The Role of ATP in Swelling-stimulated K-Cl Cotransport in Human Red Cell Ghosts

Phosphorylation–Dephosphorylation Events Are Not in the Signal Transduction Pathway

JOHN R. SACHS and DWIGHT W. MARTIN

From the Department of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794-8151

ABSTRACT Volume-sensitive K-Cl cotransport occurs in red blood cells of many species. In intact cells, activation of K-Cl cotransport by swelling requires dephosphorylation of some cell protein, but maximal activity requires the presence of intracellular ATP. We have examined the relation between K-Cl cotransport activity and ATP in ghosts prepared from human red blood cells. K-Cl cotransport activity in swollen ghosts is increased by ATP, and the increase requires Mg so that it almost certainly results from the phosphorylation of some membrane component. However, even in ATP-free ghosts residual volume-sensitive K-Cl cotransport can be demonstrated. This residual cotransport in ATP-free ghosts is greater in the presence of vanadate, a tyrosyl phosphatase inhibitor, and in ghosts that contain ATP cotransport is reduced by genistein, a tyrosyl kinase inhibitor. Okadaic acid, an inhibitor of serine and threonine phosphatases, inhibits K-Cl cotransport in ghosts as it does in intact cells. Experiments in which ghosts were preexposed to okadaic acid showed that the protein dephosphorylation that permits K-Cl cotransport can proceed to completion before the ghosts are swollen and K transport measured and therefore dephosphorylation is not a response to ghost swelling. In experiments with ATP-free ghosts we found that phosphorylation is not necessary to increase the cotransport rate when shrunken ghosts are swollen, nor is rephosphorylation necessary to decrease the cotransport rate when swollen ghosts are shrunken. Cotransport is greater in swollen than in shrunken ghosts even when the swollen and shrunken ghosts have the same concentration of cytoplasmic solutes. We conclude that, although phosphorylation and dephosphorylation modify the activity of the cotransporter in swollen and in shrunken ghosts, neither of these processes nor any other known messenger is involved in signal transduction between the cell volume sensor and the cotransporter as originally proposed by Jennings and Al-Rohil (Jennings, M. L., and N. Al-Rohil. 1990. *Journal of General Physiology.* 95:1021–1040).

Address correspondence to Dr. John R. Sachs, Division of Hematology, HSC T15-040, SUNY at Stony Brook, Stony Brook, NY 11794-8151.

INTRODUCTION

Many animal cells respond to cell swelling by losing two of their principal intracellular solutes, K and an anion, most often Cl. Loss of solute together with osmotically obligated water tends to restore cell volume to normal. In many cells the loss of K and Cl occurs through independently regulated channels, but in red blood cells from a variety of species the loss of K and Cl promoted by cell swelling appears to be mediated by a cotransporter (Dunham, 1989; Sarkadi and Parker, 1991; Lauf, Bauer, Adragna, Fujise, Zade-Oppen, Ryn, and Delpire, 1992). The best evidence for the existence of K-Cl cotransport is the observation that K can be transported against a concentration gradient as a result of the dissipation of a Cl gradient (Brugnara, Van Ha, and Tosteson, 1989).

Although a great deal is known about the ion transport process, less is known about how cell volume is sensed or how the signal is transmitted from the sensor to the cotransporter. Volume-stimulated K-Cl cotransport can be demonstrated in human red cell ghosts (Dunham and Logue, 1986; Sachs, 1988; O'Neill, 1989) and in inside-out membrane vesicles prepared from sheep red blood cells (Kracke and Dunham, 1990). In these preparations K-Cl cotransport is stimulated by ATP, probably acting by phosphorylating some membrane component. We have shown that, in human red cell ghosts, the effect of ATP is not mediated by a cAMP-dependent protein kinase, by protein kinase C, or by a Ca-dependent protein kinase (Sachs, 1988).

Recently, from an analysis of the time course of the change in K-Cl cotransport activity in response to cell swelling and cell shrinking, Jennings and Al-Rohil (1990) have concluded that, in rabbit red blood cells, change in cell size is signaled by a change in the relative activity of a protein phosphatase and of a protein kinase so that net dephosphorylation of some cell component takes place during cell swelling and signals increased activity of the K-Cl cotransporter. Support for this proposal has been obtained from studies on the inhibition of swelling-activated K-Cl cotransport by the phosphatase inhibitors vanadate, fluoride, and okadaic acid (Jennings and Schulz, 1991; Kaji and Tsukitani, 1991).

We report here the results of some experiments in which we examined the role of ATP in volume-stimulated K-Cl cotransport in human red cell ghosts. Phosphorylation of some membrane component, probably a tyrosine residue, stimulates K-Cl cotransport in both swollen and shrunken ghosts, and inhibition of the dephosphorylation of a serine or threonine residue inhibits K-Cl cotransport in both shrunken and swollen ghosts. However, ATP-free ghosts respond to swelling by increasing K influx and to shrinking by decreasing K influx. Phosphorylation and dephosphorylation cannot, therefore, be obligatory processes in the transmission of the signal from the volume sensor to the cotransporter, but rather modulate the response of the cotransporter to a signal that is transmitted directly.

METHODS

Venous blood was obtained from normal volunteers and anticoagulated either with heparin or citrate-phosphate-dextrose solution. Cells were stored at 4°C and used within 3 d for preparation of resealed ghosts.

Resealed ghosts were prepared by a gel filtration method similar to that described by Kaplan (1982). Cells were separated from plasma and washed with an ice-cold 150 mM choline chloride solution that contained 0.1 mM EDTA (ethylenediamine tetraacetic acid) and 10 mM PIPES (piperazine-N,N'-bis[2-ethanosulfonic acid]) adjusted to pH 5.5 with Tris (Tris [hydroxymethyl]aminomethane). After two washes, the cells were resuspended in the same ice-cold solution and the suspension was titrated to pH 6.0 with 2 N HCl in 160 mM choline chloride until the pH of the ice-cold suspension was stable at pH 6.0. The cells were then washed twice in a solution identical to that described above except that the pH was adjusted to 6.0. The cells were brought to 50% hematocrit with this solution and stored on ice until used in the column.

The column was 45 × 10 cm and was filled with BioGel A50 beads (BioRad Inc., Rockville Center, NY); the bed volume was 3.5 liters. The column was enclosed in a water jacket and maintained at 0°C. The gel was equilibrated with a solution that contained 10 mM PIPES, 11.2 mM choline chloride, and 0.1 mM EDTA; the solution was adjusted to pH 6.0 with Tris (buffer A). To prepare ghosts, 150 ml of a solution identical to buffer A except that the choline chloride concentration was 150 mM (buffer B) was run into the column followed by 75–100 ml of cell suspension. The cells hemolyzed on the column and intracellular contents were retained by the beads. Ghosts were eluted with buffer A and collected on ice. They were concentrated by centrifugation (40,000 g for 10 min) and aspiration of the supernatant, collected in one or two tubes, and resuspended in buffer A. The ghosts were again centrifuged, the supernatant was removed, and the ghosts were distributed to resealing solutions.

Resealing solutions were made up so that the intracellular contents of the resealed ghosts at their final volume during influx measurement were solutions of 295 mosmol/kg H_2O when the ghosts were suspended in buffer at that osmolality. If the final ghost volume was to be 1.9 times its volume at resealing, the osmolality of the resealing solution was 1.9 × 295 mosmol/kg H_2O; if the ghost volume was to be 0.8 times its volume at resealing, the osmolality of the resealing solution was 0.8 × 295 mosmol/kg H_2O. As a result, the concentration of impermeable solutes was the same in the swollen and shrunken ghosts during the influx measurements. The resealing solutions were made up so that, at their final volume, ghosts always contained 10 mM Tris HEPES (4-[2 hydroxymethyl]-1-piperazine ethanesulfonic acid) adjusted to pH 7.8 at 37°C with Tris, 0.5 mM Tris EGTA (ethyleneglycol bis-[β-aminoethylether]N,N'-tetraacetic acid), and the substances listed in the legends to the figures and tables. The resealing solutions always contained 0.2 mM ouabain and 50 mg/100 ml albumin. Ghosts accounted for 10–40% of the volume of the suspension. The ghost suspension was kept at 0°C for 5 min and then incubated at 37°C for the times stated in the legends. The ghosts were separated from the suspension, the supernatant was saved for the determination of K concentration, and the ghosts were washed three times with a solution that contained 10 mM Tris HEPES, pH 7.4, and enough choline chloride or choline nitrate to make up the osmolality of the resealing solutions. The ghosts were used for determination of influx.

Influx was measured by adding ghosts to 1 ml of appropriate ice-cold solutions made up to 295 mosmol/kg H_2O containing [86]RbCl. The suspensions were mixed and incubated at 37°C for 30 min unless otherwise stated. The influx was terminated by returning the tubes to the ice bath and adding 1 ml ice-cold 160 mM choline chloride solution. The suspension was mixed and centrifuged, and the supernatant was poured off. The ghosts were washed three times with 107 mM $MgCl_2$ solution and then resuspended in 1 ml of the same solution. A sample was taken for counting, and a sample of the solution in which the influx was measured was also counted. The K content of the counted ghost suspension and the K concentration of the resealing solution were estimated by flame photometry. The volume of ghosts used in the influx measurement at the time of resealing was calculated by dividing the K content of the ghosts by the K concentration of the resealing solution. The concentration of K in the ghosts that resealed was therefore taken to be the same as the concentration of the resealing solution

(which assumes that resealing to K is an all or none phenomenon). Influx was calculated as previously described (Sachs, 1977). In all cases, ouabain was present in resealing solutions, washing solutions, and solutions in which influx measurements were made, so all values reported represent ouabain-resistant fluxes.

Ghost ATP concentration was estimated by means of a luciferin-luciferase assay according to the directions in Sigma assay kit FL-AA (Sigma Chemical Co., St. Louis, MO). After the ghosts were resealed and washed, they were solubilized and diluted in 1% Triton X-100 which also contained choline chloride; the final choline chloride concentration of the samples used for ATP determination was either 160 or 320 mM. Standards were made up to contain Triton and choline chloride at concentrations equal to those present in the samples. The volume of ghosts assayed was estimated as described above by dividing the K content of the resealed ghost suspension by the K concentration of the resealing solution. The assay could readily detect 1 μM ATP/liter ghosts.

To calculate the concentration of Mg necessary to yield the concentration of Mg^{2+} and Mg ATP indicated in the legends to the figures and tables, the K_D for Mg ATP was taken to be 50.1 μM, for Mg creatine phosphate 5.0 mM, and for Mg EGTA 6.17 mM.

Influx determinations were made in quadruplicate. Each point in the figures and each value in the tables is the mean of four determinations, and the standard error of the mean is indicated (unless, in the figures, it is smaller than the symbol). When the curves describe an equation, they are fitted to the equation by a nonlinear least-squares method; the values are weighted by their variances.

RESULTS

To assist the reader in following the description of the experimental results, it is worthwhile to outline the design of the experiments and the nomenclature used. For all experiments, red blood cells were hemolyzed on a gel filtration column. Ghost suspensions were brought to the appropriate concentration of all impermeant solutes and incubated at 37°C. This is referred to as the resealing incubation, and it varied in duration from 0.5 to 1.5 h. In some experiments inhibitors to which the resealed ghosts are permeable were added at varying times after the beginning of the resealing incubation. After resealing, ghosts were washed with solutions of the same osmolality as that of the resealing solutions. Ghosts were added to the influx solutions whose composition was such that the ghosts immediately swelled or shrank at the same time that the influx measurement was begun.

Stimulation of K-Cl Cotransport by Mg-ATP

It is clear that K-Cl cotransport in red cell ghosts and inside-out vesicles is greater in the presence of ATP than in its absence (Dunham and Logue, 1986; Brugnara, Van Ha, and Tosteson, 1988; Sachs, 1988; O'Neill, 1989; Kracke and Dunham, 1990). Fig. 1 shows the relation between chloride-dependent K influx in swollen ghosts and the intracellular concentration of ATP. Fig. 2 gives the results of an experiment in which chloride-dependent K influx was measured in swollen ghosts with and without ATP at varying concentrations of free Mg. From Fig. 1 it is clear that ATP increases K-Cl cotransport, and from the results of Fig. 2 it is clear that the effect of ATP requires the presence of Mg. Taken together, these results mean that ATP almost certainly increases K-Cl cotransport by phosphorylating some component of the cell
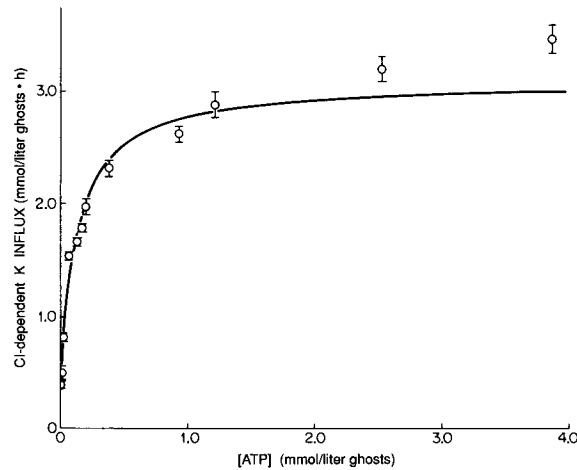
FIGURE 1. Cl-dependent K influx vs. intracellular ATP concentration. Ghosts were prepared to contain during the influx measurement the substances described in Methods and 134 mM K, 5.0 mM creatine phosphate, 10 U/ml creatine kinase, 0.8 mM Mg^{2+}, and the indicated concentrations of ATP. Ghosts were resealed by incubation at 37°C for 60 min at 561 mosmol/kg H_2O and washed in buffered choline chloride or choline nitrate solution at the same osmolality, and the influx measurements were made at 295 mosmol/kg H_2O so that during the influx measurement the ghosts were 1.9 times their volume at resealing. K influx was measured in buffered choline chloride or choline nitrate solution containing 1.97 mM K. The curve is: K influx (mmol/liter ghosts·h) = A (mmol/liter ghosts·h) + V_M (mmol/liter ghosts·h)/$(K_{1/2}$ (mmol/liter ghosts)/[ATP] + 1). A = 0.43, V_M = 2.67, and $K_{1/2}$ = 0.14 mM. Ghost ATP concentration was measured as described in Methods using samples taken just before the influx measurement was made.
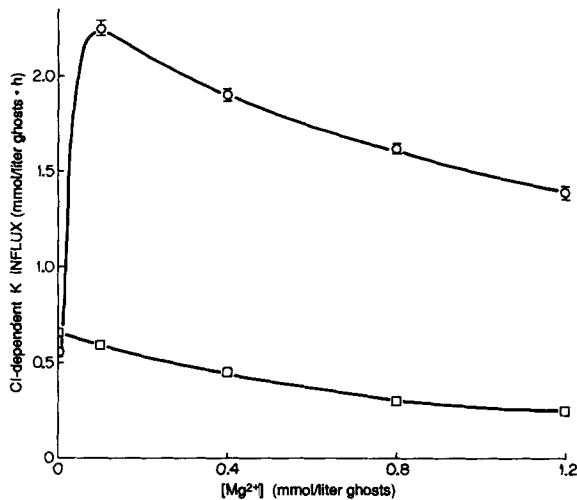
FIGURE 2. Cl-dependent K influx vs. Mg^{2+} concentration within the ghosts in ghosts with (O) and without (□) ATP. Ghosts were prepared to contain during the influx measurement the substances described in Methods, 136 mM K, either 2.0 mM ATP, 5.0 mM creatine phosphate, and 10 U/ml creatine kinase (O) or none of these substances (□), and the indicated concentrations of Mg^{2+}. Ghosts were resealed by incubation for 1 h at 37°C at 561 mosmol/kg H_2O and washed in buffered choline chloride or choline nitrate solutions at the same osmolality, and the influx measurement was made at 295 mosmol/kg H_2O so that during the influx measurement the ghosts were 1.9 times their volume at resealing. K influx was measured in buffered choline chloride or choline nitrate solutions containing 1.87 mM K. The lines were drawn by eye.

membrane. These results are similar to results we have previously reported (Sachs, 1988) except that the $K_{1/2}$ for ATP in Fig. 1 is 0.14 mM, significantly higher than we previously found (perhaps because these measurements were made at 0.8 mM Mg^{2+} and the previous measurements at 0.1 mM Mg^{2+}).

Some residual K-Cl cotransport can be seen even in the ATP-free ghosts in Fig. 1. The amount of K-Cl cotransport present in ATP-free ghosts varied from batch to batch and with intracellular Mg^{2+} concentration, but was often quite large (see, for example, Fig. 3). We had previously assumed (Sachs, 1988) that this influx was supported by residual ATP present in ghosts resealed in ATP-free solutions. However, we questioned this assumption when Kracke and Dunham (1990) reported that significant K-Cl cotransport occurred in inside-out vesicles prepared from sheep red blood cells even when the suspending solution contained no ATP. They proposed
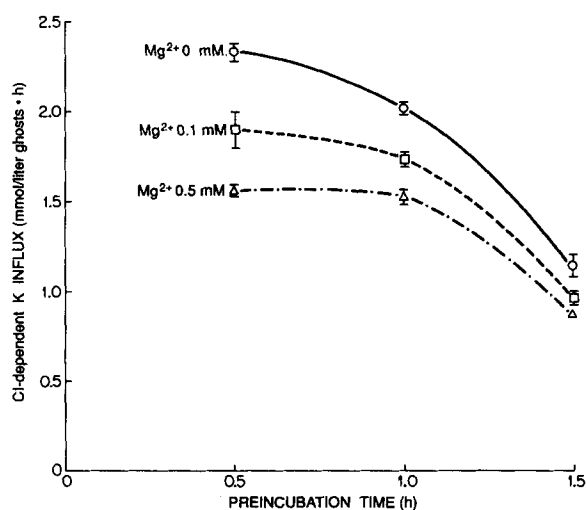

FIGURE 3. Cl-dependent K influx vs. duration of resealing at three intracellular concentrations of Mg^{2+} in ATP-free ghosts. Ghosts were prepared to contain during the influx measurements the substances described in Methods, 136 mM K^+, and 0 Mg^{2+} (O), 0.1 (□), or 0.5 (△) mM. Ghosts were resealed by incubation at 37°C and 561 mosmol/kg H_2O for the times indicated and washed in buffered choline chloride or choline nitrate solution at the same osmolality. After washing, the ghosts contained no detectable ATP. The influx measurement was made at 295 mosmol/kg H_2O so that during the influx measurement the ghosts were 1.9 times their volume at resealing. K influx was measured in buffered choline chloride or choline nitrate solution containing 2.06 mM K. The lines were drawn by eye.

that even in the absence of ATP some membrane component remains phosphorylated and able to support cotransport, and this proposal was supported by the finding that pretreatment of the vesicles with alkaline phosphatase increased the magnitude of the subsequently measured ATP stimulation of cotransport.

We measured the ATP content of ghosts resealed in ATP-free solutions and could detect no ATP; the assay we used was easily capable of detecting 1 μM ATP present in the ghosts. Fig. 3 shows the results of an experiment in which ghosts were resealed in ATP-free solutions at three concentrations of Mg^{2+}. Measurements of K influx were made after varying periods of incubation at 37°C. At each time point influx into the swollen ghosts diminished as free Mg increased. We expected that, if the residual K influx in ATP-free ghosts is supported by some phosphorylated membrane component that slowly dephosphorylates in the absence of ATP, then the flux should

decrease with the time that ATP-free ghosts are incubated at 37°C before the influx determination. The decrease was seen, especially in the ghosts with low free Mg. However, the results are complicated because a second phosphoprotein also dephosphorylates during the resealing incubation, and dephosphorylation of this protein increases cotransport activity (see below). We attempted to reduce further the residual influx by incorporating phosphatases within ATP-free ghosts, but the experiments were unsuccessful because of uncontrolled effects of phosphatase preparations on the cell membrane, and because of difficulties in maintaining ghost integrity while manipulating pH.

From the results of experiments using inhibitors of phosphoryl-transfer reactions, we obtained tentative evidence that a phosphotyrosyl group is responsible for the increased cotransport activity due to ATP. Genistein is an inhibitor of tyrosine phosphorylation in many systems; it has been shown to inhibit the activity of tyrosine kinases, but to be relatively inactive against serine and threonine kinases (Akiyama and Ogawara, 1991). We examined the effect of genistein on K-Cl cotransport in swollen ghosts; the results are given in Fig. 4. Genistein inhibited K influx in ghosts that contained ATP, although the effect was incomplete even at high concentrations. Genistein had little effect in ATP-free ghosts.

Vanadate inhibits many phosphoryl-transfer reactions, and it is thought to be an effective inhibitor of tyrosine phosphatases (Swarup, Cohen, and Garbers, 1982; Gordon, 1991). Several investigators have shown that vanadate inhibits swelling-activated K-Cl cotransport in red cells (Jennings and Al-Rohil, 1990; O'Neill, 1991). We have found that vanadate at high concentrations inhibits K-Cl cotransport in ghosts, but at low concentrations vanadate stimulates cotransport (Sachs, 1988). Table I shows the effect of vanadate at high concentrations on K-Cl cotransport in swollen ghosts that either contained ATP or were ATP free. In ghosts that contained ATP, vanadate inhibited K influx, but when the ghosts were ATP free, influx was greater in the presence of vanadate than in its absence. Table II gives the results of an experiment in which we examined the effect of vanadate on chloride-dependent K influx into swollen ATP-free ghosts measured at two concentrations of Mg^{2+}. Influx was considerably higher in the presence of vanadate at both Mg^{2+} concentrations. Since the ghosts are ATP free, the effect of vanadate cannot be attributed to its inhibition of a protein kinase, but must be due to inhibition of a phosphatase.

From these studies, it is clear that K-Cl cotransport in swollen ghosts is increased by ATP, but significant cotransport persists even in the absence of ATP. Inhibition studies suggest that the effect of ATP is exerted by phosphorylation of a tyrosine residue.

Signaling of Volume Increase Is Not Mediated by a G Protein

Before concluding that inhibition of a phosphatase is the basis of vanadate activation, we decided to eliminate one other highly unlikely possibility. Vanadate may stimulate G protein–dependent functions by binding to the GDP complexed protein and maintaining it in a state of permanent activation. Although it is not likely that our ATP-free ghosts contain GTP, it has been reported that G proteins can be found in red cell ghosts (Ikeda, Kikuchi, and Takai, 1988). To make certain that G proteins are not involved in the modulation of K-Cl cotransport, we performed the experi-

ment shown in Table III. GTPγS, a nonhydrolyzable analogue of GTP, activates G protein–dependent functions, but it did not increase K-Cl cotransport in shrunken ghosts (we compared the effect of GTPγS to the effect of ATPγS). GDPβS, which inhibits G protein–dependent functions, did not inhibit KCl cotransport in swollen ghosts. G proteins are not involved in signal transduction between the volume sensor and the K-Cl cotransporter.
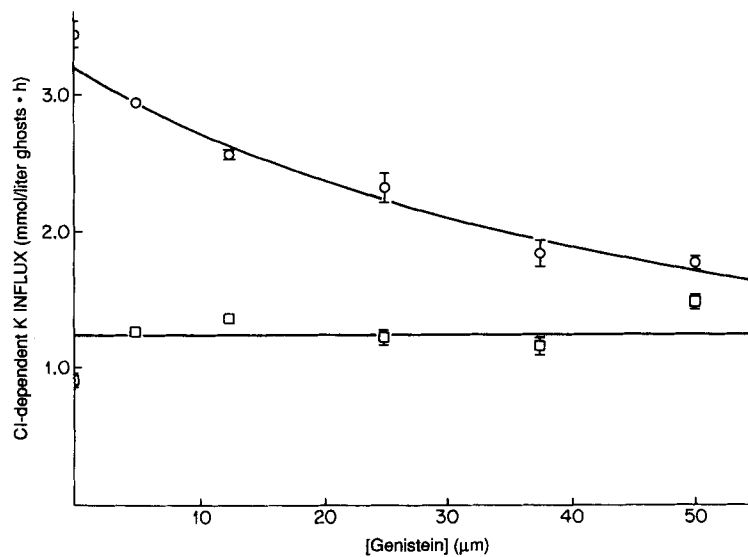

FIGURE 4. Cl-dependent K influx vs. genistein concentration in ghosts with (O) and without (□) ATP. Ghosts were prepared to contain during the influx measurement the substances described in Methods and 136 mM K, either 2.0 mM ATP, 5.0 mM creatine phosphate, and 10 U/ml creatine kinase (O) or none of these substances (□), and 0.1 mM Mg^{2+}. Ghosts were resealed by incubation for 1 h at 37°C and 561 mosmol/kg H_2O and washed in buffered choline chloride or choline nitrate solutions at the same osmolality, and the influx measurement was made at 295 mosmol/kg H_2O so that during the influx measurement the ghosts were 1.9 times their volume at resealing. K influx was measured in buffered choline chloride or choline nitrate solutions containing 2.12 mM K. Genistein at the indicated concentrations was present in resealing solutions and in the solutions in which influx was measured. The curve for the ghosts containing ATP is v(mmol/liter ghosts·h) = V_M(mmol/liter ghosts·h)/(1 + [I](μM)/K(μM)), where I is the concentration of genistein, V_M is 3.18, and K is 57.4. For the ATP-free ghosts, the straight line was drawn by eye.

Inhibition by Okadaic Acid

Okadaic acid is synthesized by dinoflagellates and concentrated by marine animals, including sponges from which it was first isolated (Tachibana, Scheuer, Tsukitani, Kikuchi, Van Engen, Clardy, Gopichand, and Schmitz, 1981). It has been shown to be a specific inhibitor of protein phosphatase 1 and protein phosphatase 2A which dephosphorylate serine and threonine residues (Cohen, Holmes, and Tsukitani,

TABLE I

Effect of Vanadate on Cl-dependent K Influx into ATP-free Ghosts and Ghosts Containing ATP

Ghost composition	Cl-dependent and ouabain-resistant K influx ± SEM	
	0 vanadate	1 mM vanadate
	mmol/liter ghosts·h	
0 Mg^{2+}, 0 ATP	1.96 ± 0.022	2.35 ± 0.127
0.1 Mg^{2+} mM, 2.0 mM ATP	4.84 ± 0.054	3.62 ± 0.075

Ghosts were prepared to contain during the influx measurement the substances described in Methods, 136 mM K$^+$, the indicated concentrations of ATP and Mg^{2+}, 5.0 mM creatine phosphate, and 10 U/ml creatine kinase in the ghosts containing ATP, and the indicated concentrations of vanadate. The ghosts were resealed at 561 mosmol/kg H$_2$O by incubation for 90 min at 37°C, and after resealing they were washed with a buffered choline chloride or choline nitrate solution at the same osmolality containing the indicated concentrations of vanadate. K influx was measured in similar solutions containing 1.86 mM KCl or KNO$_3$ and the indicated concentrations of vanadate. The osmolality of the influx solution was 295 mosmol/kg H$_2$O so that during the influx measurement the ghosts were 1.9 times their volume at resealing.

1990; Hardie, Haystead, and Sim, 1991). Low concentrations of okadaic acid inhibit swelling-activated K-Cl cotransport in red blood cells from several species (Jennings and Schulz, 1991; Kaji and Tsukitani, 1991; Orringer, Brockenbrough, Whitney, Glosson, and Parker, 1991; Parker, Colclasure, and McManus, 1991).

Table IV shows that okadaic acid also inhibits K-Cl cotransport in red cell ghosts, both swollen and shrunken. The concentration dependence of inhibition by okadaic acid is shown in Fig. 5. In this experiment, okadaic acid was included in the resealing

TABLE II

Effect of Vanadate on Cl-dependent K Influx into ATP-free Ghosts

Vanadate concentration	Cl-dependent and ouabain-resistant K influx ± SEM	
	0 mM Mg$_c^{2+}$	0.8 mM Mg$_c^{2+}$
mM	*mmol/liter ghosts·h*	
0	0.72 ± 0.017	0.34 ± 0.031
0.025	0.76 ± 0.021	0.48 ± 0.019
0.200	1.01 ± 0.040	0.48 ± 0.021
1.000	1.29 ± 0.067	0.69 ± 0.037

Ghosts were prepared to contain during the influx measurement the substances described in Methods, 149 mM K$^+$, and the indicated concentrations of vanadate and Mg^{2+}. The ghosts were resealed at 561 mosmol/kg H$_2$O and, after resealing by incubation at 37°C for 60 min, they were washed with a buffered choline chloride or choline nitrate solution at the same osmolality containing the indicated concentrations of vanadate. K influx was measured in similar solutions containing 1.89 mM KCl or KNO$_3$ and the indicated concentrations of vanadate. The osmolality of the influx solution was 295 mosmol/kg H$_2$O so that during the influx measurement the ghosts were 1.9 times their volume at resealing.

TABLE III

Effect of GTPγS and GDPβS on Cl-dependent and Ouabain-resistant K Influx

	K influx ± SEM	
	Swollen	Shrunken
	mmol/liter ghosts·h	
Control	5.64 ± 0.099	1.26 ± 0.021
20 μM GTPγS	6.23 ± 0.016	1.13 ± 0.015
100 μM GTPγS	5.63 ± 0.071	0.98 ± 0.027
20 μM ATPγS	6.66 ± 0.095	1.35 ± 0.007
100 μM ATPγS	6.37 ± 0.106	1.10 ± 0.018
Control	4.70 ± 0.218	1.00 ± 0.030
20 μM GDPβS	5.80 ± 0.125	1.06 ± 0.084
100 μM GDPβS	4.84 ± 0.083	0.94 ± 0.020
20 μM ADPβS	5.21 ± 0.050	0.81 ± 0.009
100 μM ADPβS	5.41 ± 0.073	0.71 ± 0.008

Cells were prepared to contain the substances described in Methods and 132 mM K, 2 mM ATP, 5 mM creatine phosphate, 10 U/ml creatine kinase, 0.2 mM Mg^{2+}, and the indicated concentrations of GTPγS, ATPγS, GDPβS, and ADPβS. Ghosts were resealed by incubation for 60 min at 37°C at 590 mosmol/kg H_2O or 236 mosmol/kg H_2O, washed with buffered choline chloride or choline nitrate solutions at the same osmolality, and used for measurement of K influx at 295 mosmol/kg H_2O so that the ghosts were either 2.0 times (swollen) or 0.8 times (shrunken) their volumes at resealing. During the influx measurement, extracellular K was 1.91 mM. Different batches of ghosts were used for the GTPγS and GDPβS experiments.

TABLE IV

Effect of Okadaic Acid on Cl-dependent and Ouabain-insensitive K Influx in Swollen and Shrunken Ghosts

Okadaic acid	Cl-dependent K influx ± SEM	
	Swollen ghosts	Shrunken ghosts
	mmol/liter ghosts · h	
0	6.34 ± 0.14	1.51 ± 0.02
0.25 μM	0.66 ± 0.04	0.14 ± 0.01

Ghosts were prepared to contain during the influx measurement the substances described in Methods, and 24 mM Na^+, 104 mM K^+, 1 mM ATP, 5mM creatine phosphate, 10 U/ml, creatine kinase, and 0.5 mM Mg^{2+}. Ghosts were resealed for 0.5 h at 561 mosomol/kg H_2O (swollen ghosts) or 236 mosmol/kg H_2O (shrunken ghosts) and washed in buffered choline chloride or choline nitrate solutions at the same osmolality, and the influx measurement was made at 295 mosmol/kg H_2O so that during the influx measurement the ghosts were 1.9 or 0.8 times their volume at resealing. During resealing, okadaic acid dissolved in DMSO was present at the indicated concentration, and DMSO was present at the same concentration when okadaic acid was absent. K influx was measured for 0.5 h in buffered choline chloride or choline nitrate solution containing 1.79 mM K.

solutions so that the ghosts were exposed to the inhibitor throughout the resealing period, which lasted 0.5 h; it was also present in the influx solutions. Okadaic acid inhibited K-Cl cotransport in swollen red cell ghosts just as it does in intact cells, but half-maximal inhibition is seen at much lower concentrations with ghosts than with red cells.

Time Course of Inhibition by Okadaic Acid

Although okadaic acid inhibits swelling-activated cotransport in ghosts, inhibition depends on exposure of the ghosts to okadaic acid while they are resealing. In the experiment shown in Table V, okadaic acid at a very high concentration (>60 times the concentration that resulted in half-maximal inhibition in Fig. 5) was added at the
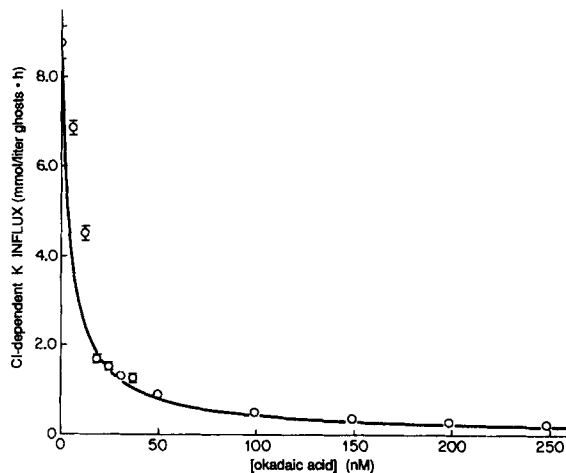

FIGURE 5. Cl-dependent K influx vs. okadaic acid concentration. Ghosts were prepared to contain during the influx measurements the substances described in Methods and 134 mM K, 2.0 mM ATP, 5.0 mM creatine phosphate, 10 U/ml creatine kinase, and 0.5 mM Mg^{2+}. Ghosts were resealed by incubation for 0.5 h at 561 mosmol/kg H_2O and washed in buffered choline chloride or choline nitrate solutions at the same osmolality, and the influx measurement was made at 295 mosmol/kg H_2O so that during the influx measurement the ghosts were 1.9 times their volume at resealing. Okadaic acid at the indicated concentrations was present during resealing and during the influx measurement; okadaic acid was dissolved in dimethylsulfoxide (DMSO) and the concentration of DMSO was kept constant. The curve is K influx (mmol/liter ghosts·h) = A(mmol/liter ghosts·h)/(1 + [okadaic acid](nM)/B(nM)), where A is 10.89 and B is 3.93.

beginning of the resealing incubation, 30 min after the beginning of resealing, or not at all during the resealing process. After resealing, ghosts were added to the influx solution. At this point they swelled to ~1.9 times their volume at resealing, and influx was measured with and without okadaic acid in the solution. When okadaic acid was present from the beginning of resealing, inhibition was nearly complete when influx was measured 60 min later; when okadaic acid was added 30 min after the resealing incubation began, only a third of the K-Cl cotransport present in the control was inhibited. In neither case did the presence of okadaic acid in the influx solutions make much difference. When okadaic acid was present only in the influx solution, little inhibition was seen. Both ATP-free ghosts and ghosts containing ATP were used in this experiment, and the results were about the same whether or not ATP was present.

The results show that dephosphorylation is necessary for maximal K-Cl cotransport, and that the dephosphorylation occurs during the resealing incubation before the ghosts are swollen and K influx measured. When ghosts are exposed to okadaic acid during the entire 60-min resealing incubation, dephosphorylation of the relevant serine or threonine is prevented and inhibition of K influx is nearly complete. When resealing proceeds for a full hour without okadaic acid, nearly complete dephosphorylation occurs, and the presence of the inhibitor in the influx solution results in little inhibition. If ghosts are allowed to incubate in the resealing solution for 30 min before okadaic acid is added, dephosphorylation proceeds during that period and partial inhibition is seen.

TABLE V

Inhibition of Cl-dependent Ouabain-insensitive K Influx in Swollen Ghosts at Varying Times after Exposure to Okadaic Acid

Okadaic acid during resealing	Okadaic acid during influx	ATP-free ghosts		2.0 mM ATP ghosts	
		K influx ± SEM	Fractional inhibition	K influx ± SEM	Fractional inhibition
		mmol/liter ghosts · h		*mmol/liter ghosts · h*	
None	None	1.01 ± 0.027		4.88 ± 0.096	
	0.25 μM	1.01 ± 0.051	—	4.27 ± 0.063	0.12
0.25 μM 30 min	None	0.86 ± 0.001	0.14	3.20 ± 0.039	0.34
after start of resealing	0.25 μM	0.90 ± 0.015	0.11	3.04 ± 0.048	0.38
0.25 μM at	None	0.21 ± 0.014	0.79	0.57 ± 0.042	0.88
start of resealing	0.025 μM	0.21 ± 0.017	0.79	0.59 ± 0.028	0.88

Ghosts were prepared to contain during the influx measurement the substances described in Methods, and 136 mM K^+ and 0.5 mM Mg^{2+}. In addition, the 2.0 mM ATP ghosts contained 2 mM ATP, 5 mM creatine phosphate, and 10 U/ml creatine kinase. Ghosts were resealed for 1 h at 561 mosmol/kg H_2O and washed in buffered choline chloride or choline nitrate solutions at the same osmolality, and the influx measurement was made at 295 mosmol/kg H_2O so that during the influx measurement the ghosts were 1.9 times their volume at resealing. K influx was measured for 0.5 h in buffered choline chloride or choline nitrate solutions containing 2.06 mM K. Okadaic acid dissolved in DMSO was either absent from the resealing solution, or added at the indicated concentration either at the beginning of the resealing incubation or 30 min later. In addition, okadaic acid was either included or excluded from the solutions in which influx was measured as indicated. DMSO was present in equal concentration when okadaic acid was absent.

Although cells are very permeable to okadaic acid, it might be argued that the results shown in Table V are due to delayed entry of okadaic acid into the ghosts when it is added after resealing has begun. We therefore repeated the experiment with ghosts containing ATP, but we used two concentrations of okadaic acid, one the same as that used in Table V and one five times higher (300 times the concentration which resulted in half-maximal inhibition in Fig. 5). The results of the experiment are shown in Table VI; there was no difference in the results at the two concentrations, and therefore limited permeability seems to be excluded as an explanation for these findings.

TABLE VI

Inhibition of Cl-dependent Ouabain-insensitive K Influx in Swollen Ghosts by Okadaic Acid at Two Concentrations

Okadaic acid concentration		K influx ± SEM	Fractional inhibition
During resealing	During influx		
		mmol/liter ghosts · h	
None	None	5.10 ± 0.114	
None	0.25 μM	4.23 ± 0.116	0.17
None	1.25 μM	4.17 ± 0.081	0.18
Added 30' after start of resealing			
0.25 μM	None	3.67 ± 0.174	0.28
1.25 μM	None	3.52 ± 0.214	0.31
Added at start of resealing			
0.25 μM	None	1.03 ± 0.164	0.80
1.25 μM	None	1.18 ± 0.085	0.77

Ghosts were prepared to contain during the influx measurement the substances described in Methods, and 1.36 mM K$^+$, 2 mM ATP, 5 mM, creatine phosphate, 10 U/ml creatine kinase, and 0.5 mM Mg^{2+}. Ghosts were resealed for 1 h at 561 mosmol/kg H$_2$O and washed in buffered choline chloride or choline nitrate solutions at the same osmolality, and the influx measurement was made at 295 mosmol/kg H$_2$O so that during the influx measurement the ghosts were 1.9 times their volume at resealing. K influx was measured for 0.75 h in buffered choline chloride or choline nitrate solutions containing 1.83 mM K. Okadaic acid dissolved in dimethyl formamide was either absent from the resealing solution or added at the indicated concentration either at the beginning of the resealing incubation or 30 min later. In addition, okadaic acid was either included or excluded from the solutions in which influx was measured as indicated. Dimethyl formamide was present in equal concentration when okadaic acid was omitted.

TABLE VII

Effect of Okadaic Acid on Cl-dependent K Influx in Swollen and Shrunken Ghosts in Solutions with and without Na

	Cl-dependent and ouabain-resistant K influx ± SEM	
	138 mM Choline$_o$	138 mM Na$_o$
	mmol/liter ghosts · h	
Swollen ghosts	6.34 ± 0.14	5.95 ± 0.18
Okadaic acid	0.66 ± 0.04	0.52 ± 0.05
Shrunken ghosts	1.51 ± 0.02	1.48 ± 0.04
Okadaic acid	0.14 ± 0.01	0.13 ± 0.01

Ghosts were prepared to contain during the influx measurement the substances described in Methods 24 mM Na, 104 mM K, 1.0 mM ATP, 0.05 mM Mg^{2+}, 5.0 mM creatine phosphate, and 10 U/ml creatine kinase. For the ghosts with okadaic acid, 200 ng/ml of the inhibitor was included in the resealing solutions. The ghosts were resealed at 561 mosmol/kg H$_2$O (swollen ghosts) or 236 mosmol/kg H$_2$O (shrunken ghosts) by incubation for 30 min at 37°C, and, after resealing, they were washed with a buffered choline chloride or choline nitrate solution at the same osmolality. K influx was measured in buffered choline chloride, choline nitrate, NaCl, or NaNO$_3$ solutions containing 1.97 mM K. The osmolality of the influx solution was 295 mosmol/kg H$_2$O so that during the influx measurement the ghosts were 1.9 or 0.8 times their volume at resealing.

We have been unable to demonstrate Na,K,2Cl cotransport in column ghosts even though it is present in human red blood cells. Na,K,2Cl cotransport is known to be activated by ATP, and recently several groups have reported that transport is increased in avian blood cells (Pewitt, Hegde, Hass, and Palfrey, 1990), shark rectal gland (Lytle and Forbush, 1990), and human red blood cells (Mairbäurl and Hoffman, 1992) by okadaic acid. It seemed possible that Na,K,2Cl cotransport is absent in ghosts because the phosphoserine or phosphothreonine that supports Na,K,2Cl cotransport is dephosphorylated during resealing just as the phosphoserine or phosphothreonine that inhibits K-Cl cotransport is dephosphorylated. Table VII

TABLE VIII

Ouabain-insensitive K Influx in ATP-free Ghosts

Ghosts	Ouabain-insensitive K influx ± SEM
	mmol/liter ghosts · h
1. Shrunken → swollen	2.69 ± 0.06
2. Shrunken → shrunken	0.93 ± 0.03
3. Swollen → swollen	2.35 ± 0.02
4. Swollen → shrunken	0.81 ± 0.01

Ghosts were prepared to contain during the influx measurement the substances described in Methods, and 0.1 mM Mg^{2+} and 150 mM K^+ (295 mosmol/kg H_2O). The ghosts were resealed by incubation at 37°C for 30 min at either 2.0 (590 mosmol/kg H_2O) or 0.8 times (236 mosmol/kg H_2O) these final concentrations. The ghosts were then washed in buffered choline chloride solutions of varying osmolalities so that half the ghosts in each batch were swollen and half shrunken. At the final wash, the ghosts were incubated in these wash solutions for 0.5 h at 37°C. In all cases, ghosts were removed from these solutions and K influx was measured at 295 mosmol/kg H_2O. Ghosts 1 (shrunken → swollen) were resealed at 590 mosmol/kg H_2O, washed, and incubated at 738 mosmol/kg H_2O (0.8 times their volume at resealing), and uptake was measured at 295 mosmol/kg H_2O (2.0 times their volume at resealing). Ghosts 2 (shrunken → shrunken) were resealed at 236 mosmol/kg H_2O, washed, and incubated at 295 mosmol/kg H_2O (0.8 times their volume at resealing), and uptake was measured at 295 mosmol/kg H_2O (0.8 times their volume at resealing). Ghosts 3 (swollen → swollen) were resealed at 590 mosmol/kg H_2O, washed, and incubated at 295 mosmol/kg H_2O (2.0 times their volume at resealing), and uptake was measured at 295 mosmol/kg H_2O (2.0 times their volume at resealing). Ghosts 4 (swollen → shrunken) were resealed at 236 mosmol/kg H_2O, washed, and incubated at 118 mosmol/kg H_2O (2.0 times their volume at resealing), and uptake was measured at 295 mosmol/kg H_2O (0.8 times their volume at resealing). During the uptake measurement external K concentration was 1.76 mM. ATP concentration was measured before influx was measured; ATP was not detectable.

shows the results of an experiment in which ghosts were resealed with and without okadaic acid, and K influx was measured in choline and in Na solutions; the presence of an Na,K,2Cl cotransporter should have resulted in higher influxes in Na solutions than in choline solutions. Measurements were made in shrunken and in swollen ghosts. In Na solutions, we expected to see greater influx in the okadaic acid–treated ghosts than in the control ghosts, especially in the shrunken ghosts. There was, in fact, no difference between K influx measured in Na and choline solutions; okadaic acid did not stimulate the flux in Na solution, and we conclude that the absence of

Na,K,2Cl cotransport must have some other explanation. We have no evidence for coordinate regulation of swelling-stimulated and shrinkage-inhibited fluxes which has been found with other systems (Starke and McManus, 1990; Parker et al., 1991).

Swelling-activated Cotransport in ATP-free Ghosts

We do not believe that either phosphorylation of a tyrosine by ATP or dephosphorylation of a phosphothreonine or phosphoserine by a phosphatase is in the pathway
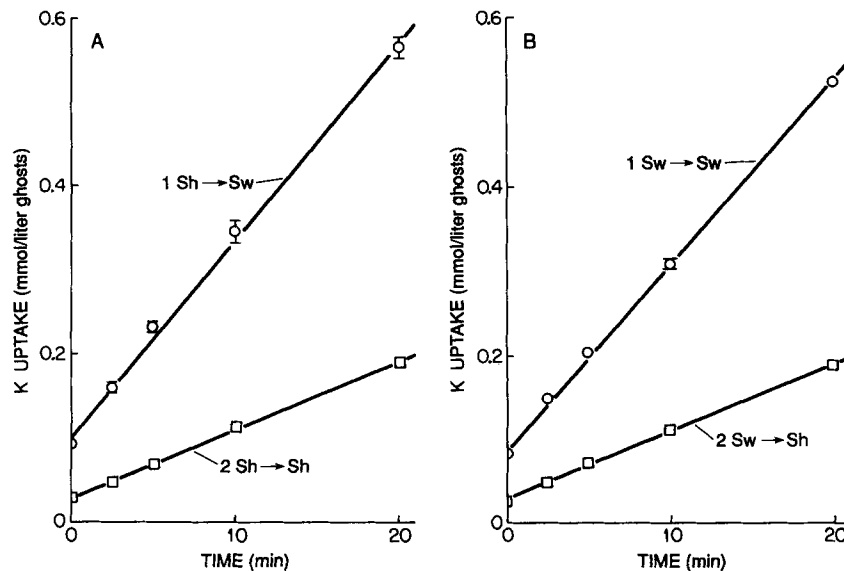

FIGURE 6.   Ouabain-insensitive K uptake vs. time in ATP-free ghosts. The experiment was performed exactly as described in the caption to Table VIII except that samples were taken for uptake determinations at the indicated times. During the uptake measurement, external K concentration was 1.89 mM. The lines are: K uptake (mmol/liter ghosts) = X(mmol/liter ghosts) + Y(mmol/liter ghosts·min) × t(min). For ghosts 1 of panel A (shrunken to 0.8 times their original size during washing and incubation and swollen to 2.0 times their original size during the uptake measurement), X = 0.099 and Y = 0.024, and for ghosts 2 of panel A (shrunken to 0.8 times their original size both during washing and incubation and uptake measurement), X = 0.028 and Y = 0.0082. For ghosts 1 of panel B (swollen to 2.0 times their original size during both washing and incubation and uptake measurement), X = 0.087 and Y = 0.022, and for ghosts 2 of panel B (swollen to 2.0 times their original size during washing and incubation and shrunken to 0.8 times their original size during the uptake measurement), X = 0.028 and Y = 0.0080.

of signal transduction from the sensor which detects changes in cell volume to the cotransporter. Table VIII shows the results of an experiment in which we prepared two batches of ATP-free ghosts resealed at different osmolalities and then incubated them for 30 min at 37°C in solutions of osmolalities such that half of each batch were swollen and half shrunken. During this incubation K influx in the swollen ghosts increased and K influx in the shrunken ghosts decreased (we performed a separate

experiment, which is not shown, to verify that the influx changed as predicted). After the incubation we separated the ghosts from the solutions and measured K influx in solutions whose composition was adjusted so that both the swollen and shrunken ghosts in the first batch were swollen and both the shrunken and swollen ghosts in
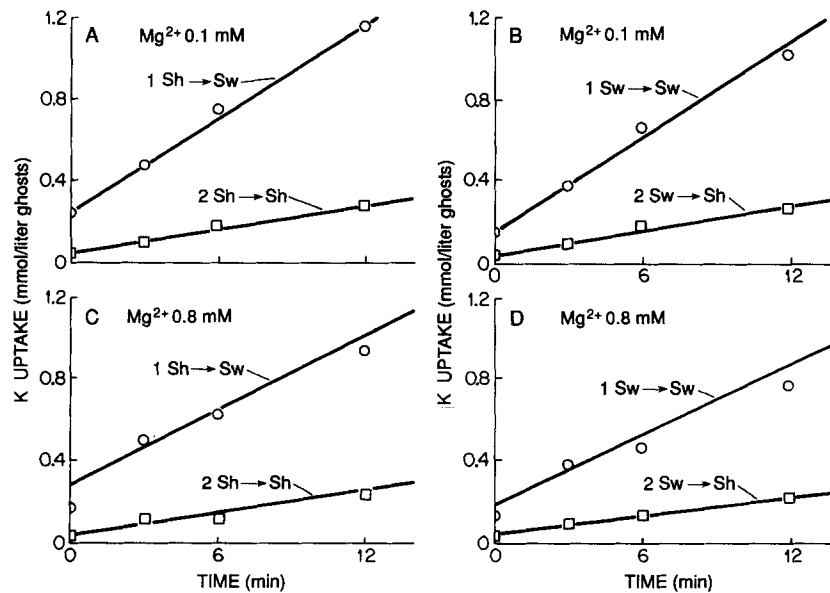

FIGURE 7.   Ouabain-insensitive K uptake vs. time at two concentrations of Mg^{2+}. Ghosts were prepared to contain during the uptake measurement the substances described in Methods, and 0.1 mM Mg^{2+} (A and B) or 0.8 mM Mg^{2+} (C and D), 2.0 mM ATP, 5.0 mM creatine phosphate, and 136 mM K (295 mosmol/kg H_2O). The ghosts were resealed by incubation at 37°C for 30 min at either 2.0 times (590 mosmol/kg H_2O) or 0.8 times (236 mosmol/kg H_2O) these final concentrations. The ghosts were then washed as described in the legend to Fig. 6. The ghosts that were swollen during washing (1 and 2 of panel B and 1 and 2 of panel D) were 2.0 times their volume at resealing and the ghosts that were shrunken during washing (1 and 2 of panel A and 1 of 2 of panel C) were 0.8 times their volume at resealing. The ghosts that were swollen during the uptake measurement (1 of panels A–D) were 2.0 times their volume at resealing and the ghosts that were shrunken during the uptake measurement (2 of panels A–D) were 0.8 times their volume at resealing. During the uptake measurement, external K was 2.0 mM. The lines are: K uptake (mmol/liter cells) = X(mmol/liter cells) + Y(mmol/liter cells·min) × t(min). For ghosts 1 of panel A, X = 0.25 and Y = 0.076, and for ghosts 2 of panel A, X = 0.048 and Y = 0.019. For ghosts 1 of panel B, X = 0.16 and Y = 0.039, and for ghosts 2 of panel B, X = 0.039 and B = 0.020. For ghosts 1 of panel C, X = 0.029 and Y = 0.061, and for ghosts 2 of panel C, X = 0.044 and Y = 0.019. For ghosts 1 of panel D, X = 0.020 and Y = 0.056, and for ghosts 2 of panel D, X = 0.054 and Y = 0.014.

the second batch were shrunken; all measurements were made at 295 mosmol/kg H_2O and the concentration of intracellular solutes was the same for the shrunken and swollen ghosts. Regardless of their history, the fluxes in the swollen ghosts were about the same and higher than those in the shrunken ghosts, which were also about the same, regardless of their history.

When ATP-free ghosts were swollen in this experiment, they increased their influx rates, which demonstrates that phosphorylation is not in the transduction pathway. If dephosphorylation is in the transduction pathway, then the relevant group would have been dephosphorylated in the ghosts that were preincubated for 30 min in the swollen state. But when these swollen ghosts were shrunken, their influx rate returned to that characteristic of shrunken ghosts. If dephosphorylation of some group is responsible for activating influx in swollen ghosts, then rephosphorylation must be necessary to reduce influx in shrunken ghosts; since no ATP is available to rephosphorylate a dephosphorylated group, it is clear that dephosphorylation cannot be part of the signal transduction pathway.

Although a delay in the activation of K-Cl cotransport by cell swelling has been demonstrated in red cells from several species (Kregenow, 1971; Kim, Sargeant, Forte, Sohn, and Im, 1989; Jennings and Al-Rohil, 1990; Kaji and Tsukitani, 1991; Parker et al., 1991; Dunham, Klimczak, and Logue, 1993), we found that, in human red cell ghosts, there was no delay in either the increase in K influx that results from ghost swelling or the decrease in K influx that results from ghost shrinkage (Sachs, 1988). In the experiment we reported, however, the first time point was taken at 5 min, and there is a remote possibility that we missed the delay. We therefore repeated the experiment of Table VIII using ATP-free ghosts, but we measured the time course of K influx (Fig. 6); the first sample was taken immediately after adding ghosts to the influx solutions. There was no delay in the increase in K influx in response to swelling or in the decrease in K influx in response to shrinkage.

Dunham et al. (1993) found that, in sheep red cells, the lag in the increase in K-Cl cotransport induced by cell swelling varies with the intracellular Mg^{2+} concentration; at very low Mg^{2+} concentration there is no detectable lag, but at higher cell Mg^{2+} a lag is clearly detectable. Since most of our experiments are performed at low Mg^{2+}, we measured the time course of K uptake in ghosts that contained ATP and either high or low Mg^{2+}, and that were suddenly swollen or shrunken. The results are shown in Fig. 7; there was no sign of a lag in response to change in ghost volume in either low or high Mg^{2+} ghosts.

DISCUSSION

Although swelling-activated K-Cl cotransport is stimulated by MgATP, significant increase in cotransport occurs even when ATP-free ghosts are swollen. Transmission of the signal from the volume sensor to the cotransporter cannot, therefore, require protein phosphorylation. This is consistent with our previous findings that in human red cell ghosts swelling-activated cotransport does not involve the participation of a cAMP-activated protein kinase, a Ca-activated protein kinase, or protein kinase C (Sachs, 1988), and with the finding of Kracke and Dunham (1990) that swelling stimulates K-Cl cotransport in inside-out vesicles of sheep red blood cells in the absence of ATP. From our experiments with the inhibitors vanadate and genistein, we tentatively conclude that stimulation of cotransport by ATP results from phosphorylation of a tyrosine group. Both tyrosyl kinase activity (Boivin, Galand, and Bertrand, 1986) and tyrosyl phosphatase activity (Clari, Brunati, and Moret, 1987) are known to be associated with the red cell membrane.

Jennings and Schulz (1991) and Kaji and Tsukitani (1991) have demonstrated that exposure of red cells to okadaic acid before they are swollen greatly reduces

swelling-activated K-Cl cotransport. The cells they used showed a lag between swelling and the appearance of swelling-activated cotransport, and low concentrations of okadaic acid increased the duration of the lag. From these results, and from an analysis of the kinetics of the response of cotransport rate to cell shrinkage and swelling, they concluded that swelling-induced increase in K-Cl cotransport results from dephosphorylation of a membrane protein due to swelling-induced inhibition of a protein kinase. Our results with okadaic acid in red cell ghosts show that K-Cl cotransport is enhanced by dephosphorylation of a membrane protein, but the dephosphorylation takes place during the resealing incubation, before the ghosts are swollen. Red cell membrane-bound serine and threonine phosphatases have been reported (Clari et al., 1987). The difference in the observations with intact red cells and red cell ghosts would occur if, during ghosting, the ghosts retain the membrane-bound phosphatase but lose a cytoplasmic serine-threonine kinase. These findings show that dephosphorylation is not in the signal transduction pathway, and this conclusion is confirmed by the finding that swollen ATP-free ghosts rapidly reduce their K influx rate when shrunken.

We have eliminated phosphorylation or dephosphorylation as a signal-transducing mechanism, we have previously presented evidence that inositol phosphates are not involved (Sachs, 1988), and we show here that there is no role for G proteins in the process. We know of no evidence that red cells contain nitric oxide synthase, nor is it likely that column ghosts contain arginine (the substrate for the synthase) or NADPH (a necessary cofactor) (Moncada, Palmer, and Higgs, 1989). Nevertheless, we demonstrated that neither nitric oxide nor nitroprusside (which generates nitric oxide in solution [East, Batchelor, and Garthwaite, 1991]) stimulated K-Cl cotransport in shrunken ghosts nor did L-N^G-monomethylarginine (a competitive inhibitor of arginine) inhibit K-Cl cotransport in swollen ghosts (results not shown). Similarly, human red cells apparently do not contain the enzymes of arachidonic acid metabolism. Nevertheless, we examined the effect of some inhibitors of these enzymes on K-Cl cotransport (Needleman, Turk, Jokschik, Morrison, and Lefkowitz, 1986). Indomethacin and aspirin inhibit cyclooxygenase activity, and nordihydroguaiaretic acid inhibits lipoxygenases; these inhibitors did not inhibit K-Cl cotransport in swollen ghosts (results not shown). In the course of these studies we found that eicosatetraynoic acid and ketoconazole inhibited K influx in swollen ghosts, but only at high concentrations. Both of these substances inhibit cytochrome P450 activity, but since cytochrome P450 requires NADH or NADPH, which are not likely to be present in column ghosts, this is probably not the mechanism by which they inhibit K-Cl cotransport. Inhibition by eicosatetraynoic acid is interesting, but has little apparent mechanistic significance. None of the recognized mechanisms of signal transmission is involved in the transmission of the signal from the volume sensor to the cotransporter.

Dilution of some intracellular component is often suggested as the signal produced by cell swelling which leads to increased cotransport. Jennings and Schulz (1991) proposed that dilution of cytosolic Mg^{2+} may be important in regulating cotransport activity in rabbit red cells, and Colclasure and Parker (1992) have published evidence that K-Cl cotransport in dog red cells is activated by dilution of intracellular hemoglobin or some other protein. We have previously reported (Sachs, 1988) that

ghosts demonstrate volume-stimulated increases in K-Cl cotransport when they were prepared so that their volumes varied in solutions of 295 mosmol/kg H_2O although their intracellular solute concentration was the same at each volume; similarly, Kracke and Dunham (1990) showed that inside-out vesicles prepared from sheep red cells demonstrated volume-sensitive increases in K-Cl cotransport at constant cytoplasmic solute concentration. The experiments reported here were designed so that K-Cl cotransport was measured in swollen and shrunken ghosts with the same concentration of intracellular solute, and there was very little protein present in these ghosts. Ghosts respond to swelling and shrinkage as do intact cells, and the response cannot be mediated by changes in ghost solute concentration.

Jennings and Al-Rohil (1990) and Colclasure and Parker (1992) have questioned the results from ghosts and from inside-out vesicles, primarily because of their calculation that swelling increases cotransport only twofold in these preparations. The fractional stimulation of influx in ghosts is less than the stimulation in intact cells, but it varies from preparation to preparation; stimulation by swelling was about fourfold in the experiments recorded in Table IV. Furthermore, the absolute value of the stimulated influx is quite large; for technical reasons, we measure K influx at ~ 2 mM K, but the $K_{1/2}$ for influx in these ghosts is 26.7 mM (Sachs, 1988) so that the values we measure at 2 mM K are $\sim 7\%$ of the maximal value (Lauf et al. [1992] have collected values for these parameters from experiments with ghosts and with intact red cells). The ratio of the fluxes in the swollen ghosts to those in the shrunken ghosts is low primarily because K-Cl cotransport in the shrunken ghosts is large; we suggest a reason for this below. At any rate, it is hard for us to believe that the fundamental mechanisms by which swelling stimulates K-Cl cotransport in ghosts and inside-out vesicles are different from those in intact cells. In addition, ghosts allow one to manipulate experimental conditions to a far greater extent than is possible with intact cells.

A major difference between ghosts and red cells is the relation between swelling and the dephosphorylation of the serine or threonine necessary for optimal demonstration of K-Cl cotransport. In ghosts, this dephosphorylation occurs before swelling and in cells it may occur in large part after swelling. We suggest that this may be due to the relative activity of a membrane-bound phosphatase and a cytosolic kinase. In ghosts, the kinase is gone and the balance of activity is shifted toward dephosphorylation even in shrunken ghosts, and as a result K-Cl cotransport is relatively high. In intact mature red cells, access of the kinase to its substrate may be reduced due to a change in the conformation of the substrate induced by cell swelling. But on the basis of the ghost experiments this cannot be the primary effect of swelling; in order for increased cotransport to appear, swelling must in some way activate the cotransporter directly, and it is possible that whatever change occurs in the cotransporter alters access of the kinase to its serine or threonine substrate. This phenomenon may be responsible for the observation that K-Cl cotransport is higher in young than in old red cells (Ellory, Hall, and Stewart, 1985; Canessa, Fabry, Blumenfeld, and Nagel, 1987) and cells with abnormal hemoglobins, HbC and HbS (Brugnara, Kopin, Bunn, and Tosteson, 1985; Brugnara, Bunn, and Tosteson, 1986; Berkowitz and Orringer, 1987; Canessa et al., 1987). In these cells it may be that balance of phosphatase and kinase activity is poised toward dephosphorylation, perhaps because of variations in

the molecular structure of the membrane, and as a result K-Cl cotransport is apparent even in cells of normal volume. In mature red cells both dephosphorylation and dilution of hemoglobin may provide mechanisms for modulating the response to the primary signal elicited by cell swelling.

Differences between ghosts and cells in the regulation of protein phosphorylation may also provide an explanation for the absence of a lag in the onset of a swelling-induced increase in cotransport activity in ghosts and its presence in intact cells. Dunham et al. (1993) have been able to dissect the timed response of K-Cl cotransport to cell swelling by varying intracellular Mg^{2+} concentration. They concluded that there is a fast step in cotransport stimulation directly resulting from cell swelling, and a slow step caused by dilution of cell Mg^{2+}; in Mg^{2+}-free cells there was no lag, but in high Mg^{2+} cells a lag was apparent. We propose that, when lags are seen, they result from a slow step caused by some mechanism secondarily related to cell swelling which occurs in series with a rapid step caused by direct interaction of the sensor with the cotransporter. For instance, in the experiments with rabbit red cells reported by Jennings and Al-Rohil (1990), we suggest that the slow step is dephosphorylation of a phosphoprotein which enhances K-Cl cotransport in swollen cells and which occurs after cell swelling; this step is in series with a rapid step directly coupled to cell swelling and requiring direct communication between the sensor and the cotransporter. This explanation is consistent with the observation that low doses of okadaic acid increase the duration of the lag. If this is true then reducing the phosphorylation level of the protein before swelling (perhaps by inhibiting the relevant kinase) as occurs in ghosts removes the slow step and reduces the lag in the swelling-activated increase in cotransport. Similarly, dilution of hemoglobin might increase the rate of some slow step in series with a fast process initiated by swelling. In ghosts neither dephosphorylation nor dilution of hemoglobin is necessary to observe maximal K-Cl cotransport and so there is no slow step and no lag.

Consideration of the effects of kinase and phosphatase inhibitors on the response of cotransport and countertransport systems to cell swelling has led to an attractive hypothesis which proposes that volume regulatory ion fluxes are coordinated by swelling-dependent dephosphorylation and shrinkage-dependent phosphorylation (Cossins, 1991; Parker et al., 1991; Grinstein, Furuya, and Bianchini, 1992). Thus, swelling results in dephosphorylation which activates the K-Cl cotransporter and inactivates the Na-H exchanger and the Na,K,2Cl cotransporter, resulting in a net loss of ions and cell shrinkage. Shrinkage results in phosphorylation which inactivates the K-Cl cotransporter and activates the Na-H exchanger and Na,K,2Cl cotransporter, resulting in a net gain of ions and cell swelling. The results presented here suggest that in red cell ghosts at least activation of K-Cl cotransport can occur without intervention of a phosphorylation event, and Grinstein, Woodside, Sardet, Pouysse-gur, and Rotin (1992) reported that, in human bladder carcinoma cells and Chinese hamster ovary cells, stimulation of Na-H exchange by swelling is not associated with phosphorylation of the antiporter although stimulation by growth promoters is. If phosphorylation–dephosphorylation events coordinate the response of ion transporters to volume changes, they must do so by modulating the response of the transporters to other, more primary signals of volume change.

In conclusion, swelling-activated K-Cl cotransport in human red cell ghosts is

promoted by phosphorylation by ATP of some membrane component, probably a tyrosine group, and requires dephosphorylation of a phosphoserine or phosphothreonine. However, neither of these events is part of the pathway of signal transduction from the sensor of ghost volume to the cotransporter, nor is any other known messenger. Swelling activation of cotransport in these ghosts is not mediated by dilution of any cytoplasmic solute. Remaining possibilities are that the sensor interacts directly with the cotransporter, or that the cotransporter itself senses changes in cell volume. Phosphorylation and dephosphorylation or changes in the concentration of intracellular solutes then modulate the response of the cotransporter to the primary signal from the volume sensor. This mechanism no doubt occurs in intact red cells, and may well occur in other cells that respond to increases in cell volume by losing K and Cl.

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