Kinetics of Activation and Inactivation of Swelling-stimulated K^+/Cl^- Transport

The Volume-sensitive Parameter is the Rate Constant for Inactivation

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ABSTRACT Red blood cells of several species are known to exhibit a ouabain-insensitive, anion-dependent K^+ (Rb^+) flux that is stimulated by cell swelling. We have used rabbit red cells to study the kinetics of activation and inactivation of the flux upon step changes in tonicity. Sudden hypotonic swelling (210 mosmol) activates the flux after a lag period of 10 min at 37°C and 30–50 min at 25°C. In cells that were preswollen to activate the transporter, sudden shrinkage (by addition of hypertonic NaCl) causes a rapid inactivation of the flux; the time lag for inactivation is <2 min at 37°C. A minimal model of the volume-sensitive KCl transport system requires two states of the transporter. The activated (A) state catalyzes transport at some finite rate (turnover number unknown because the number of transporters is unknown). The resting (R) state has a much lower or possibly zero transport rate. The interconversion between the states is characterized by unimolecular rate constants

$$R \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A .$$

The rate of relaxation to any new steady state is equal to the sum of the rate constants $k_{12} + k_{21}$. Because the rate of transport activation in a hypotonic medium is lower than the rate of inactivation in an isotonic medium, we conclude that the volume-sensitive rate process is inactivation (the A to R transition); that is, cell swelling activates transport by lowering k_{21}. Three phosphatase inhibitors (fluoride, orthovanadate, and inorganic phosphate) all inhibit the swelling-activated flux and also slow down the rate of approach to the swollen steady state. This finding suggests that a net dephosphorylation is necessary for activation of the flux and that the net dephosphorylation takes place as a result of swelling-induced inhibition of a kinase rather than stimulation of a phosphatase.

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Volume 95 June 1990 1021–1040

INTRODUCTION

Many cells exhibit solute transport systems that are influenced by changes in cell volume. In general, the volume-sensitive fluxes cause cell volume to return toward normal after an acute swelling or shrinkage. Several different volume-sensitive transport processes have been identified in nature, and there seems to be no general single mechanism for cell volume regulation (Kregenow, 1981; Pierce, 1982; Parker, 1983; Cala, 1980, 1983; Grinstein et al., 1984).

Little is known about the mechanisms by which a change in cell volume activates or inactivates transport. The main information available on this issue comes from studies in which transport is activated in the absence of a volume change. For example, Na-K-Cl cotransport in avian red cells can be activated either by cell shrinkage or by catecholamines (see McManus et al., 1985). Amiloride-sensitive Na^+-H^+ exchange is activated either by cell shrinkage or by low intracellular pH, growth factors, or, in some cells, elevation of intracellular Ca^{++} (see Grinstein et al., 1989). Hypoxia can activate the same process in fish red cells (see Mahe et al., 1985). Na-independent KCl cotransport is activated by cell swelling or by slight intracellular acidification (Brugnara et al., 1985), N-ethylmaleimide (NEM) treatment (Lauf, 1985), or high hydrostatic pressure (Hall and Ellory, 1986*a*).

In addition to interventions that activate transport in the absence of volume change, there are also interventions that prevent cell volume from affecting transport. For example, glutaraldehyde treatment can "fix" the dog red cell Na^+-H^+ exchanger in a volume-insensitive state (Parker, 1984). Norepinephrine treatment prevents cell swelling from activating KCl cotransport in duck red cells (Haas and McManus, 1985). All these studies provide interesting information about possible mediators of volume effects on transport, but the level of understanding of the signaling mechanisms is still very primitive and certainly no general principles have emerged.

The present study concerns the swelling-activated, anion-dependent K^+ transport system of mammalian red cells (see Dunham, 1990, for recent review). The transport is Na-independent, and recent work by Brugnara et al. (1989) indicates that a Cl^- gradient can drive an uphill K^+ flux; accordingly, we will refer to the swelling-stimulated K^+ (Rb^+) flux as "KCl cotransport." The swelling-stimulated KCl cotransport flux is detectable in unfractionated human red cells (Kaji, 1986), but the effect of cell volume is much more pronounced in the younger cells (Canessa et al., 1987; Brugnara and Tosteson, 1987; Hall and Ellory, 1986*b*; O'Neill, 1987, 1989). The KCl cotransport flux is much larger in human SS and CC cells than in AA cells (Brugnara et al., 1985, 1986; Canessa et al., 1986; Berkowitz and Orringer, 1987). In the LK sheep red cell, and presumably in other species, the swelling-stimulated and NEM-stimulated fluxes are very likely catalyzed by the same transport protein (Lauf, 1984). In rabbit, LK sheep, and human SS cells, NEM treatment removes most of the volume sensitivity of KCl cotransport. Human red cell KCl cotransport, however, is still sensitive to cell volume after NEM treatment (Dunham and Benjamin, 1985; Lauf et al., 1985). Raising the intracellular [Mg^{++}] inhibits KCl cotransport in human and LK sheep red cells (Brugnara and Tosteson, 1987; Lauf, 1985; Lauf et al., 1985; Sachs, 1988).

Although there have been many dozen studies of the effects of cell volume on various transport processes, there have been very few studies of the rates of change

of transport after step changes in cell volume. Sarkadi et al. (1984) showed that volume-sensitive conductances in lymphocytes have complex time dependences, with a rapid, time-dependent inactivation under some conditions. Kim et al. (1989) recently demonstrated that swelling-activated KCl cotransport in pig red cells requires a time lag of several minutes. Kregenow (1971) had originally observed a slight time lag in the activation of the same process in duck red cells. Dunham (1989), Lauf (personal communication) and McManus (personal communication) have also observed a time lag for activation of the flux in LK sheep and duck red cells, respectively.

The work described below is a study of the rates of activation (by cell swelling) and inactivation (by an imposed cell shrinkage) of KCl cotransport in young rabbit red cells. These cells have the advantage that the swelling-stimulated fluxes are large enough to measure accurately (Al-Rohil and Jennings, 1989) but still slow enough that the cell does not shrink back down to normal volume before a steady-state KCl cotransport flux can be measured. We find that the time lag for activation of KCl cotransport by swelling is much longer than that for inactivation of transport by sudden shrinkage of preswollen cells. Analysis of a simple kinetic model of the system indicates that cell swelling stimulates transport by decreasing the rate constant for inactivation. The effects of three inhibitors of phosphatases indicate that the activation of transport by cell swelling involves a net dephosphorylation caused by the inhibition of a kinase rather than the activation of a phosphatase. This is to our knowledge the first published analysis of the rates of activation and inactivation of a volume-sensitive transport system. A preliminary report of this work has been presented (Jennings and Al-Rohil, 1989).

MATERIALS AND METHODS

Rabbit Red Blood Cells

Blood was drawn by cardiac puncture from New Zealand white rabbits immediately after a lethal injection of pentobarbital sodium through a marginal ear vein. (The rabbits were killed to obtain not only the blood, but also the intestine, urinary bladder, skeletal muscle, and brain for studies in other laboratories; the animals were killed in accordance with National Institutes of Health guidelines.) The anticoagulant was ethylenediaminetetraacetate (EDTA).

Cells were usually used on the same day or after at most 2 d of storage as whole blood at 4°C. To maximize the swelling-stimulated flux, the least dense one-third of the cells were isolated on a Percoll-Renografin gradient (Speiser and Etlinger, 1982). In previous work (Al-Rohil and Jennings, 1989), we had discarded reticulocytes, but in the present study reticulocytes were included in the "light" cell fraction. The rabbits had not been previously bled, and the reticulocyte count in the "light" fraction was generally 2–3%. After density gradient centrifugation, cells from the top third of the gradient were washed twice and suspended in 10 vol of *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES)-buffered physiological saline (HPS): 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM NaH_2PO_4, 10 mM glucose, 10 mM HEPES, pH 7.5 (measured at 23°C). The suspension was incubated 1 h at 37°C in order to attempt to establish a reproducible physiological steady state.

Other Materials

Salts and buffers were from either Sigma Chemical Co., St. Louis, MO or Fisher Scientific Co., Pittsburgh, PA. ^{86}RbCl was purchased from DuPont-New England Nuclear, Boston, MA.

Sodium orthovanadate (Na_3VO_4) was from Fisher Scientific Co.; a stock solution of 100 mM was prepared and titrated to pH 7.4 with morpholine propane sulfonic acid (MOPS).

Influx Measurements

Influx of $^{86}Rb^+$ was measured essentially as described previously (Al-Rohil and Jennings, 1989), but with different flux media. The standard starting medium in these experiments was HEPES-buffered saline with ouabain (HBSO): 160 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, at 37°C 10^{-4} M ouabain. In early experiments the NaCl concentration was 150 mM; the concentration was 160 mM in later experiments because the basal flux is smaller in the slightly hypertonic solution and the relative effect of cell swelling is larger. The protocols for altering the tonicities of the suspensions during flux measurements are given in the figure legends. Bumetanide was not included in the flux media; rabbit red cells appear to lack the Na-K-Cl cotransporter (Al-Rohil and Jennings, 1989). Unidirectional influx was calculated assuming that $^{86}Rb^+$ is an ideal tracer for K^+. In the plots of intracellular $^{86}Rb^+$ vs. time, 1 ml of cells refers to the number of cells that would occupy 1 ml in an isotonic medium.

Data Analysis

Influx time courses were fit to the two-state model (see below) using a nonlinear least squares iteration according to the Marquardt algorithm (Bevington, 1969). The computation was performed on a personal computer with "NFIT" (Island Products, Galveston, TX). The program was written by Bruce Simon and Bill Little of this department. The parameters derived from the curve-fitting are indicated in the text and figure legends. For some experiments, not enough data points were taken after cell swelling for a quantitative analysis.

THEORY

Time Course of Shifts from One Steady-State Flux to Another

The purpose of the studies in this paper is to test a simple theoretical model for the activation and inactivation of anion-dependent K^+ transport by cell swelling and shrinkage. The experiments are not concerned with the kinetics of transport itself; all measurements were performed at a single extracellular K^+ concentration. At this single K^+ concentration, the ouabain-insensitive unidirectional $K^+/^{86}Rb^+$ flux is assumed to be a measure of the number of functioning KCl cotransporters. The rate of change of this flux thus reflects the rate of activation or inactivation of transporters.

A minimal model of the swelling-activated transport system requires two states of the transporter: resting (R) and activated (A). These states do not refer to intermediates in the catalytic cycle for transport itself, but rather are regulatory states. The R state catalyzes transport slowly or not at all, and the A state catalyzes transport at some appreciable rate. According to the minimal model, the transitions between these two states are characterized by unimolecular rate constants, which in general are affected by cell volume through unknown signaling mechanisms:

$$R \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A.$$

Cell swelling induces a net conversion of R states to A states; according to the model, this increase in A states must be caused by an increase in k_{12} and/or a

decrease in k_{21}. It can be shown very easily that in two-state systems the rate of approach to any new steady state is given by the sum of the forward and reverse rate constants, evaluated at the new steady state. The following derivation is essentially identical to that for the relaxation kinetics of any two-state system (see Czerlinski, 1966).

Start by assuming that each cell contains an ensemble of independently functioning KCl cotransporters, each of which can be in either of the two states. For any given steady state the actual proportions of the A and R states are not known, but it is still possible to write an expression for the time course of the transition from one steady state to another after an abrupt change in cell volume. Begin at a steady state with initial flux J_0. In this steady state there is some fraction A_0 of transporters in the A state. The remainder $(1 - A_0)$ are in the R state. Suddenly the cell volume is changed by adding water or hypertonic NaCl. The change in cell volume alters one or both the rate constants k_{12} and k_{21}, and the system relaxes to a new steady state, in which a new fraction A_1 of the transporters is in the A state. In the new steady state, the fraction A_1 is related to the rate constants as follows:

$$k_{12}(1 - A_1) = k_{21}A_1 . \tag{1}$$

At any time during the approach to the new steady state, let $A = A_1 + x$, where x represents the departure from the final new steady state. From the definition of the forward and reverse rate constants.

$$dx/dt = k_{12}(1 - A_1 - x) - k_{21}(A_1 + x) . \tag{2}$$

In this equation the rate constants k_{12} and k_{21} have the magnitudes that apply to the new conditions (i.e., after the sudden swelling or shrinkage). Substituting Eq. 1 into Eq. 2 gives a very simple differential equation with the following solution:

$$x = (A_0 - A_1) \exp [-k_{12} + k_{21})t] . \tag{3}$$

So the time course of the fraction of transporters in the activated state is

$$A = A_1 + (A_0 - A_1) \exp [-(k_{12} + k_{21})t] . \tag{4}$$

Thus, the relaxation to any new steady state, after a sudden swelling or shrinkage of the cells, proceeds at a rate given by the sum of the forward and reverse rate constants.

It is now possible to express the unidirectional $^{86}Rb^+$ flux (at a given K/Rb concentration) as a function of the proportions of R and A states. Let J_R be the total KCl cotransport flux if all copies of the system were in the R state; this flux could be zero but need not be. Let J_A be the flux that would be mediated by the system if all copies were in the A state. The actual flux, then, for any given steady state, is

$$J = (1 - A)J_R + (A)J_A . \tag{5}$$

Eqs. 4 and 5 can be combined and rearranged to the following:

$$J = J_1 + (J_0 - J_1) \exp [-(k_{12} + k_{21})t] , \tag{6}$$

where J_0 is the flux before the step change and J_1 is the final steady state flux. The accumulated $^{86}Rb^+$ (under conditions of zero back flux) is given by the time integral

of the unidirectional influx:

$$^{86}Rb_{in} = J_1 t + (J_1 - J_0)(k_{12} + k_{21})^{-1}\{\exp[-(k_{12} + k_{21})t] - 1\}. \quad (7)$$

Eq. 7 describes the time course of the $^{86}Rb^+$ accumulation after any abrupt change (increase or decrease) in cell volume at $t = 0$. There are in general three unknowns: the initial steady-state flux, the final steady-state flux, and the relaxation time $(k_{12} + k_{21})^{-1}$. A three-parameter fit of the data is possible, but in most experiments we have independent estimates of the initial flux. Therefore, most of the curve-fits were with two adjustable parameters: the final steady-state flux and the relaxation time.

RESULTS

Stability of Cell Volume

The experiments were designed to measure the rates of transition from one steady-state flux to another. That is, cell volume is viewed as the independent variable, to

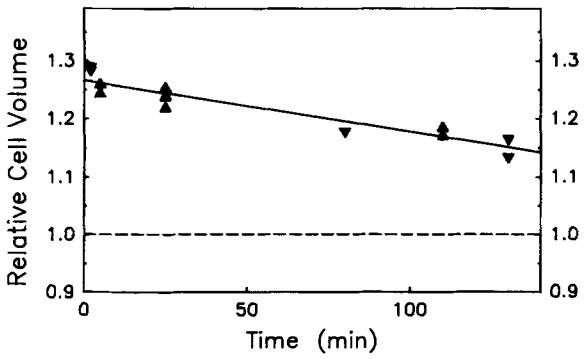

FIGURE 1. Time course of cell volume under the conditions of the $^{86}Rb^+$ influx measurements. Young rabbit red cells were prepared as described in Materials and Methods and preincubated 1 h at 37°C in HPS plus glucose. The cells were centrifuged and resuspended in HBSO (160 mM NaCl, 5 KCl, 10 mM HEPES, pH 7.5, at 23°C), 10^{-4} M ouabain. The suspension was diluted with distilled water to a final osmolality of 210 mosmol, and the cells were incubated further at 37°C. At the indicated times, aliquots were removed and centrifuged, and the cell water was determined from the wet weight, dry weight, and hematocrit of an 80–90% suspension. The different symbols refer to two different preparations of cells. Cell water is expressed relative to that of cells in 300 mosmol medium.

be stepped to various values. The experiments would be very difficult to interpret if the cell volume itself were not stable over the times needed for the flux to reach a new steady state. Fortunately, rabbit red cell volume is relatively stable for time periods of up to 1 h under the conditions of the transport experiments. Fig. 1 shows the time course of cell water for cells suspended in a hypotonic medium under the same conditions as were used to measure $^{86}Rb^+$ influx. The hypotonicity causes the expected 25% increase in cell volume, and, after 1 h in the hypotonic medium, the cells are still 20% larger than normal. These cells do of course have a substantial swelling-stimulated net KCl efflux (Davson, 1937; Al-Rohil and Jennings, 1989). The rate constant for $^{86}Rb^+$ efflux under these conditions is ~0.2/h in swollen cells. On the basis of the $^{86}Rb^+$ efflux, a somewhat more rapid volume regulatory

decrease would be expected. However, ouabain is included in the flux medium, and the net NaCl influx partially offsets the swelling-induced KCl efflux. We have not made a systematic study of the rate of net water loss compared with the $^{86}Rb^+$ efflux in the same cells. The important point for the present purposes is that, after acute cell swelling in the presence of ouabain, cell volume is reasonably stable at 37°C for at least 60 min.

Possible Effects of Heterogeneous Cell Population

It is well established that, in both human and rabbit red cells, the least dense cells have the highest swelling-sensitive fluxes (e.g., Canessa et al., 1987; Brugnara and Tosteson, 1987; O'Neill, 1989; Al-Rohil and Jennings, 1989). Our experiments were all performed on a cell preparation in which the densest two-thirds of the cells were discarded so as to eliminate the cells that have only small swelling-stimulated fluxes. In our cell preparations, most of the cells undergo a slow regulatory volume decrease, as demonstrated by the time course of the water content (Fig. 1) and by osmotic fragility measurements after incubation in hypotonic medium (Al-Rohil, 1988). Thus, although there are undoubtedly variations in the flux among cells of different ages even within a density-separated fraction, these variations are much less pronounced than in unfractionated human AA cells (O'Neill, 1989).

Time Lag for Activation of Transport

It is difficult to use efflux measurements in a study of the time course of activation of the swelling-stimulated transport. In the first few minutes after cell swelling, 2% of the intracellular radioactivity has left the cells, and even a trace of lysis in the hypotonic medium could cause a significant overestimate of the initial efflux. Slight errors in the early time points after cell swelling make it difficult to detect possible time lags in the onset of the swelling-stimulated efflux. In contrast, the same degree of lysis causes only a trivial error in the influx determination. For this reason, all the results presented here were obtained from tracer ($^{86}Rb^+$) influx measurements. The influx is measured over times in which there is negligible backflux (the intracellular specific activity is <5% of the extracellular specific activity).

Fig. 2 shows the time course of entry of $^{86}Rb^+$ into young rabbit red cells from a medium containing 5 mM K^+ and 10^{-4} M ouabain. Initially the medium is isotonic and the influx is relatively slow. At the arrow, the tonicity of the suspension is lowered from 310 to 215 mosmol. The flux begins to increase until a new steady state is reached after about 25 min. (The time course of the influx for longer incubations is more complicated because the cells do shrink slowly and the flux declines.) The time course of activation of transport after sudden hypotonic swelling clearly displays a lag that can be estimated visually from the point of intersection of the lines representing the initial and final steady-state fluxes. The lag can be estimated more objectively by fitting the data to the two-state model. Curve-fitting gives a slightly larger time lag than would be estimated graphically, probably because the eye tends to underestimate the final steady-state flux. In 14 separate preparations of cells, we have found that the time lag for approach to the swollen steady state is 10 ± 4 min (mean ± SD) under these conditions (37°C, 210 ± 5 mosmol). In Br^- medium, in which the flux itself is more rapid than in Cl^- (Al-Rohil and Jennings, 1989), the

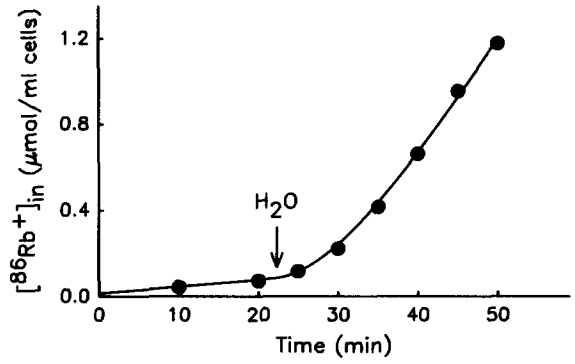

FIGURE 2. Time course of ouabain-insensitive $^{86}Rb^+$ influx before and after a sudden increase in cell volume. Cells were preincubated in HPS plus glucose for 1 h, then centrifuged, and resuspended at 37°C, 2.5% Hct, in HBSO (150 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.5, at 23°C), 10^{-4} M ouabain. At $t = 0$, $^{86}RB^+$ was added, and the intracellular $^{86}Rb^+$ in l-ml aliquots of the suspension was measured at the indicated times. At $t = 22$ min, 5 mM KCl plus $^{86}RbCl$ was added to lower the osmolality from 300 to 215 mosmol while keeping the extracellular concentration of K^+ and $^{86}Rb^+$ constant. The curve through the data is fit to the equivalent of Eq. 7 (with axes offset because the swelling is not at $t = 0$). The fitting procedure fixed the initial flux at the value measured over the first 20 min. The derived time lag $[(k_{12} = k_{21})^{-1}]$ is 10.5 min. A single time course is shown, typical of at least 15 others.

time lag for activation is indistinguishable from that in Cl^- (two experiments; data not shown).

Effect of Degree of Cell Swelling

In three experiments the extent of cell swelling was varied to determine the effect of final cell volume on the rate of approach to the new steady state (Fig. 3). The final steady-state flux is of course larger in cells that are more swollen, and the time lag appears to be larger for higher degrees of swelling. The longer time lag for the higher final flux is consistent with the idea that cell swelling causes a decrease in k_{21}, the rate constant for the transition from the A state to the R state (see below).

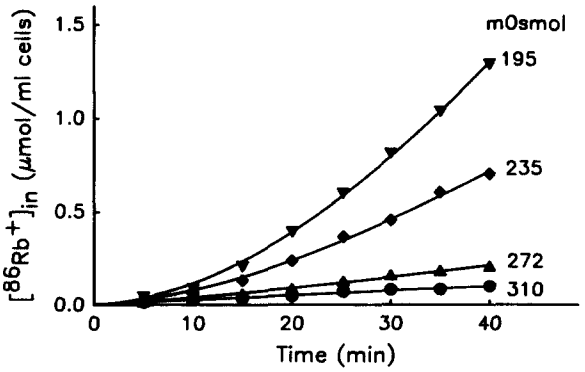

FIGURE 3. Time course of ouabain-insensitive influx into cells suspended at 37°C in HBSO plus varying amounts of 5 mM KCl to give the indicated final osmolality. The cell swelling and $^{86}Rb^+$ addition were both at $t = 0$. Intracellular $^{86}Rb^+$ was determined as in Fig. 1. The curves through the data were fit to Eq. 7, with the initial flux derived from that in the 310 mosmol medium. The derived time lags for cells in 272, 235, and 195 mosmol media were 14, 26, and 45 min, respectively. In two other experiments the derived time lags at the same three osmolalities were 2.1, 10, and 14 min, and 10, 28, and 30 min, respectively. Although there is considerable variability in the absolute values of the time lags among different experiments, the time lag was an increasing function of cell volume in all three experiments.

Time Course of Inactivation by Cell Shrinkage

Fig. 4 depicts the time course of $^{86}Rb^+$ influx into cells that were swollen hypotonically (210 mosmol) 20 min before the addition of tracer. The 20-min preincubation is sufficiently time to activate the anion-dependent transport to roughly 90% of the steady-state value, and the initial tracer influx, as expected, is large. At the indicated time, the cells were shrunk back to normal volume by the addition of hypertonic NaCl, which raised the osmolality from 210 to 310 mosmol. The flux abruptly returned to a low level, with a time lag not exceeding 2 min. In six cell preparations, the time lag for inactivation of the flux after shrinkage was 1.4 ± 0.4 min at 37°C. The large uncertainty in the delay time reflects the difficulty of the measurements; on the time scale of the inactivation of the transport, there is little change in the intracellular radioactivity and it is therefore hard to obtain an exact time course of the change in flux. Although the precise time lag for inactivation of transport is uncertain, we are confident that the relaxation time for inactivation in an isotonic or hypertonic medium is shorter than that for activation in a hypotonic medium.

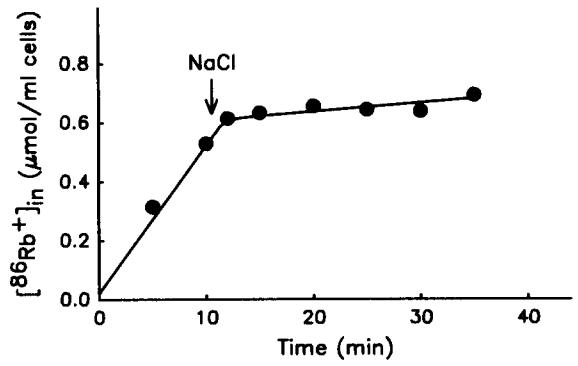

FIGURE 4. Time course of ouabain-insensitive $^{86}Rb^+$ entry into preswollen cells before and after sudden shrinkage by the addition of concentrated NaCl. Cells were prepared exactly as in Figs 1–3 and were swollen in HBSO plus water (210 mosmol) for 20 min to allow the KCl cotransport to be activated. At t = 0, $^{86}Rb^+$ was added and the time course of tracer influx measured as usual. At the arrow, NaCl was added from a 1.5 M stock to raise the osmolality to 310 mosmol. The curve through the data, starting at the NaCl addition, is derived from Eq. 7 with a time lag of 1.0 min. A single time course is shown, representative of six others.

Effect of Temperature

Ellory et al. (1985) have shown that the swelling-stimulated KCl flux is very temperature-dependent: lowering the temperature from 40°C to 30°C causes a 10-fold reduction of the maximum flux. The large temperature effect could have at least three origins. The transport process itself (i.e., the number of ions transported per second by a single activated transporter) would be expected to have a finite temperature dependence. In addition, both the rate and the extent of activation at a given cell volume could depend on temperature. Fig. 5 depicts the time course of $^{86}Rb^+$ influx into cells suspended in the same hypotonic medium at two different temperatures. Cell volume is approximately the same at each temperature. (The Donnan ratio for Cl^- will not change much with temperature under these conditions because the extracellular and intracellular hydrogen ion buffers have similar temperature dependences.) At 37°C in this experiment, the influx activated with a delay time of

about 11 min. At 25°C, the steady-state flux was about threefold lower than at 37°C, and the approach to the new steady state was also about threefold slower. In five separate preparations of cells the delay time for activation of the flux in a 210 msmol medium averaged 55 min at 25°C (range 23–101 min). Although there is large uncertainty in the activation rate at 25°C, it is clear that lowering the temperature from 37°C to 25°C has a sizable effect on the rate; the apparent E_a (activation energy) of the activation process is 20–25 kcal/mol.

As mentioned above, the temperature dependence of the steady-state flux in a hypotonic medium might not reflect the temperature dependence of the flux through an activated transporter, because the number of A states in a given medium could depend on temperature. That is, the forward and reverse rate constants k_{12} and k_{21} could have different temperature dependences. We attempted to measure the effect of temperature on the transport process itself, independent of the effects of cell volume, by activating the transport with NEM (see Lauf, 1985). With maximal treatment with NEM, the anion-dependent flux in rabbit red cells is not

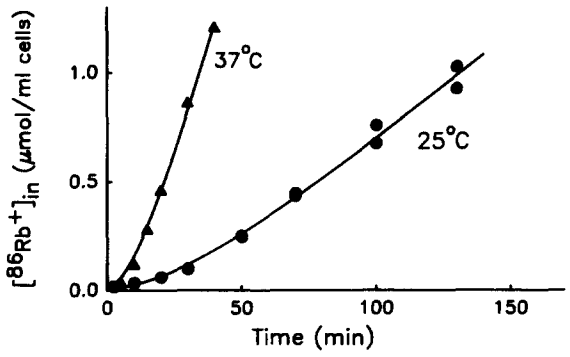

FIGURE 5. Temperature dependence of the rate of activation of swelling-stimulated $^{86}Rb^+$ influx. Cells were prepared as in Figs. 1–3 and suspended in 6 ml of HBSO plus 3 ml of 5 mM KCl (210 mosmol). Cell swelling and ^{86}Rb addition were both at t = 0. One pair of fluxes was performed at 37°C; another (same cells, same medium) was performed at 25°C. The time lag at 37°C in this experiment is ~11 min; that at 25°C is ~35 min.

affected by cell swelling, presumably because NEM has locked the system into a swelling-independent, activated state (Al-Rohil and Jennings, 1989). The temperature dependence of the NEM-stimulated flux is shown in Fig. 6. The flux is only slightly affected by temperature between 25°C and 37°C; the apparent activation energy of the flux is ~9 kcal/mol. The small temperature dependence of the NEM-activated flux suggests that the transport process itself has a low activation energy. The higher temperature dependence of the swelling-activated flux could be explained by a temperature dependent shift in the proportion of active and inactive transporters at a given cell volume.

Time Course of Shrinkage-induced Inactivation of 25°C

Although the time course of the activation of transport after hypotonic swelling is considerably slower at 25°C than at 37°C, the time course of inactivation after sudden shrinkage is still quite rapid at 25°C (Fig. 7). The flux was activated for 60 min

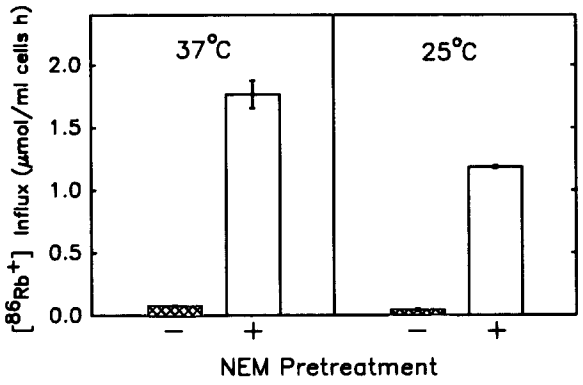

FIGURE 6. Temperature dependence of NEM-stimulated $^{86}Rb^+$ influx. Cells were incubated in HBS, then split in half, and one half was exposed to 2 mM NEM, 15 min, 37°C. Both halves were washed and then resuspended in HBSO at either 37°C or 25°C, and the influx of $^{86}Rb^+$ was measured over a 20-min interval (during which the time course is linear). The data shown are from a single experiment (mean and range of duplicate determinations). The identical effect of temperature was observed in a separate experiment.

at 25°C in a 205 mosmol medium before the addition of hypertonic NaCl to inactivate the transport. The lag time for inactivation at 25°C is 2.8–3.5 min (range, two experiments).

Approach to the Same Steady State from Two Different Directions

To test further the two-state model of transport regulation, we attempted experiments in which the cells approach the same steady-state flux from two different directions. If the transport regulation can be modeled as a two-state process, then the rate of approach to any new steady state should depend only on the final conditions, and not on the direction of the approach. (In algebraic terms, the parameter x

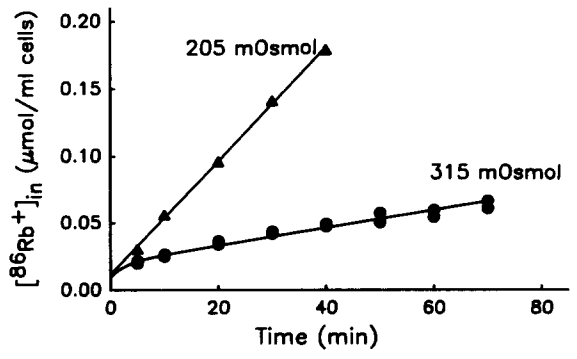

FIGURE 7. Time course of inactivation of swelling-activated $^{86}Rb^+$ influx at 25°C by a sudden increase in osmolality. Cells were prepared as usual and preincubated in 105 mM NaCl, 7 mM HEPES, pH 7.5, 10^{-4} M ouabain, 205 mosmol, for 1 h at 25°C to activate the swelling-stimulated flux. At t = 0, 5 mM KCl/^{86}RbCl was added to all the suspensions. In two of the suspensions, concentrated NaCl was also added at t = 0 to raise the osmolality to 315 mosmol. In the cells that continued to be swollen, the influx was reasonably linear, but in the cells that were returned to normal volume, the influx was inactivated rapidly. Indistinguishable results were obtained in two other experiments at this temperature.

in Eq. 2 can be either positive or negative, and the rate of decay of x is independent of the sign.) The steady state in this case is the following: swollen cells (210 mosmol), pH 7.4, 25°C. One set of cells was initially at 25°C in isotonic medium and then swollen (with $^{86}Rb^+$ added simultaneously) to measure the time lag for activation of transport as in Figs. 2 and 5. Another aliquot of the same cells in the same hypotonic medium was preincubated 20 min at 37°C to activate a larger proportion of the transporters, and then the temperature was quickly shifted (within 2 min) to 25 ± 1°C immediately before adding $^{86}Rb^+$. In the cells that had been at 37°C, the initial flux is rapid and then decays to a lower value with a time lag of ~30 min. In the cells that were simply exposed to a hypotonic medium at 25°C, the flux starts out small and increases to a new steady state, with a similar time lag (Fig. 8). To a first approximation, therefore, the rate of approach to the 25°C steady state in

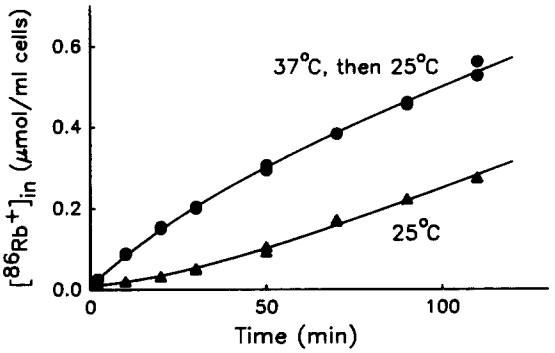

FIGURE 8. Time course of the approach to the same steady state from two different directions. One half of a preparation of cells was suspended at 25°C in hypotonic medium made from 6 ml of HBSO plus 3 ml 5 mM KCl; cell swelling and $^{86}Rb^+$ addition were both at $t = 0$ (▲). The other half of the preparation was suspended in hypotonic medium without $^{86}Rb^+$ and incubated 20 min at 37°C to activate the swelling-stimulated transport. Then the cells were chilled to 25°C over a time period of <2 min, and $^{86}Rb^+$ was added at $t = 0$ (●). The initial flux was more rapid in the warm preincubated cells; a finite amount of time was needed for the system to return to the 25°C steady state. The curves through the data represent Eq. 7, with the final steady-state flux equal to 0.20 μmol/ml cells per hour for both sets of data. The time lag for the flux increase was 39.8 min (▲); the time lag for the flux decrease was 34.0 min (●). Essentially identical results were obtained in two other experiments.

swollen cells is the same from either direction, in keeping with a simple two-state model.

Effects of Phosphatase Inhibitors

Given the sizable literature on the regulation of various transport processes by phosphorylation/dephosphorylation cycles (e.g., Beam et al., 1979; Grinstein et al., 1986, 1989; Altamirano et al., 1988), we tested the effect of three known phosphatase inhibitors: fluoride, vanadate, and inorganic phosphate (Li, 1982). All three inhibit the swelling-stimulated flux (Fig. 9). Cells were preincubated with vanadate or inorganic phosphate to allow entry into the cells. No preincubation was necessary for F^-, because it is rapidly permeant (Tosteson, 1959). The phosphatase inhibitors lowered the basal flux very slightly, but in the 310 mosmol medium in which the basal flux was measured, the KCl cotransporter is activated only to a very small extent, and inhibition of this flux is hard to detect.

The inhibitory effect of the above agents on the swelling-stimulated flux appears to be caused by a decrease in k_{12}, as indicated by the time course of activation of the flux in the presence of low concentrations of the phosphatase inhibitors (Fig. 9, *lower panels*). In four experiments with 2 mM F^- and two experiments with 10 mM phosphate, the inhibitors decreased the rate of activation of the flux. Moreover, in swollen cells, the continued presence of 8 mM F^- (added after transport was activated) causes a slow decline in the flux (Fig. 10). These findings are consistent with

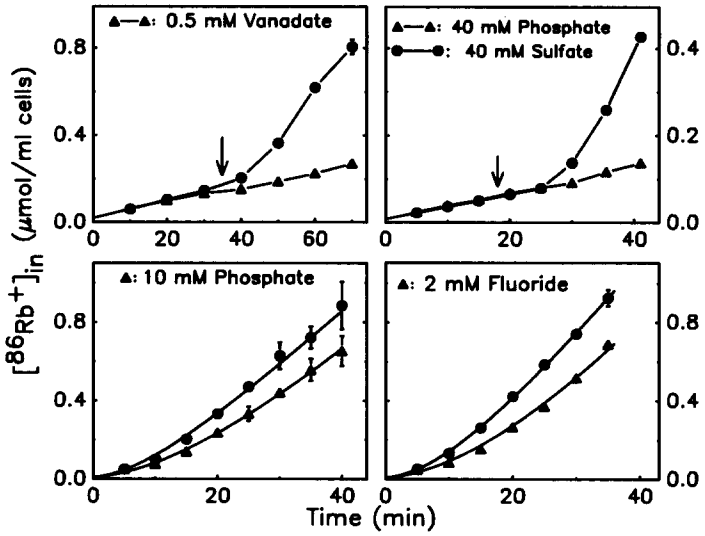

FIGURE 9. Inhibition of swelling-stimulated $^{86}Rb^+$ influx by orthovanadate, phosphate, and fluoride. *Upper left:* Cells were incubated 15 min in 150 mM NaCl, 10 mM HEPES, 10^{-4} M ouabain, with or without 0.5 mM vanadate before addition of $^{86}RbCl$ with 5 mM KCl at $t = 0$. At the arrow, the osmolality was lowered to 210 mosmol. Triangles represent the suspensions with vanadate. The intracellular vanadate concentration is unknown but is less than the extracellular concentration, because the Donnan ratio is less than unity and because of reduction of vanadate (Macara et al., 1980). Data shown are mean and range of duplicate determinations. *Upper right:* Same as upper left, except that 55 mM of the NaCl was replaced with either 40 mM Na_2SO_4 or 40 mM Na_2HPO_4/NaH_2PO_4. The comparison of sulfate with phosphate was performed to ensure that the phosphate inhibition is not caused by removal of some of the chloride. Single experiment representative of three. *Lower left:* Cells were swollen simultaneously with $^{86}Rb^+$ addition. The triangles represent suspensions in which 10 mM NaCl was replaced by 8 mM Na_2HPO_4/2 mM NaH_2PO_4 40 min before the beginning of the influx measurement. *Lower right:* Same as lower left, except 2 mM NaF replaced 2 mM NaCl 1 min before the influx began. Similar results were obtained in three other experiments.

the idea that fluoride inhibits swelling-activated transport by way of a decrease in k_{12} rather than an increase in k_{21}. Thus, cell volume appears to affect k_{21} whereas phosphatase inhibitors affect k_{12} (see below).

DISCUSSION

The goal of this study was to derive information from the rates of activation and inactivation of the volume-dependent KCl cotransport flux in rabbit red cells.

According to a very simple two-state model, the rate of relaxation to any new steady state is equal to the sum of the unimolecular rate constants for activation and inactivation of the transport system. We find that the rate of transport activation in swollen cells is much slower than that for inactivation of the flux by shrinking preswollen cells. Cell swelling must cause an increase in the ratio k_{12}/k_{21}, because swelling increases the proportion of activated states. But the rate of approach to the swollen steady state ($k_{12} + k_{21}$) is smaller than the rate of approach to the steady state in cells of normal volume. The only way that cell swelling can cause an increase in k_{12}/k_{21} and a decrease in ($k_{12} + k_{21}$) is if cell swelling causes a decrease in k_{21} rather than an increase in k_{12}. Moreover, the time lag for activation is largest at high cell volumes (Fig. 3). At high cell volume the ratio k_{12}/k_{21} is largest, and the rate of approach to the steady state is the slowest, as expected if k_{21} becomes progressively smaller at higher degrees of swelling.

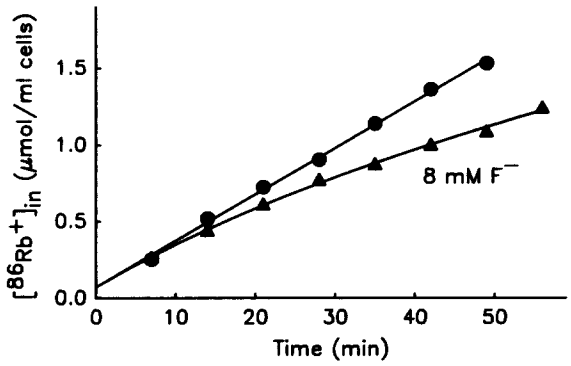

FIGURE 10. Progressive inhibition of $^{86}Rb^+$ influx by fluoride in preswollen cells. Cells were prepared as usual, suspended in 105 mM NaCl, 7 mM HEPES, pH 7.5, 10^{-4} M ouabain, and incubated 20 min at 37°C to activate the swelling-stimulated transport system. To one suspension, NaF was added from a 100 mM stock solution to a final concentration of 8 mM. 1 min later, ^{86}RbCl/5 mM KCl was added and the time course of influx was measured as usual. Similar results, but with less inhibition, were obtained in a separate experiment with 2 mM NaF.

Phosphatase Inhibitors

Rate analysis provides insight into the effects of phosphatase inhibitors. Three inhibitors (fluoride, vanadate, and inorganic phosphate) all lower the swelling-stimulated flux. The effects are not likely caused by metabolic depletion, because the exposures were short, and inorganic phosphate would not be expected to cause ATP depletion. The effects are also not likely to be caused by changes in intracellular [Mg^{++}], because a decrease in [Mg^{++}] would be expected to stimulate the flux, not inhibit it (Brugnara and Tosteson, 1987; Sachs, 1988). Although fluoride, vanadate, and phosphate are certainly not pharmacologically specific, we believe that the most reasonable interpretation of the effect of all three is that they act by inhibiting a phosphatase. Several phosphatases are known to be in red cells (Usui et al., 1983; Clari et al., 1986; Boivin and Galand, 1986; Cooke et al., 1989), but it is not yet clear which of these is the most likely candidate for the phosphatase of interest here. The phosphatase need not be a protein phosphatase.

The fact that low concentrations of phoshpatase inhibitors slow down the rate of swelling activation of transport (Fig. 9) indicates that the agents cause a decrease in k_{12} rather than an increase in k_{21}. This conclusion, though simple, is an illustration of the power of knowing the rates of change of transport. Fluoride lowers the steady-state flux and also lowers the rate of approach to the steady state. The only way (within the two-state model) that this could happen is if phosphatase inhibition lowers the forward rate constant k_{12}.

Swelling-inhibited Kinase Can Explain the Data

A very simple hypothesis that can explain our data is that cell swelling inhibits a kinase and has little or no effect on the conjugate phosphatase (Fig. 11). The kinase inhibition would cause a net dephosphorylation over time, and this would cause transport activation. In normal cells the system is poised such that in the steady state most of the crucial sites are phosphorylated. It is only when the kinase is inhibited

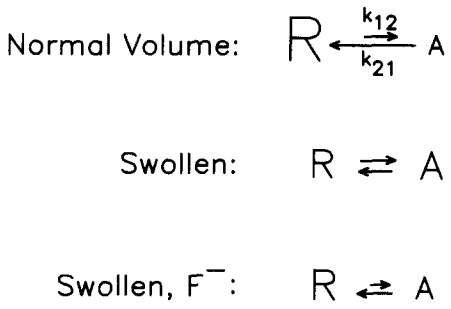

FIGURE 11. Schematic of two-state model for swelling-stimulated KCl transport. The resting state, R, is converted to the activated state, A, at a rate k_{12}. The A state is converted to the R state at a rate k_{21}. In cells of normal volume, k_{21} is much larger than k_{12} and there are very few A states. Cell swelling lowers k_{21} and causes a slow increase in the number of A states. In the presence of a phosphatase inhibitor such as F^-, the rate and extent of activation of the flux are both lower, indicating that phosphatase inhibition lowers k_{12}. Because k_{12} appears to be linked to a phosphatase activity, it is likely that k_{21} is associated with a kinase.

by cell swelling that the dephosphorylation rate becomes comparable to the phosphorylation rate. If cells are swollen in the presence of a phosphatase inhibitor, then the net dephosphorylation is slower, and both the rate and extent of transport activation are smaller, as observed.

Magnesium Effects

The notion of a phosphorylation-dephosphorylation cycle regulating KCl cotransport is consistent with the known inhibitory effect of high [Mg^{++}] on KCl cotransport (Sachs, 1988; Brugnara et al., 1988). The inhibitory effect of Mg^{++} could be explained if high [Mg^{++}] either inhibited the phosphatase or activated the kinase. Dunham (1989) has recently shown that Mg^{++} depletion activates the KCl flux and also eliminates the time lag; these effects are consistent with Mg^{++} inhibition of a phosphatase. Removal of that inhibition would increase k_{12} and thereby activate the flux and shorten the relaxation time.

Relation to Studies on Red Cell Ghosts

In the context of discussing kinases and phosphatases that may regulate KCl cotransport, it is relevant to mention studies in red cell ghosts. Anion-dependent K^+ (Rb^+) transport has been demonstrated in human red cell ghosts (Dunham and Logue, 1986; Brugnara et al., 1988; Sachs, 1988). In ghosts prepared by batch hemolysis/reversal, Dunham and Logue (1986) found that the furosemide-inhibitable K^+ influx is doubled by a 20% cell swelling. Ghosts prepared by this method retain nearly 10% of the original cytoplasm. Sachs (1988) used ghosts prepared by the gel filtration method; these ghosts retain only a very small fraction of cytoplasm. In these ghosts the K^+ influx is volume-dependent, but somewhat less so than those in the Dunham/Logue study. The influx is roughly doubled by a doubling of ghost volume. The observation of a volume-sensitive influx in these ghosts indicates that at least some elements of the volume sensing/signal transduction apparatus are preserved in the absence of cytoplasm. However, a separate study using the same kind of ghosts showed that the K^+ efflux, though anion-dependent, is not affected by cell volume (Brugnara et al., 1988); the reasons for the differences between the two studies are not clear.

Sachs (1988) showed that there is no detectable time lag for either activation or inactivation of the swelling-stimulated KCl flux in human red cell ghosts. This finding is in marked contrast with the present study and those in five other laboratories using intact red cells of pig (Kim et al., 1989), duck (Kregenow, 1971; McManus, personal communication), and LK sheep (Dunham, 1989; Lauf, personal communication). The lack of time lag may be caused by a low Mg^{++} concentration in the ghosts (Dunham, 1989). In any case, the regulation of KCl cotransport is significantly different in intact cells and resealed ghosts.

Relationship to Work Showing ATP Requirement

The suggestion that a net dephosphorylation may activate KCl cotransport seems at odds with the clearly demonstrated requirement of the system for low concentrations of ATP (Dunham and Logue, 1986; Sachs, 1988; Brugnara et al., 1988). However, there are protein phosphatases known that require ATP (Li, 1982; Clari et al., 1986). Complete removal of ATP could inactivate both the kinase and phosphatase associated with transport regulation. With both enzymes inactive, the transport could be frozen in the phosphorylated, inactive state. Therefore, the ATP requirement does not rule out the possibility that kinase inhibition is involved with swelling activation of transport.

Relation to NEM-activated Transport

It is well known that pretreatment of several species of red cells with NEM causes activation of KCl cotransport (see Lauf, 1985); in rabbit red cells the flux appears to be locked by NEM into a volume-independent state (Al-Rohil and Jennings, 1989). In the context of the present data, the stimulatory effect of NEM in principle could be explained by either a strong, irreversible inhibition of the putative swelling-inhibited kinase or an acceleration of the phosphatase. If the latter were true, then phosphatase inhibition should still inhibit the flux following NEM activation. However,

20 mM fluoride does not inhibit the flux in NEM-pretreated cells (Al-Rohil, 1988). This finding is consistent with a direct effect of NEM on the transporter, but given the multiplicity of sulfhydryl groups related to KCl cotransport (see Lauf, 1987), it is not possible at present to distinguish between a direct action of NEM on the transporter and an action on the regulatory machinery. Conversely, though, it is worth pointing out that the lack of effect of fluoride on the NEM-stimulated flux suggests that fluoride does not act directly on the transporter itself, but rather affects regulation.

Temperature Dependence

The temperature dependence of NEM-activated $^{86}Rb^+$ influx is remarkably low: ~9 kcal/mol. (The temperature of the flux measurement, not the NEM pretreatment, is varied.) The only other measurement of temperature dependence of NEM-activated transport is that by Lauf (1983), who found an apparent activation energy of 15 kcal/mol for NEM-stimulated efflux from LK sheep red cells. The E_a of 9 kcal/mol measured here is an underestimate of the E_a of the translocation event for KCl cotransport, because all our measurements were at a single extracellular K^+ concentration. The affinity for extracellular K^+ rises as the temperature drops (see Dunham, 1989). Therefore, the effect of temperature on the influx is not as large as it would be at higher substrate concentration. Similarly, the temperature dependence of the swelling-stimulated influx measured here at 5 mM extracellular K^+ is smaller than that measured by Ellory et al. (1985) at higher concentrations.

The point of our temperature study was not to estimate a true activation energy of a well-defined kinetic event, but rather to learn how temperature affects the relative numbers of transporters in the resting vs. activated state in swollen cells. The most important result is that the steady-state KCl cotransport flux in swollen cells is more temperature-dependent than that of NEM-treated cells. The former changes by a factor of about 3.5 between 37°C and 25°C, and the latter changes by a factor of 1.6 between the same two temperatures. The simplest interpretation of this finding is that, at a given cell volume, there are more transporters in the activated state at 37°C than at 25°C, even if enough time is allowed at each temperature for a steady state to be reached.

The authors acknowledge helpful discussions with John M. Russell. The authors thank Philip B. Dunham, Peter K. Lauf, and Thomas J. McManus for providing data before publication. We thank Dr. Dunham for informing us that the first observation of a time lag was published by Floyd M. Kregenow in 1971, and we thank Mark Allen and Richard Schulz for expert technical assistance.

This work was supported by research grant R01 HL-37479 from the National Institutes of Health. Dr. Jennings was supported by Research Career Development Award DK-01137 during the initial phases of the work. Dr. Al-Rohil was supported by the Jordan University of Science and Technology.

Original version received 9 August 1989 and accepted version received 27 December 1989.

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