for a volume-insensitive protein phosphatase and suggested that k_{21} is the rate constant for a volume-sensitive (swelling-inhibited) protein kinase. In preliminary experiments, we showed that a protein phosphatase inhibitor, okadaic acid, inhibits K-Cl cotransport in sheep red cells (Dunham, 1992),

and this may be the A → B reaction. B → C in sheep cells is unlikely to be promoted by the same kind of protein phosphatase envisioned for A → B in rabbit cells if the latter is volume insensitive; we proposed above that B → C in sheep cells is promoted by a swelling-activated reaction.

The increase in J_{max}, which we suggest is associated with the A → B conversion, must be due to a decrease in k_{21}, the rate constant for a reaction requiring Mg, presumably a swelling-inhibited, Mg-dependent protein kinase. As argued above, swelling inhibition of this Mg-dependent reaction cannot be accounted for by dilution of a^c_{Mg}, so there is some other signal of swelling that inhibits this reaction, perhaps by modifying the binding of Mg to something, though to what and how this could work are not understood. Even less can be said about the proposed decrease in $K_{1/2}$ for K_o associated with the B → C conversion, except that there is no strong evidence for a role of a kinase or a phosphatase (Sachs, J. R., and D. W. Martin, manuscript submitted for publication). We hope that our proposal for a mechanism for signal transduction for volume-sensitive K-Cl cotransport in sheep red cells will lead to experiments that will help illuminate regulation of transport pathways involved in the control of cell volume.

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