

Volume-sensitive K Influx in Human Red Cell Ghosts

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ABSTRACT K influx into resealed human red cell ghosts increases when the ghosts are swollen. The influx demonstrates properties similar to volume-sensitive K fluxes present in other cells. The influx is, for the most part, insensitive to the nature of the major intracellular cation and therefore is not a K-K exchange. The influx is much greater when the major anion is Cl than when the major anion is NO₃; Cl stimulates the flux and, at constant Cl, NO₃ inhibits it. Increase in the influx rate is rapid when shrunken ghosts are swollen or when NO₃ is replaced by Cl. The volume-sensitive K influx requires intracellular MgATP at low concentrations, and ATP cannot be replaced by nonhydrolyzable ATP analogues. The volume-sensitive influx is inhibited by Mg²⁺ and by high concentrations of vanadate, but is stimulated by low concentrations of vanadate. It is not modified by cAMP, the removal of Ca²⁺ by EGTA, substances that activate protein kinase C, or by inhibition of phosphatidylinositol kinase. The influx is inhibited by neomycin and by trifluoperazine.

INTRODUCTION

Many cells restore their volume toward normal after exposure to anisotonic solutions (Eveloff and Warnock, 1981; Siebens, 1985). Volume regulatory mechanisms may be important for processes such as the maintenance of epithelial cell volume during solute absorption and the adjustment of cell volume during differentiation. When cells swell in hypoosmotic solutions, readjustment of volume takes place by the loss of two of the principal intracellular solutes, K and Cl, along with osmotically obligated water. However, the pathway by which solute is lost varies from cell to cell. In Ehrlich ascites tumor cells (Hoffman et al., 1984; Thornton and Laris, 1984) and peripheral blood lymphocytes (Grinstein et al., 1982) KCl loss takes place through separate conductive pathways for K and Cl; in *Amphiuma* red blood cells (Cala, 1983) K is lost by means of K-H exchange and Cl loss occurs through the anion exchanger capnophorin; the volume-sensitive loss of KCl in duck red cells (Kregenow, 1971) seems to take place by electroneutral K-Cl cotransport (Lytle and McManus, 1987).

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Volume-sensitive K fluxes have been demonstrated in the mature red cells of many mammalian species (Ellory et al., 1985). In most cases the fluxes have been shown to be Cl dependent, but K movement through a conductive pathway or by means of K-H exchange may be Cl dependent, as is K loss by means of K-Cl cotransport. Mature human red blood cells demonstrate Cl-dependent (Dunham et al., 1980) and volume-sensitive (Kaji, 1986) K fluxes similar to those present in the red cells of other species, and the fluxes are much more pronounced in young than in old red cells (Hall and Ellory, 1986; Brugnara and Tosteson, 1987; Canessa et al., 1987). Volume-sensitive K fluxes have also been demonstrated in resealed ghosts prepared from human red cells by a one-step hemolysis technique (Dunham and Logue, 1986), and Cl-dependent K fluxes have been described in ghosts prepared by a gel-filtration method (Ha et al., 1987), although in the latter case the fluxes were not volume-sensitive.

Many of the characteristics of the volume-sensitive ion movements have been described, but little is known about the way in which volume change is sensed, or the way in which the signal is transmitted to the transport mechanism. In the course of experiments that characterized the nucleotide requirement of cation fluxes carried out by the Na,K pump of gel-filtered red cell ghosts, we observed ouabain-resistant and volume-sensitive K fluxes similar to those described in intact cells. Since the gel-filtered ghosts are virtually free of cytoplasmic components, and since their composition can be fixed at will, they provide an opportunity for defining the requirements for expression of the volume-sensitive fluxes and for identifying the steps in the sequence between cell swelling and increased K flux. This paper reports the results of some experiments designed with those goals in mind.

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 were used within 3 d in the preparation of resealed ghosts. For the preparation of broken membranes, cells stored at 4°C for as long as 35 d were used.

Resealed ghosts were prepared by a gel-filtration method similar to that described by Kaplan (1982). Cells were separated from plasma and washed with a 150-mM choline chloride solution that contained 0.1 mM EDTA (ethylenediamine tetraacetic acid) and 10 mM PIPES (piperazine-*N,N'*-bis [2-ethanesulfonic acid]) adjusted to pH 5.5 with Tris (Tris [hydroxymethyl] aminomethane); the cells were washed repeatedly until the pH of the cell suspension was 6.0. The cells were then brought to 50% hematocrit in the wash solution and stored on ice until run into the column. The column was 45 × 10 cm and was filled with Bio Gel A50 beads (Bio Rad Inc., Rockville Center, NY); the bed volume was 3.51. The column was enclosed in a water jacket and maintained at -1°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, 200 ml of solution that was 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 B 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. These contained 2% by volume (final volume including ghosts) of a 500-mM Tris HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) solution (500 mM HEPES adjusted to pH 8.0 at 37°C with Tris), 0.5 mM Tris EGTA (ethylene glycol bis- $[\beta$ -aminoethylether] *N,N'*-tetraacetic acid), 0.2 mM ouabain, 50 mg/100 ml albumin, and the substances listed in the legends to the figures and tables; for some experiments the resealing solution contained trace amounts of $^{54}\text{MnCl}_2$. Ghosts accounted for 10–40% of the volume of the suspension. The ghost suspension was kept at 0°C for 5 min and was then incubated at 37°C for 60 min. The ghosts were separated from the suspension, the supernatant was saved for determination of Na and K concentration and, where appropriate, the concentration of ^{54}Mn , and the ghosts were washed three times with solutions described in the legends to the figures and tables.

K influx was measured by adding ghosts to appropriate ice-cold solutions containing $^{86}\text{RbCl}$ or ^{42}KCl . The suspensions were mixed and incubated at 37°C for 45 min, unless otherwise stated in the figure legends. The influx was terminated by returning the tubes to the ice bath and adding 4 ml of ice cold 160 mM choline chloride solution. The suspension was mixed, 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 concentration of the ghosts were measured by flame photometry, or the ^{54}Mn content was measured after decay of ^{42}K . From the K (or ^{54}Mn) content of the ghosts and the K (or ^{54}Mn) concentration of the resealing solution, the volume of resealed ghosts used in the influx measurement was calculated; the concentration of K (or ^{54}Mn) in the ghosts that resealed was, therefore, taken to be the same as the concentration in the resealing solution (which assumes that resealing to K [or ^{54}Mn] 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 measurements were made so all values reported represent ouabain-resistant fluxes.

Broken red cell membranes were prepared by osmotic lysis followed by freezing and thawing, and ATPase activity of the membranes was measured by an assay coupled to the oxidation of NADH; both procedures have been described in detail (Sachs, 1980). The assay solutions contained 500 mM Tris HEPES 2% by volume, 0.5 mM Tris EGTA, 0.2 mM ouabain, 1.75 mM phosphoenolpyruvate, 1.0 mM dithiothreitol, 1.7 IU/ml pyruvate kinase, and appropriate concentrations of KCl, KNO_3 , ATP, $\text{Mg}(\text{NO}_3)_2$, and vanadate; pH was adjusted to 7.4.

To calculate the concentration of Mg necessary to yield the concentration of Mg^{2+} and MgATP indicated in the legends to the figures and tables, the K_D for MgATP was taken to be 50.1 μM , for Mg creatine phosphate (CP) 5.0 mM, for MgEGTA 6.17 mM, for Mg phosphoenolpyruvate 25.1 mM, for MgTNP-ATP 50.1 μM , for Mg TNP-ADP 0.51 mM, for Mg AMP-PNP 5 μM , and for Mg AMP-PCP 50.1 μM .

Determinations were made in quadruplicate. Each point in the figures and each value in the tables is the mean of four determinations, and the SE of the mean is indicated; except, in the figures, not if it is smaller than the symbol. When the curves describe a rate equation, they were fitted to the data by a nonlinear least-squares method; the points were weighted by their variances.

RESULTS

The phenomena with which this paper is concerned are illustrated in the experiment shown in Fig. 1. In this experiment, ghosts either contained ATP or they were ATP-free. In the ghosts that contained ATP, creatine phosphokinase and phospho-

creatine were included to maintain the ATP concentration relatively constant; the ATP-free ghosts contained hexokinase, NADP, and glucose-6-phosphate dehydrogenase, and both the ghosts and the solution in which the measurements were made contained 50 mM glucose. Under these circumstances resealed gel-filtered ghosts have been found to contain low, but not zero-level, concentrations of ATP, under 4 μM , and usually < 1 μM (Sachs, 1987). Either Cl or NO_3 was the principal anion

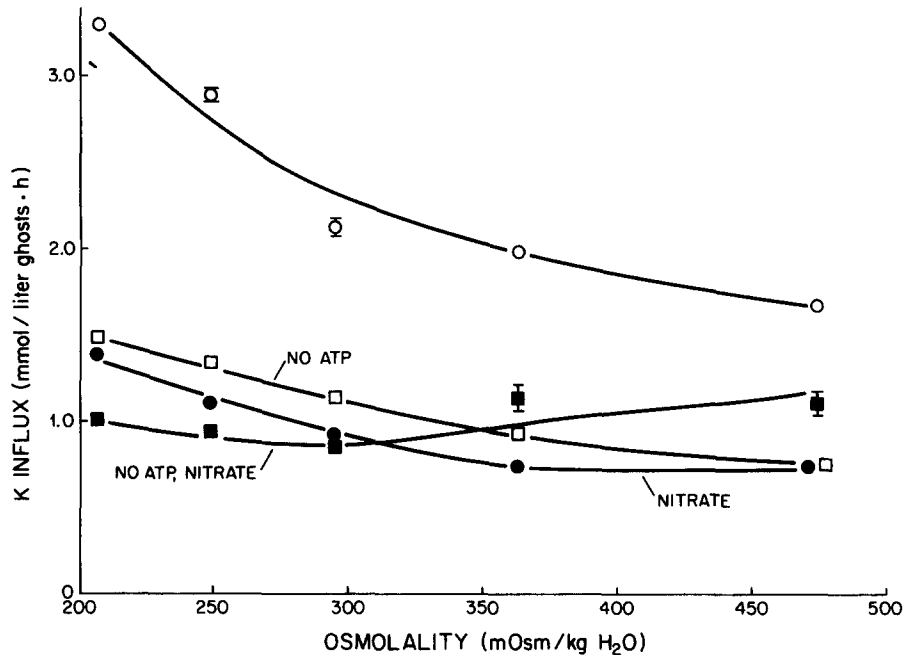


FIGURE 1. Ouabain-resistant K influx vs. osmolality of the solution in which the influx was measured. Ghosts were prepared and resealed in solutions containing the substances described in the Methods section and 136 mM KCl (O, □) or KNO_3 (●, ■), and either 2 mM ATP, 5 mM creatine phosphate (CP), 5 IU/ml creatine kinase (CK), 1.46 mM $\text{Mg}(\text{NO}_3)_2$ (O, ●); or 50 mM glucose, 3.2 IU/ml hexokinase (HK), 1.7 mM Tris NADP, 1.9 IU/ml glucose 6 phosphate dehydrogenase (G6PDH), and 0.11 mM $\text{Mg}(\text{NO}_3)_2$ (□, ■). In each case the concentration of $\text{Mg}(\text{NO}_3)_2$ was calculated to yield a concentration of Mg^{2+} of 0.1 mM. Influx was measured (using ^{86}Rb as tracer) in solutions that contained Tris-glycylglycine buffer (500 mM glycylglycine brought to pH 7.4 at 37°C with Tris) 2% by volume, 2 mM KCl (O, □) or KNO_3 (●, ■), choline chloride (O, □), or choline nitrate (●, ■) to make up the indicated osmolality, 20 mg/100 ml albumin, and 0.2 mM ouabain.

inside and outside the ghosts, and the measurements were made at several osmolalities so that the ghosts shrank when exposed to solutions more concentrated than the solution in which they were resealed, and they swelled when exposed to less concentrated solutions; Hoffman (1958) has shown that resealed ghosts behave as perfect osmometers. In the ghosts that contained ATP, K influx was higher at all osmolalities when measurements were made in Cl solutions than when measurements were made in the ATP-free ghosts or in NO_3 solutions; the influx increased as

the ghosts swelled. The ghosts showed, then, a volume-sensitive K influx that was ATP and Cl dependent.

The experiment shown in Table I was designed to show that the increased K influx was in response to volume and not osmolality. In this experiment, ghosts were resealed in solutions that differed only in the concentration of KCl or KNO₃ they contained. The ghosts, therefore, contained varied amounts of solute after they were resealed. The ghosts were used to measure Cl-dependent K influx, and all measurements were made at the same osmolality. The ghosts resealed at high solute concentration swelled and the ghosts resealed at low solute concentrations shrank. Table I shows that K influx increased with ghost volume when osmolality was held constant.

TABLE I
Ouabain-resistant K Influx at Varying Ghost Volumes but Constant Osmolality

Relative ghost volume	Ouabain-resistant K influx \pm SEM (mmol/liter ghosts \cdot h)		
	Chloride solution	Nitrate solution	Δ
2.09	4.21 \pm 0.05	1.41 \pm 0.02	2.79 \pm 0.05
1.57	3.19 \pm 0.03	1.00 \pm 0.01	2.19 \pm 0.03
1.25	2.45 \pm 0.03	0.89 \pm 0.02	1.57 \pm 0.04
1.05	2.04 \pm 0.02	0.74 \pm 0.01	1.30 \pm 0.02
0.84	1.42 \pm 0.03	0.57 \pm 0.01	0.85 \pm 0.03

Ghosts at the largest relative ghost volume were resealed in solutions that contained 500 mM Tris HEPES buffer 4.2% by volume, 1.04 mM Tris EGTA, 4.18 mM ATP, 10.4 mM CP, 10.4 IU/ml CK, 280 mM KCl or KNO₃, 4.0 mM Mg(NO₃)₂, 0.42 mM ouabain, and 104 mg/100 ml albumin. The calculated Mg²⁺ concentration was 0.47 mM. The ghosts at the smaller relative volumes were resealed in the same solutions diluted with water to contain 75% (relative volume 1.57), 60% (relative volume 1.25), 50% (relative volume 1.05), or 40% (relative volume 0.84) of the concentrations of substances listed above. After resealing the ghosts, they were washed with either Tris chloride or Tris nitrate (174 mM Tris) solution, which contained 0.2 mM ouabain and 20 mg/100 ml albumin. Influx (⁸⁶Rb was used as tracer) was measured in solutions similar to the wash solutions, but with 2 mM KCl or KNO₃. Relative ghost volume was estimated by dividing the calculated osmolality of the resealing solution by the calculated osmolality of the influx solution.

Characteristics of the Cl-dependent K Influx

Fig. 2 shows the results of an experiment in which Cl-dependent K influx was measured at varied external K concentrations. K influx, measured in chloride solutions and in nitrate solutions, is plotted along with the difference between the two values, the Cl-dependent influx. The Cl-dependent influx was fit to a Michaelis-Menten equation, and the best fitting parameters were found to be V_M 27.0 mmol/liter ghosts per h and $K_{1/2}$, 26.7 mM. The Michaelis-Menten curve is strictly hyperbolic and there is no sign of sigmoidicity. In ghosts $K_{1/2}$ is somewhat greater (1.6 times) and V_M is markedly greater (22 times) than values for these parameters measured in intact red blood cells under similar conditions (Kaji and Kahn, 1985). Marked stimulation of Cl-dependent K influx during the process of hemolyzing and resealing human red cells has been observed before (Dunham and Logue, 1986).

Table II shows the results of an experiment in which ^{42}K uptake by swollen ghosts was measured at a fixed concentration of external K and high fixed concentrations of various internal and external monovalent cations; the value of the rate constant for K influx is given. Variation of the principal intracellular monovalent cation did not greatly alter ^{42}K uptake in nitrate solutions, nor did variation of the principal

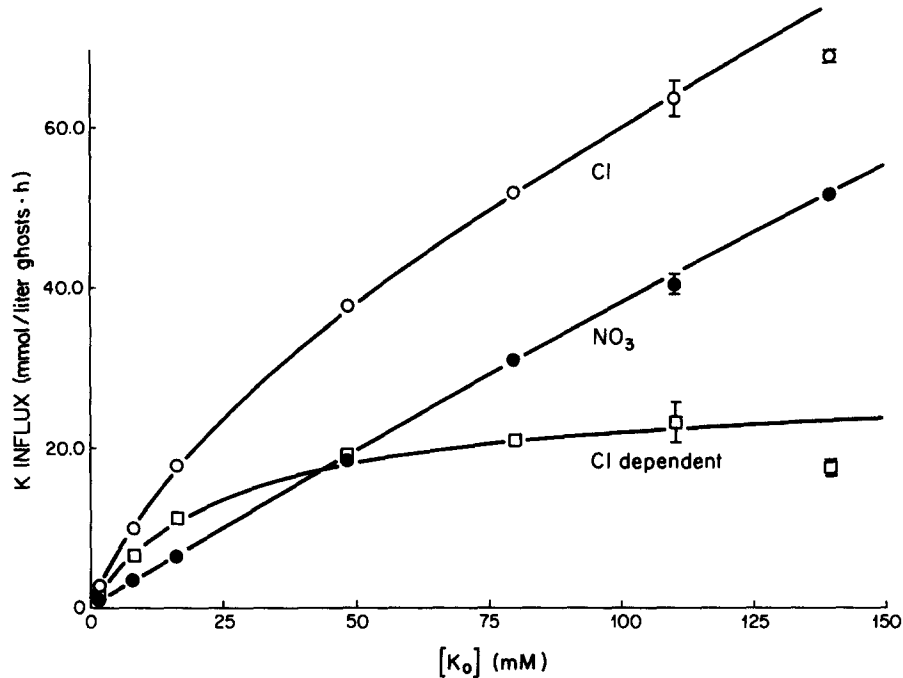


FIGURE 2. Ouabain-resistant K influx vs. extracellular K concentration. Ghosts were prepared and resealed in solutions that contained the substances described in the Methods section and 136 mM KCl or KNO_3 , 2 mM ATP, 5 mM CP, 5 IU/ml CK, and 1.46 mM $\text{Mg}(\text{NO}_3)_2$; the calculated concentration of Mg^{2+} was 0.1 mM. After resealing, the ghosts were washed in a solution that contained 160 mM glucamine chloride or glucamine nitrate 0.2 mM ouabain, and 20 mg/100 ml albumin. Influx was measured (using ^{86}Rb as tracer) in solutions containing the indicated concentrations of KCl or KNO_3 , glucamine chloride or glucamine nitrate to make up a total of 160 mM, 0.2 mM ouabain, and 20 mg/100 ml albumin. Values obtained in chloride (○) and nitrate (●) solutions, and the difference between them (Cl-dependent influx) (□), are plotted. The curve for the Cl-dependent influx is $v = 27.0 \text{ mmol/liter} \cdot \text{ghosts per h} / (1 + 26.7 \text{ mM}/[\text{K}_o])$, and the curve for the influx measured in nitrate solution is $v = 666 \text{ m mol/liter} \cdot \text{ghosts per h} / (1 + 1660 \text{ mM}/[\text{K}_o])$; it differs little from a straight line. the curve for the influx in the chloride solution is the sum of the curves for the Cl-dependent influx and the influx measured in nitrate solution.

extracellular cation. The top part of the table shows the results of experiments in which the principal extracellular cation was choline and the principal intracellular cation was varied. Cl-dependent K uptake was higher when the ghosts contained K, Rb, and Cs than it was when the principal intracellular cation was choline. This suggests that, although there is some exchange of extracellular K for intracellular K,

Rb, and Cs, nevertheless, the bulk of the Cl-dependent K influx takes place through a pathway that is indifferent to the nature of the intracellular cation (similar results were obtained when Tris replaced K). On the other hand, K uptake by ghosts in which the major intracellular cation was Na or Li was less than it was when the principal intracellular cation was choline. Na and Li at the inside surface of the ghost inhibit the Cl-dependent K uptake.

The bottom of Table II shows the results obtained when the principal intracellular cation was K, and the principal extracellular cation was varied. The rate constant at high external K or high external Rb was much less than the rate constant when

TABLE II
Effect of Intracellular and Extracellular Monovalent Cations on Cl-dependent K Influx

Principal cation		Rate coefficient for ouabain-resistant K influx \pm SEM (mmol/liter ghosts \cdot h per mM K_o)		
Intracellular	Extracellular	Chloride solution	Nitrate solution	Δ
K	Choline ↓	1.37 \pm 0.005	0.46 \pm 0.004	0.91 \pm 0.006
Rb		1.54 \pm 0.026	0.56 \pm 0.054	0.98 \pm 0.060
Cs		1.42 \pm 0.038	0.56 \pm 0.007	0.86 \pm 0.039
Na		1.23 \pm 0.042	0.65 \pm 0.017	0.58 \pm 0.045
Li		1.16 \pm 0.050	0.57 \pm 0.018	0.59 \pm 0.053
Choline		1.20 \pm 0.012	0.44 \pm 0.011	0.76 \pm 0.016
K	K ↓ Rb ↓ Cs ↓ Na ↓ Li ↓ Choline	0.72 \pm 0.019	0.38 \pm 0.007	0.34 \pm 0.021
		0.67 \pm 0.011	0.41 \pm 0.005	0.26 \pm 0.012
		0.37 \pm 0.013	0.39 \pm 0.007	-0.02 \pm 0.015
		1.14 \pm 0.039	0.37 \pm 0.037	0.77 \pm 0.054
		1.06 \pm 0.009	0.29 \pm 0.004	0.77 \pm 0.010
		1.37 \pm 0.005	0.46 \pm 0.004	0.91 \pm 0.006

Ghosts were prepared and resealed in the solutions described in the Methods section and 168 mM of XCl or XNO₃ (where X is the intracellular cation indicated), 2.0 mM ATP, 5.0 mM CP, 5.0 IU/ml CK, 1.46 mM Mg(NO₃)₂, and a trace amount of ⁵⁴Mn. Calculated Mg²⁺ was 0.1 mM and calculated osmolality was 354 mosmol/kg \cdot H₂O. After resealing, ghosts were washed with a solution which contained Tris-glycylglycine 500 mM 4.8% by volume, 168 mM choline chloride or choline nitrate, ouabain 0.2 mM, and albumin 20 mg/100 ml. Influx was measured (⁴²K was used as tracer) in solutions which contained Tris-glycylglycine 500 mM 4.8% by volume, 116 mM XCl or XNO₃ (where X is the indicated extracellular cation) 1.09 mM KCl or KNO₃, 0.2 mM ouabain, and 20 mg/100 ml albumin. The calculated osmolality was 260 mosmol/kg \cdot H₂O so that the ghosts must have swelled to 1.36 times their resealed volume during the influx measurement. The rate coefficient for K influx is defined as K influx/[K_o].

choline was the principal extracellular cation. The decreased rate constants result from competition from the unlabeled cation (K or Rb) when it is present at high concentrations and when isotopically labeled K is present at low concentrations. These results indicate, therefore, that the transport system has about the same high affinity for the two ions. Cs depressed the rate constant even more than K or Rb, which may mean that the affinity of the transport system for Cs is even greater than its affinity for the other two ions, but inhibition by Cs by some other mechanism cannot be excluded. The rate constant in the presence of high external concentrations of Na or Li is also less than when choline is the principal extracellular cation. These ions may also interact with the K transport site. Together with the evidence

that Na and Li at the inside inhibit influx, this shows that the transport system is not indifferent to these two ions. The specificity of the transport system for K is not great.

Rapidity with which K Influx Responds to Changes in Osmolality and Anion Composition

Fig. 3 shows the results of two experiments of similar design. In the first experiment (A) we measured the rate of uptake of ^{86}Rb after the ghost volume was suddenly changed. Ghosts were incubated in low osmolality solutions (swollen) and high osmolality solutions (shrunken). Ghosts from each batch were then placed in both low and high osmolality solutions and ^{86}Rb uptake was measured at fixed times. It

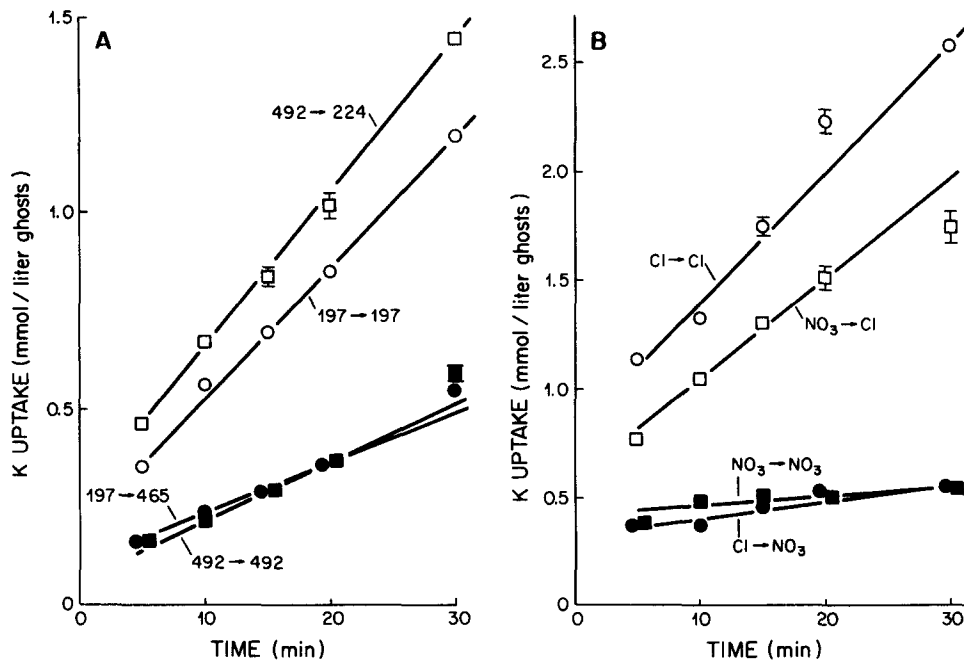


FIGURE 3.

can be seen that when shrunken ghosts are suddenly swollen, the rate of ^{86}Rb uptake reached that of the swollen ghosts placed in hypoosmotic media as rapidly as measurements could be made. Similarly, when swollen ghosts were suddenly shrunk, the rate of ^{86}Rb uptake rapidly decreased to that characteristic of shrunken ghosts. In all cases the uptake extrapolated to $t = 0$ intersected the ordinate above the abscissa. Response of the K influx to volume change is very rapid and without detectable lag.

For the experiment shown in B, ghosts were preincubated either in Cl or NO₃ solutions. The experiment was started by adding ghosts to solutions that were hypoosmotic relative to the solutions in which the ghosts were resealed so that the ghosts would swell. Ghosts incubated in Cl or NO₃ solutions were added to both Cl

and NO_3 solutions. ^{86}Rb uptake was then measured at fixed times. The rate of ^{86}Rb uptake by ghosts preincubated in Cl solution and added to NO_3 solution was about the same as the rate of uptake by ghosts preincubated in NO_3 solution and added to NO_3 solution (the solution that resulted from the $\text{Cl} \rightarrow \text{NO}_3$ solution change contained a final concentration of 20 mM Cl). When measurements were made in the Cl solution, the ghosts preincubated in NO_3 solution took up ^{86}Rb at a rate compa-

FIGURE 3. (*opposite*) Ouabain-resistant K uptake vs. time. For the experiment shown in A, ghosts were prepared and resealed in solutions containing the substances described in the Methods section and 136 mM KCl, 2 mM ATP, 5 mM CP, 5 IU/ml CK, and 1.46 mM $\text{Mg}(\text{NO}_3)_2$. The calculated concentration of Mg^{2+} was 0.1 mM and the calculated osmolality was 295 mosmol/kg· H_2O . After resealing, the ghosts were separated from the resealing solution and divided in two; half (○, ●) were suspended in a solution that contained 500 mM Tris-glycylglycine (2% by volume), 97 mM choline chloride, 0.2 mM ouabain, and 20 mg/100 ml albumin, and incubated for 30 min at 37°C. The calculated osmolality of this solution was 197 mosmol/kg· H_2O so that the cells swelled to 1.5 times their size after resealing. The other half of the ghosts (□, ■) were suspended and incubated for 30 min at 37°C in a solution identical to the first except that it contained, in addition, 160 mM choline nitrate. Its calculated osmolality was 492 mosmol/kg· H_2O , so that the ghosts must have shrunk to 0.6 times their size after resealing. After the incubation, the ghosts were washed in the same solutions, and then K uptake (^{86}Rb used as tracer) was measured in the same solutions as the wash solutions, except that they contained 2 mM KCl; 0.1 ml of ghost suspension was added to 1 ml of solution. Half of the swollen cells were added to the hypotonic solution (○) (197 → 197 mosmol/kg· H_2O) and half to the hypertonic solution (●) (197 → 465 mosmol/kg· H_2O), and half of the shrunken cells were added to the hypotonic solution (□) (492 → 224 mosmol/kg· H_2O) and half to the hypertonic solution (■) (492 → 492 mosmol/kg· H_2O). At the times indicated, ghosts were separated from the suspension, washed, and the uptake of ^{86}Rb determined. The lines are v (mmol/liter ghosts) = A (mmol/liter ghosts) + B ([mmol/liter ghosts]/min) × t (min). The values are: (○) $A = 0.192$, $B = 0.033$; (●) $A = 0.105$, $B = 0.0127$; (□) $A = 0.265$, $B = 0.039$; (■) $A = 0.068$, $B = 0.0150$. For the experiment shown in B, ghosts were prepared and resealed in solutions that contained 500 mM Tris HEPES 1.25% by volume, 0.625 mM Tris EGTA, 5 mM ATP, 6.25 mM CP, 6.25 IU/ml CK, 2.89 mM $\text{Mg}(\text{NO}_3)_2$, 220 mM K, 220 mM Cl or NO_3 , 0.25 mM ouabain, and 62.5 mg/100 ml albumin; the calculated osmolality of the solution was 461 mosmol/kg· H_2O . The ghosts were washed in solutions that contained 500 mM Tris-glycylglycine 6% by volume, 220 mM choline chloride or choline nitrate, 0.25 mM ouabain and 62.5 mg/100 ml albumin. K uptake (^{86}Rb was used as tracer) was measured in solutions that contained 500 mM Tris-glycylglycine 4.4% by volume, 176 mM choline chloride or choline nitrate, 2 mM KCl or KNO_3 , 0.2 mM ouabain, and 20 mg/100 ml albumin; the calculated osmolality of these solutions was 365 mosmol/kg· H_2O . To start the uptake measurements, 0.1 ml of ghost suspension was added to 1 ml of solution; the ghosts must have swelled to 1.23 times their volume after resealing. Half of the ghosts resealed in Cl were added to Cl solutions (○) (220 mM Cl → 180 mM Cl), and half to the NO_3 solutions (●) (220 mM Cl → 20 mM Cl + 160 mM NO_3), and half of the ghosts resealed in NO_3 solutions were added to Cl solutions (□) (220 mM NO_3 → 160 mM Cl + 20 mM NO_3) and half to NO_3 solutions (■) (220 mM NO_3 → 180 mM NO_3). At the times indicated, ghosts were separated from the suspension, washed, and the uptake of ^{86}Rb determined. The lines are v (mmol/liter ghosts) = A (mmol/liter ghosts) + B ([mmol/liter ghosts]/min) × t (min). The values are (○) $A = 0.796$, $B = 0.0595$; (●) $A = 0.314$, $B = 0.00822$; (□) $A = 0.576$, $B = 0.0462$; (■) $A = 0.404$, $B = 0.00448$.

erable to, but less than, that at which the ghosts preincubated in Cl solution took up the isotope. The final solution that resulted from the $\text{NO}_3 \rightarrow \text{Cl}$ change contained 20 mM NO_3 . The reason for the discrepancy between the uptake rate in this experiment and the uptake rate in the $\text{Cl} \rightarrow \text{Cl}$ experiment will be seen in the next section. The response of the ghosts to a change from $\text{Cl} \rightarrow \text{NO}_3$ or $\text{NO}_3 \rightarrow \text{Cl}$ was very rapid and without detectable delay (exchange of Cl and NO_3 across the ghost membrane was completed within a few seconds).

Cl Dependence of K Influx

Fig. 4 A shows the results of an experiment in which K influx was measured in swollen ghosts at a number of different Cl concentrations; Cl was replaced with NO_3 so that the sum was constant. The curve was concave upward. Similar results have been reported when K influx was measured as a function of Cl concentration, with Cl replaced by NO_3 , in unmodified human (Dunham et al., 1980) and sheep (Dunham and Ellory, 1981) red cells, and in human (Lauf et al., 1984) and sheep (Lauf, 1983) red cells exposed to NEM (*N*-ethylmaleimide) (which promotes a Cl-dependent K flux with characteristics similar to the volume-sensitive K fluxes present in ghost preparations). The upwardly concave curve has been attributed to a mechanism in which multiple Cl ions must interact with the system before K influx is stimulated.

It is possible, however, that the curve is upwardly concave because of inhibition of the influx by NO_3 instead of, or in addition to, stimulation by Cl. We were able to resolve the question by preparing swollen ghosts with a number of Cl concentrations; in half the ghosts at each Cl concentration Cl was replaced by NO_3 to maintain a constant total anion concentration, and half were NO_3 -free. The results of the experiment in which we measured ^{86}Rb uptake by these ghosts is shown in Fig. 4 B. Even in the absence of NO_3 , Cl stimulated the K influx and the curve relating influx to Cl concentration is hyperbolic. When NO_3 replaces Cl the curve is, as before, concave upwards and the K influx at any Cl concentration is less in the presence of NO_3 than in its absence. Table III gives the results of an experiment of which K influx was measured at constant Cl concentration and varying NO_3 concentration; NO_3 inhibited the K influx. For technical reasons we were not able to determine whether NO_3 inhibition was competitive with Cl. The results clearly show that, although Cl stimulated K influx, NO_3 inhibits it. Similar results have been reported (Lauf, 1984) with Cl-dependent K transport in sheep red cells; a plot of K influx against Cl concentration was upwardly concave when NO_3 replaced Cl, but hyperbolic when HCO_3 replaced Cl.

ATP Dependence of the Volume-sensitive K Influx

The Cl-dependent and volume-sensitive influx of K was shown in Fig. 1 to be dependent on the presence of ATP within the ghosts. Fig. 5 shows an experiment in which Cl-dependent K influx into ghosts swollen to 1.12 times their resealed volume was measured as a function of the MgATP concentration within the ghosts. In this experiment, MgATP concentration was varied by varying both the total Mg concentration and the total ATP concentration while Mg^{2+} was kept constant at 0.1 mM. The affinity for ATP is quite high, within the range of ATP concentrations found in

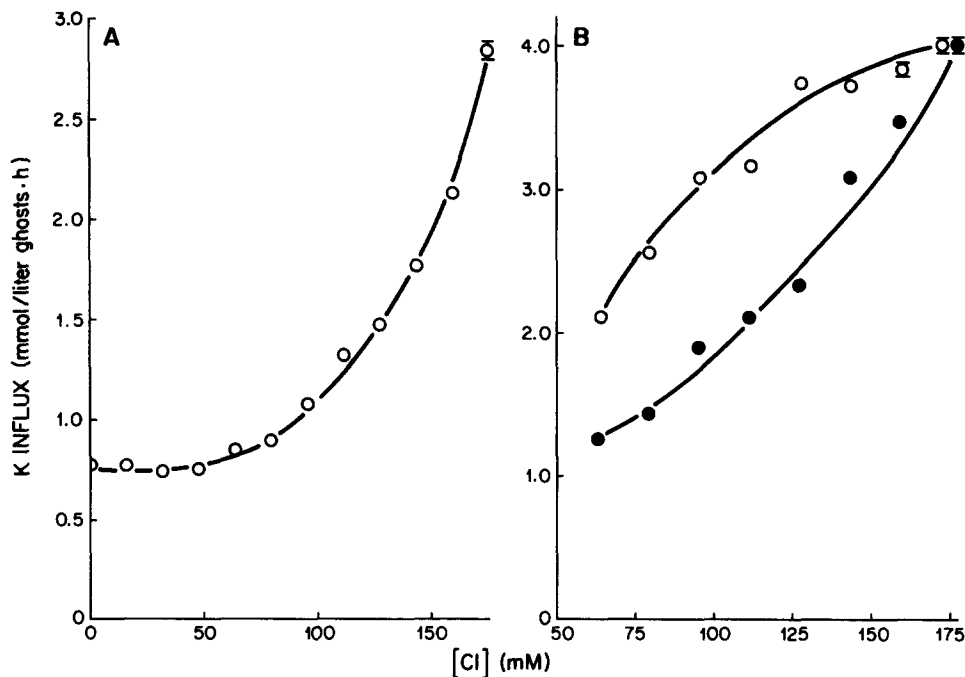


FIGURE 4. Ouabain-resistant K influx vs. Cl concentration of the solution in which K influx was measured. In the experiment shown in A, Cl was replaced by NO_3 , so that the total Cl + NO_3 remained constant. Ghosts were prepared as described in the Methods section and resealed in solutions which contained 500 mM Tris HEPES 1.25% by volume, 0.625 mM Tris EGTA, 5 mM ATP, 6.25 mM CP, 6.25 IU/ml CK, 2.89 mM $\text{Mg}(\text{NO}_3)_2$, 0.25 mM ouabain, 62.5 mg/100 ml albumin, 240 mM K, and a total concentration of Cl + NO_3 of 240 mM; the lowest concentration of nitrate was 20 mM and the highest 240 mM. The calculated concentration of Mg^{2+} was 0.125 mM, and the calculated osmolality of the resealing solutions was 492 mosmol/kg H_2O . The resealed ghosts were washed in solutions containing choline chloride and choline nitrate concentrations equal to those of the resealing solutions, 500 mM Tris-glycylglycine 6% by volume, 0.25 mM ouabain and 12.5 mg/100 ml albumin. Influx was measured (using ^{86}Rb as tracer) in solutions that contained 500 mM Tris-glycylglycine 4.8% by volume, combinations of choline chloride and choline nitrate sufficient to yield a total of 192 mM Cl + NO_3 with the concentration of Cl indicated on the abscissa, 2 mM KNO_3 , 0.2 mM ouabain, and 10 mg/100 ml albumin. Final calculated osmolality of the influx solution was 394 mosmol/kg H_2O so that the ghosts swelled in the influx solution to 1.25 times their resealed volume. In the experiment shown in B, Cl was (●) or was not (○) replaced by NO_3 . Ghosts were prepared as described in the Methods section and resealed in solutions that contained 500 mM Tris HEPES 2.5% by volume, 0.625 mM Tris EGTA, 2.5 mM ATP, 6.25 mM CP, 6.25 IU/ml CK, 80 mM KCl, 1.82 mM $\text{Mg}(\text{NO}_3)_2$, 0.25 mM ouabain, and 62.5 mg/100 ml albumin. The calculated concentration of Mg^{2+} was 0.125 mM. The solutions contained 0–140 mM choline chloride, and the choline chloride was (●) or was not (○) replaced by choline nitrate to make up a total of 140 mM. After resealing, the ghosts were washed in solutions made up of 500 mM Tris-glycylglycine 4.4% by volume, 0.2 mM ouabain, 10 mg/100 ml albumin, and 80–220 mM choline chloride; choline chloride was (●) or was not (○) replaced by choline nitrate to make up a total of 220 mM. Influx was measured (^{86}Rb was used as tracer) in solutions identical to the wash solutions except that they were diluted with 0.25 volume H_2O /volume wash solution, and with 1.74 mM KCl. During the influx measurement the ghosts were 1.25 times their volume after resealing.

ghosts resealed in the absence of ATP. We assumed that all the Cl-dependent K influx is dependent on MgATP, and that the relation between K influx and intracellular MgATP is described by the Michaelis-Menten relation. V_M , $K_{1/2}$ for MgATP, and residual MgATP present in the ghosts resealed in the absence of ATP were estimated by a nonlinear least-squares procedure as described in the legend to Fig. 5. The estimated $K_{1/2}$ for MgATP was low, $< 2 \mu\text{M}$.

In the experiment shown in Fig. 5, Mg^{2+} was held constant, but both MgATP and total ATP were increased, and the Cl-dependent K influx may have varied with either. Fig. 6 shows the results of an experiment in which total ATP concentration was held constant, while the concentrations of both MgATP and Mg^{2+} were increased; the calculated MgATP concentration at each concentration of Mg^{2+} is indicated in the figure. As Mg^{2+} (and MgATP) were increased, Cl-dependent K influx at first increased; very little MgATP, $\sim 15 \mu\text{M}$, was present when K influx was

TABLE III
Inhibition of Ouabain-resistant K Influx by Nitrate

Nitrate concentration	Ouabain-resistant K influx \pm SEM (mmol/liter ghosts \cdot h)
<i>mM</i>	
0	2.86 \pm 0.05
16	2.55 \pm 0.03
32	2.33 \pm 0.02
48	2.03 \pm 0.01
64	1.50 \pm 0.01

Ghosts were prepared as described in the Methods section and resealed in solutions that contained 500 mM Tris-glycylglycine 2.5% by volume, 0.625 mM Tris EGTA, 2.5 mM ATP, 6.25 mM CP, 6.25 IU/ml CK, 1.82 mM $\text{Mg}(\text{NO}_3)_2$, 0.25 mM ouabain, 62.5 mg/100 ml albumin, 120 mM KCl, and 0, 20, 40, 60, or 80 mM choline nitrate. After resealing the ghosts, they were washed in solutions that contained 500 mM Tris HEPES 5.5% by volume, 0.25 mM ouabain, 12.5 mg/100 ml albumin, 120 mM choline chloride, and 0, 20, 40, 60, or 80 mM choline nitrate. Influx (^{86}Rb was used as tracer) was measured in solutions identical to the wash solutions except that they contained 0.25 volume H_2O /volume wash solution, and 1.74 mM KCl. During the influx measurement the ghosts were 1.25 times their volume at resealing.

half maximal. As the concentration of Mg^{2+} was further increased, K influx decreased. The estimate of $K_{1/2}$ for MgATP from this experiment must be a minimal value since the peak of the curve underestimates V_M because of inhibition by Mg^{2+} superimposed on the stimulatory effects of MgATP. Fig. 7 shows the results of a complementary experiment in which the concentration of MgATP was kept constant and the concentration of Mg^{2+} was increased. As Mg^{2+} was increased, the total ATP concentration decreased, and the total concentration of ATP at each concentration of Mg^{2+} is indicated in Fig. 7. There was little change in K influx over the range of Mg^{2+} concentrations at which K influx was stimulated in the experiment shown in Fig. 6. From the results shown in Figs. 5–7, we conclude that K influx increases with MgATP concentration rather than with the concentration of Mg^{2+} or total ATP, and that the $K_{1/2}$ for MgATP must be very low, $< 20 \mu\text{M}$. In addition, the

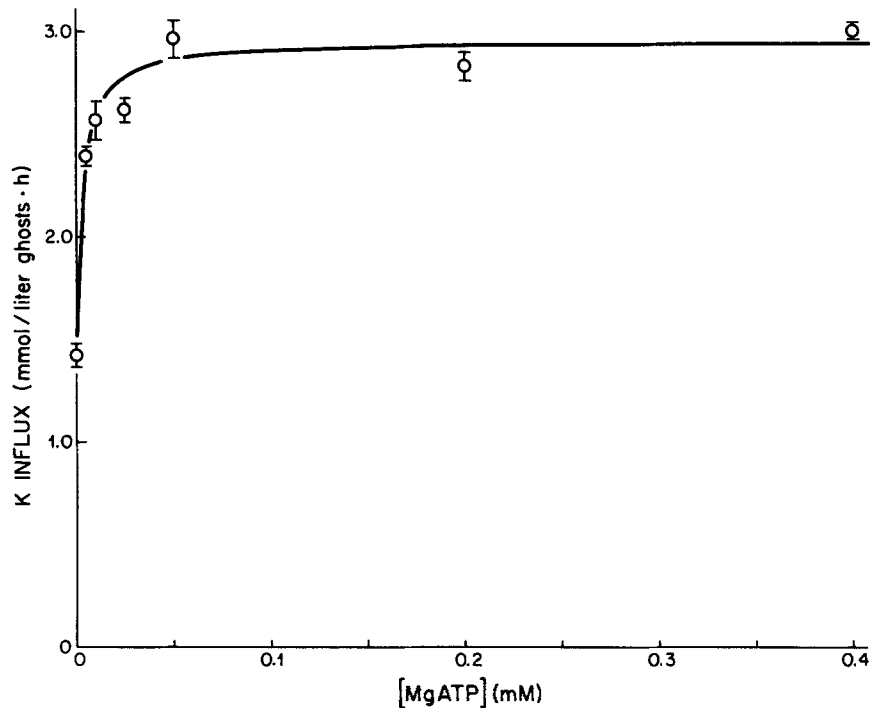


FIGURE 5. Ouabain-resistant K influx vs. intracellular concentration of MgATP. Ghosts were prepared as described in the Methods section and resealed in solutions that contained 500 mM Tris HEPES 2.5% by volume, 0.60 mM Tris EGTA, 0.24 mM ouabain, 60 mg/100 ml albumin, 169 mM KCl or KNO₃, 5.7 mM CP, 5.1 IU/ml CK and the amounts of ATP and Mg(NO₃)₂ needed to give 0.1 mM Mg²⁺ and the indicated concentrations of MgATP. In the ATP-free ghosts, CP and CK were omitted and the solutions contained 60 mM glucose, 3.1 IU/ml HK, 4.6 IU/ml G6PDH, and 2.1 mM Tris NADP. The calculated osmolality of the resealing solutions was 336 mosmol/kg·H₂O. After resealing, the ghosts were washed with solutions that contained 500 mM Tris-glycylglycine 2.4% by volume, 184 mM choline chloride or choline nitrate, 0.24 mM ouabain, and 24 mg/100 ml albumin. Influx was measured (with ⁸⁶Rb as tracer) in solutions which contained Tris-glycylglycine 2.0% by volume, 150 mM choline chloride or choline nitrate, 1.98 mM KCl or KNO₃, 0.2 mM ouabain, and 20 mg/100 ml albumin; the calculated osmolality was 299 mosmol/kg·H₂O so that during the influx measurement the ghosts were 1.12 times their volume after resealing. The values shown are the differences between the values found in Cl solutions and those found in NO₃ solutions; the influx in NO₃ solutions varied from 0.84 ± 0.022 to 1.00 ± 0.012 mmol/liter ghosts × h. The curve is $v = V_M / (1 + K_{1/2} / [MgATP + C])$ where v is the value of the K influx (mmol/liter ghosts × h); V_M is the maximal velocity at saturating MgATP (mmol/liter ghosts × h), $K_{1/2}$ is the concentration of MgATP when v is half maximal (micromolar), and C (micromolar) is a constant that estimates the residual MgATP content of the nominally ATP-free ghosts. V_M was 2.96, $K_{1/2}$ was 1.71, and C was 1.59.

experiments of Figs. 6 and 7 show that Mg^{2+} inhibits the Cl-dependent K influx even at relatively low concentrations. Most of the experiments reported in this paper were performed at 0.1 mM Mg^{2+} .

Table IV shows the results of an experiment in which we tested the ability of several ATP analogues either to support the Cl-dependent influx or to compete with ATP. None of the analogues substituted for MgATP, and only one, TNP-ADP, showed much ability to reduce the ATP-dependent flux. The ability of MgATP to

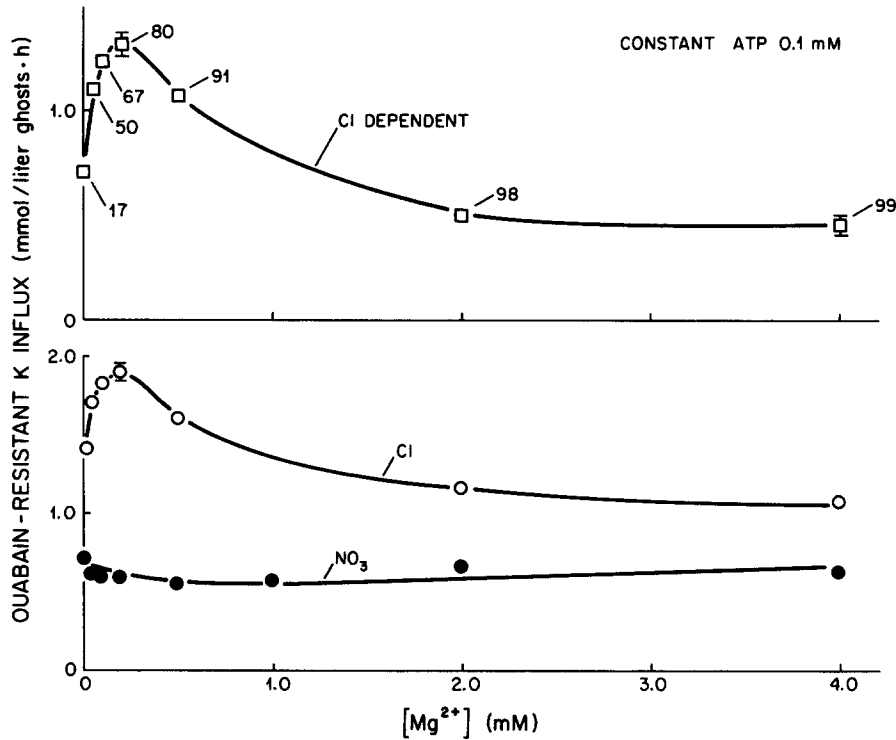


FIGURE 6. Ouabain-resistant K influx vs. intracellular Mg^{2+} concentration. Ghosts were prepared and resealed in solutions that contained the substances described in the Methods section and 136 mM KCl (○) or KNO_3 (●), 0.1 mM ATP, 5.0 mM CP, 5.0 IU/ml CK, and the concentration of $Mg(NO_3)_2$ necessary to give the indicated concentration of Mg^{2+} . The calculated osmolality of the solution was 295 mosmol/kg· H_2O . After resealing, the ghosts were washed with solutions that contained 500 mM Tris-glycylglycine 2% by volume, 150 mM glucamine chloride or glucamine nitrate, 0.2 mM ouabain, and 20 mg/100 ml albumin. Influx was measured (^{86}Rb was used as tracer) in solutions that contained Tris-glycylglycine 2% by volume, 150 mM glucamine chloride or glucamine nitrate, 190 mM KCl or KNO_3 , 0.2 mM ouabain, and 20 mg/100 ml albumin. The calculated osmolality of the influx solution was 295 mosmol/kg· H_2O so that during the influx the ghosts were about the same size as they were after resealing. This figure shows the K influx in the presence of Cl (○) or NO_3 (●), and the difference between the two values (□) at each calculated concentration of Mg^{2+} . The numbers in this figure give the calculated concentration of MgATP (micromolar) at each Mg^{2+} concentration.

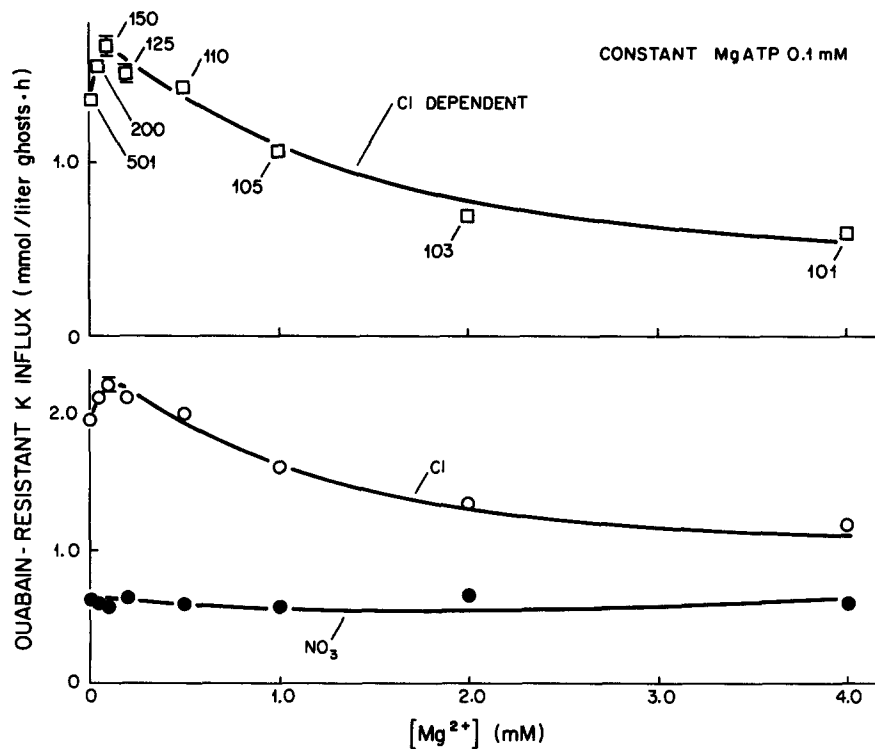


FIGURE 7. Ouabain-resistant K influx vs. intracellular Mg^{2+} concentration. Ghosts were prepared and resealed in solutions that contained the substances described in the Methods section and 136 mM KCl (○) or KNO_3 (●), 5.0 mM CP, 5.0 IU/ml CK, and the concentrations of ATP and $Mg(NO_3)_2$ necessary to yield the indicated concentrations of Mg^{2+} and constant 0.1 mM MgATP. The calculated osmolality of the solution was 295 mosmol/kg· H_2O . After resealing the ghosts, they were washed with solutions that contained 160 mM glucamine chloride or glucamine nitrate, 0.2 mM ouabain, and 20 mg/100 ml albumin. Influx was measured (^{86}Rb was used as tracer) in solutions that were the same as the wash solutions except that they contained 1.99 mM KCl or KNO_3 . The calculated osmolality of the solutions was 295 mosmol/kg· H_2O so that during the influx the ghosts were about the same size as they were after resealing. This figure shows the K influx in the presence of Cl (○) or NO_3 (●), and the difference between the two values (□) at each calculated concentration of Mg^{2+} . The numbers in this figure give the ATP concentrations within the ghosts.

support the volume-sensitive influx seems to be specific, and nonhydrolyzable analogues are not able to stimulate. On the other hand, the analogues are not very effective competitors, either because of the very high affinity for MgATP, or because the analogues are not able to interact with the nucleotide binding site.

Fig. 8 shows the results of an experiment in which Cl-dependent K influx was measured at several vanadate concentrations. The effect of vanadate was biphasic; K influx increased at low vanadate concentrations, but was inhibited at higher concentrations. The effect of vanadate depended on the concentration of Mg^{2+} . Fig. 9 shows the results of an experiment in which we measured the effect of 5 μM vana-

date on Cl-dependent influx at several concentrations of Mg^{2+} . It is clear that $5 \mu M$ vanadate stimulated Cl-dependent influx only when the concentration of Mg^{2+} is low.

The Effect of Some Modifiers of the Activity of Protein Kinases and Phosphatidylinositol Kinases on Volume-sensitive K Influx

The results of the preceding section suggest that phosphorylation of some component of the ghost membrane is necessary for the demonstration of the volume-sensitive Cl-dependent K influx. A variety of protein kinases have been demonstrated in

TABLE IV
Effect of ATP Analogues on Cl-dependent K Influx

Nucleotide analogue	Cl-dependent and ouabain-resistant K influx \pm SEM (mmol/liter ghosts \cdot h)			
	Swollen		Shrunk	
A	ATP 0	ATP 1.0 mM	ATP 0	ATP 1.0 mM
0	0.29 \pm 0.02	1.04 \pm 0.03	0.15 \pm 0.01	0.52 \pm 0.01
TNP-ATP 1.0 mM	0.35 \pm 0.02	1.16 \pm 0.05	0.33 \pm 0.02	0.45 \pm 0.01
TNP-ADP 1.0 mM	0.25 \pm 0.04	0.64 \pm 0.02	0.27 \pm 0.04	0.35 \pm 0.01
B	ATP 0	ATP 0.5 mM	ATP 0	ATP 0.5 mM
0	0.57 \pm 0.01	0.97 \pm 0.02	0.33 \pm 0.01	0.49 \pm 0.01
AMP-PNP 0.5 mM	0.67 \pm 0.02	1.13 \pm 0.01	0.36 \pm 0.01	0.58 \pm 0.01
AMP-PCP 0.5 mM	0.41 \pm 0.02	1.32 \pm 0.02	0.21 \pm 0.01	0.60 \pm 0.01

Ghosts were prepared and resealed in the solutions containing the substances described in the Methods section and 162 mM KCl or KNO_3 , 24 mM Tris, the indicated concentration of nucleotide analogue TNP-ATP, TNP-ADP, AMP-PNP, or AMP-PCP, and an amount of $Mg(NO_3)_2$ sufficient to yield a concentration of Mg^{2+} of 0.3 mM. When the ghosts contained ATP, the resealing solutions contained, in addition to the indicated ATP concentrations, 5.0 mM CP and 5.0 IU/ml CK; when the ghosts were ATP-free, the resealing solutions contained 50 mM glucose, 3.2 IU/ml HK, 2.0 IU/ml G6PDH, and 2.0 mM Tris NADP. The calculated osmolality of the solution was 343 mosmol/kg \cdot H_2O . After resealing the ghosts, they were washed with solutions that contained 500 mM Tris-glycylglycine 2% by volume, 184 mM choline chloride or choline nitrate, 0.2 mM ouabain, and 20 mg/100 ml albumin. Influx was measured (^{86}Rb was used as tracer) in solutions that contained 500 mM Tris-glycylglycine 2% by volume, 1.40 mM KCl or KNO_3 for the experiment in A and 1.88 mM for the experiment in B, 0.2 mM ouabain, 20 mg/100 ml albumin, and enough choline chloride or choline nitrate to make up a total osmolality of 227 or 426 mosmol/kg \cdot H_2O . Ghost volume in hypotonic influx solutions relative to that in the resealing solution was 1.51, and in hypertonic solutions 0.80. The values given are the difference between values obtained in Cl solutions and values obtained in NO_3 solutions.

human red cells, and we therefore attempted to determine whether one of them is involved in increasing Cl-dependent K influx when cells are swollen.

Shrinkage of duck red cells in hyperosmotic solutions increases Na, K, Cl cotransport. The same cotransport system is activated by cAMP (Riddick et al., 1971) or β adrenergic catecholamines (Schmidt and McManus, 1977), and both norepinephrine and cAMP abolish net K loss from hypoosmotically swollen duck red cells (Haas and McManus, 1985). Mature human red cells possess little or no adenylate cyclase or cAMP phosphodiesterase (Thomas et al., 1979), but they do possess cAMP-stimulated protein kinases that are bound to the cell membrane and phosphorylate specific membrane components (Fairbanks and Avruch, 1974). We measured the effect

of cAMP, cAMP and a phosphodiesterase inhibitor, and cGMP on the Cl-dependent K influx. Ghosts were resealed in the presence cAMP, cAMP and isobutylmethylxanthine, and cGMP. After resealing, ghosts were washed, and Cl-dependent K influx was measured at several ghost volumes; the results of the experiment are shown in Table V. The nucleotides did not stimulate the Cl-dependent influx in shrunken

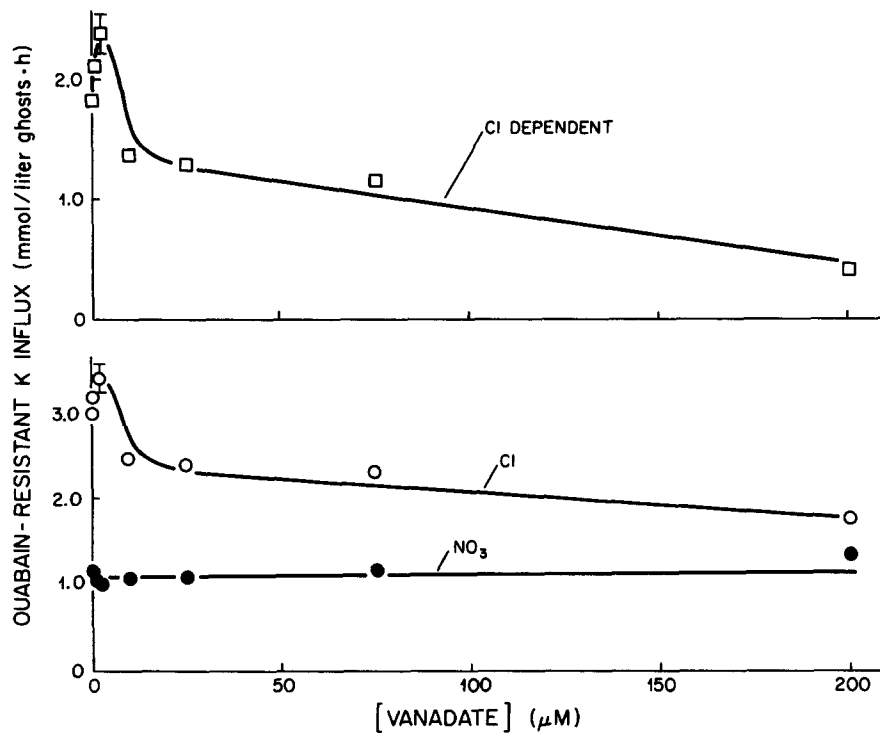


FIGURE 8. Ouabain-resistant K influx vs. vanadate concentration. Ghosts were prepared and resealed in solutions that contained the substances described in the Methods section and 136 mM KCl (○) or KNO₃ (●), 2.0 mM ATP, 5.0 mM CP, 5.0 IU/ml CK, 1.92 mM Mg(NO₃)₂, and the indicated concentrations of vanadate. The calculated concentration of Mg²⁺ was 0.22 mM, and the calculated osmolality was 295 mosmol/kg·H₂O. After resealing the ghosts, they were washed with solutions that contained 160 mM Tris chloride or Tris nitrate, 0.2 mM ouabain, 20 mg/100 ml albumin, and vanadate equal in concentration to that present in the resealing solutions. Influx was measured (⁸⁶Rb was used as tracer) in solutions that were the same as the wash solutions except that they contained 1.95 mM KCl or KNO₃. The calculated osmolality of the influx solutions was 295 mosmol/kg·H₂O so that during the influx measurement the ghosts were about the same size as they were after resealing. This figure shows the K influx in the presence of Cl (○) or NO₃ (●), and the difference between the two values. (□)

cells, and it is, therefore, unlikely that swelling activates K influx by a pathway that includes cAMP- or cGMP-dependent protein phosphorylation. On the other hand, it is not possible to disregard the possibility that phosphorylation of some component of the ghost membrane by a cAMP-dependent kinase is necessary for the expression of the volume-sensitive influx since such a cAMP-dependent process

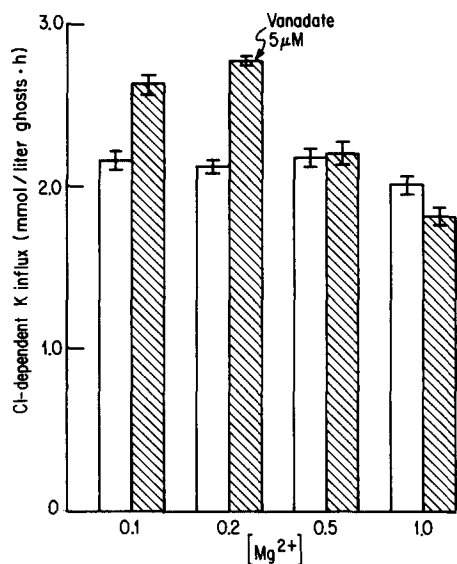


FIGURE 9. Ouabain-resistant K influx measured at several intracellular Mg^{2+} concentrations with and without $5 \mu M$ vanadate. Ghosts were prepared and resealed in solutions that contained the substances described in the Methods section and 136 mM KCl or KNO_3 , 2.0 mM ATP, 5.0 mM CP, 5.0 IU/ml CK, and an amount of $Mg(NO_3)_2$ sufficient to yield the indicated concentrations of Mg^{2+} . The calculated osmolality of the resealing solutions was 295 mosmol/kg $\cdot H_2O$. The resealed ghosts were washed with solutions that contained 160 mM glucamine chloride or glucamine nitrate, 0.2 mM ouabain, 20 mg/100 ml albumin, and the concentration of vanadate present in the resealing solutions. Influx was measured

(^{86}Rb was used as tracer) in solutions identical to the wash solutions except that they contained 1.99 mM KCl or KNO_3 . The calculated osmolality of the solution in which influx was measured was 295 mosmol/kg $\cdot H_2O$, so that during the influx measurements the ghosts were about the same size as they were after resealing. This figure gives the difference between the value of the influx in Cl and NO_3 solutions. Open symbols give Cl-dependent K influx in the absence of vanadate, and hatched symbols in the presence of $5 \mu M$ vanadate.

TABLE V
Effect of cAMP and cGMP on Cl-dependent K Influx

Relative ghost volume	Cl-dependent and ouabain-resistant K influx \pm SEM (mmol/liter ghosts \cdot h)		
	1.59	1.00	0.60
Control	1.88 ± 0.06	0.87 ± 0.02	0.18 ± 0.07
cAMP (1 mM)	1.74 ± 0.03	0.67 ± 0.05	0.22 ± 0.02
cAMP (1 mM + 1 mM IBX)	1.70 ± 0.03	0.32 ± 0.03	0.03 ± 0.03
cGMP (1 mM)	1.98 ± 0.04	0.70 ± 0.02	0.12 ± 0.04

Ghosts were prepared and resealed in solutions containing the substances indicated in the Methods section and 136 mM KCl or KNO_3 , 2 mM ATP, 5 mM CP, 5 IU/ml CK, 1.46 mM $Mg(NO_3)_2$, and 1 mM c-AMP, 1 mM 3 isobutyl-1-methyl xanthine (IBX), 1 mM cGMP as indicated. The resealing solutions also contained 1% by volume dimethylsulfoxide (DMSO). The calculated Mg^{2+} concentration was 0.1 mM and the calculated osmolality was 295 mosmol/kg $\cdot H_2O$. After resealing the ghosts, they were washed with solutions that contained Tris-glycylglycine 2% by volume, 150 mM choline chloride or choline nitrate, 0.2 mM ouabain, and 20 mg/100 ml albumin. Influx was measured (^{86}Rb was used as tracer) in solutions that contained 500 mM Tris-glycylglycine 2% by volume, 1.66 mM KCl or KNO_3 , 0.2 mM ouabain, 20 mg/100 ml albumin, and enough choline chloride or choline nitrate to make up total osmolality of 186, 295, or 492 mosmol/kg $\cdot H_2O$. The values given are the differences between values obtained in Cl solution and values obtained in NO_3 solutions.

might take place in ghosts resealed in the presence of ATP if a small amount of adenylate cyclase was present in the ghost membrane.

There are some indications that the phosphoinositide pathway may be involved in activating the ionic events that result in an increase in cell volume when cells are shrunk in hyperosmotic solutions (Eveloff and Warnock, 1987). Mature human red cells contain protein kinase C (Palfrey and Waseem, 1985), and the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate) and the synthetic diacylglycerol OAG (1-oleoyl-2-acetyl-glycerol) both initiate rapid phosphorylation of cytoskeletal proteins when added to intact cells (Faquin et al., 1986). However, in the absence of activators, protein kinase C is located in the red cell cytoplasm, and membranes prepared by hypoosmotic lysis contain no protein kinase C activity (Palfrey and Waseem, 1985); therefore, it is unlikely that the enzyme is present in our gel-filtered ghosts. Nevertheless, we examined the effect of the activators at concentrations (50 μ M TPA and 0.5 mM OAG) far higher than those shown to simulate protein kinase C in intact cells (Faquin et al., 1986) on the volume-sensitive K influx in an experiment similar to that shown in Table V. Neither TPA nor OAG increased Cl-dependent K influx into shrunken ghosts, but TPA slightly increased and OAG slightly decreased Cl-dependent K influx into swollen ghosts (not shown).

The loss of K and Cl that occurs when Ehrlich ascites tumor cells, *Amphiuma* red blood cells, or lymphocytes return to their original volume after swelling in hypoosmotic solutions is probably initiated by Ca^{2+} (Eveloff and Warnock, 1987). Mature human red cells contain large amounts of calmodulin and several calmodulin-binding proteins (Agre et al., 1983). In the presence of Ca^{2+} , red cell calmodulin is associated with the cell membrane, and slowly dissociates when Ca^{2+} is removed. Our gel-filtered ghosts, prepared at 0°C in Ca^{2+} -free solutions that contained 0.1 mM EDTA, may have retained significant amounts of calmodulin. Although Palfrey et al. (1984) could find no evidence for calmodulin-activated protein kinase in human red cells, Huestis et al. (1981) reported Ca^{2+} -stimulated phosphorylation of spectrin that depended on the presence of calmodulin. Our measurements of volume-sensitive K influx were made using ghosts resealed in Ca^{2+} -free solutions that contained 0.5 mM EGTA, but to be certain that Ca^{2+} was not involved in the activation of the volume-sensitive flux, we compared Cl-dependent K influx into swollen and shrunken ghosts that contained either 0.5 or 10 mM EGTA. The high concentration of EGTA did not alter the Cl-dependent influx at either ghost volume (not shown), so that it seems unlikely that Ca^{2+} initiates volume-sensitive K influx in human red cells.

Although mature human red cells are unable to synthesize phosphatidylinositol *de novo*, their membranes contain phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate; and phosphatidylinositol kinases, phosphokinases, and phosphomonoesterases have been shown to be present in red cell ghosts (Downes and Michell, 1981; Ferrell and Huestis, 1984; Dale, 1985). Although ATP-dependent shape changes of red cell ghosts have been reported to be associated with changes in the phosphorylation state of the membrane phosphoinositides (Quist and Reece, 1980; Ferrell and Huestis, 1984), Patel and Fairbanks (1986) reported experiments that make such involvement unlikely. We have made use of some of their findings to test the possibility that the volume sensitivity of

Cl-dependent K influx may be determined by the phosphorylation state of the phosphoinositides. Adenosine competitively inhibits phosphatidylinositol kinase activity (Buckley, 1977), and Patel and Fairbanks (1986) found that 5 mM adenosine markedly inhibits incorporation of ^{32}P into phosphatidylinositol phosphates of red cell membranes incubated with 1 mM $\gamma[^{32}\text{P}]\text{ATP}$. Table VI shows that 5 mM adenosine had little effect on Cl-dependent K influx into swollen or shrunken ghosts; at the concentration of ATP we used, 5 mM adenosine should have reduced phosphatidylinositol kinase activity to 16% of its activity in the absence of adenosine (Buckley, 1977). Patel and Fairbanks (1986) also found that CoATP (cobalt-ATP) is a good substrate for the phosphatidylinositol kinases and that it supports the incorpo-

TABLE VI
Effect of Adenosine and CoATP on Cl-dependent K Influx

	Cl-dependent and ouabain-resistant K influx \pm SEM (mmol/liter ghosts \cdot h)		
Relative ghost volume	1.50	1.00	0.60
Control	1.71 \pm 0.05	0.76 \pm 0.02	0.30 \pm 0.03
Adenosine (5 mM)	2.06 \pm 0.06	0.61 \pm 0.05	0.24 \pm 0.02
CoATP (1.7 mM)	0.55 \pm 0.01	0.28 \pm 0.03	0.11 \pm 0.01

Ghosts were prepared and resealed in solutions that contained the substances described in the Methods section and 2.0 mM ATP, 5.0 mM CP, 5.0 IU/ml CK, and 136 mM KCl or KNO_3 . The control ghosts contained 1.46 mM $\text{Mg}(\text{NO}_3)_2$, and the adenosine ghosts contained 1.46 mM $\text{Mg}(\text{NO}_3)_2$ and 5 mM adenosine; the calculated concentration of Mg^{2+} in these solutions was 0.1 mM. The CoATP ghosts were Mg-free and contained 1.92 mM CoCl_2 ; calculated Co^{2+} was 0.1 mM. The calculated osmolality of the resealing solutions was 295 mosmol/kg \cdot H_2O . After resealing the ghosts, they were washed with solutions that contained Tris-glycylglycine 2% by volume, 150 mM choline chloride or choline nitrate, ouabain 0.2 mM, and 20 mg/100 ml albumin. Influx was measured (^{86}Rb was used as tracer) in solutions that contained 500 mM Tris-glycylglycine 2% by volume, 0.2 mM ouabain, 20 mg/100 ml albumin, and 1.70 mM KCl or KNO_3 ; for the measurements made in Cl solutions, the influx solution contained 97 mM choline chloride and enough choline nitrate to make up 197, 295, or 492 mosmol/kg \cdot H_2O , and for the measurements in NO_3 solutions, the solutions contained enough choline nitrate to make up the same osmolalities. For the measurements made in ghosts containing adenosine, the influx solutions contained 5 mM adenosine. Relative volume given in the table is the volume of the ghosts during the influx measurement relative to the ghost volume after resealing. The values given are the difference between influx measured in chloride solution and influx measured in nitrate solution.

ration of ^{32}P from $\gamma[^{32}\text{P}]\text{ATP}$ into phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-biphosphate. Table VI shows that CoATP did not support the increase in Cl-dependent K influx that resulted when ghosts that contained MgATP were hypoosmotically swollen.

Although we did not obtain evidence that the activity of either a calmodulin-dependent protein kinase or a phosphatidylinositol kinase accounts for the ATP dependence of the volume-sensitive K influx, we did obtain some unexpected results when we examined the effect on the influx of two substances that are known to modify the behavior of the systems. Trifluoperazine is known to inhibit calmodulin-dependent processes in human red cells (Luthra, 1982) as it does in other tissues. Neomycin has been shown to bind with high affinity to phosphatidylinositol 4,5-biphosphate (Schacht, 1976) and, with lesser affinity, to phosphatidylinositol (McLaughlin and Whitaker, 1988); it inhibits the breakdown of phosphatidylinositol

4-phosphate and phosphatidylinositol 4,5-biphosphate (Lang et al., 1977). Table VII shows that both neomycin and trifluoperazine inhibited the Cl-dependent K influx into swollen ghosts markedly, and, to a lesser extent, also inhibited influx into the shrunken ghosts. Half maximal inhibition of the Cl-dependent K influx into swollen ghosts occurred at ~0.44 mM neomycin (not shown), much higher than the concentration at which binding of neomycin to phosphatidylinositol 4,5-biphosphate is half maximal (Schacht, 1976). We were unable to define the concentration of trifluoperazine at which inhibition is half maximal because concentrations of the

TABLE VII
Inhibition of Cl-dependent K Influx by Neomycin and by Trifluoperazine

	Cl-dependent and ouabain-resistant K influx \pm SEM (mmol/liter ghosts \cdot h)	
	Swollen	Shrunken
Neomycin		
0	3.69 \pm 0.11	1.36 \pm 0.03
10 (μ M)	3.18 \pm 0.05	1.21 \pm 0.03
1 (mM)	0.46 \pm 0.04	0.09 \pm 0.02
Trifluoperazine		
0	4.09 \pm 0.10	2.60 \pm 0.03
10 (μ M)	3.02 \pm 0.05	1.65 \pm 0.01
25 (μ M)	2.32 \pm 0.04	1.69 \pm 0.03

Ghosts were prepared and resealed in solutions that contained the substances described in the Methods section and 2.0 mM ATP, 5.0 mM CP, 5.0 IU/ml CK, 1.46 mM $\text{Mg}(\text{NO}_3)_2$, 168 mM KCl or KNO_3 , and the indicated concentrations of neomycin or trifluoperazine. The calculated concentration of Mg^{2+} was 0.1 mM, and the calculated osmolality was 354 mosmol/kg \cdot H_2O . After resealing the ghosts, they were washed with solutions that contained 500 mM Tris-glycylglycine 4.8% by volume, 168 mM choline chloride or choline nitrate, 0.2 mM ouabain, and 20 mg/100 ml albumin. Influx was measured (^{86}Rb was used as tracer) in solutions that contained 500 mM Tris-glycylglycine 4.8% by volume, 0.2 mM ouabain, and 20 mg/100 ml albumin, and, for the shrunken cells, 235 mM choline chloride or choline nitrate (the calculated osmolality was 478 mosmol/kg \cdot H_2O so that the ghosts were 0.74 times their resealed volume during the influx measurement), and, for the swollen cells, 104 mM (calculated osmolality 236 mosmol/kg \cdot H_2O , ghost volume 1.50 times resealed volume). The influx solutions contained the concentrations of neomycin and trifluoperazine present within the ghosts and, for the neomycin experiment, 2.02 mM KCl or KNO_3 , and, for the trifluoperazine experiment, 1.87 mM KCl or KNO_3 . The table gives the differences between the influx measured in Cl solutions and the influx measured in NO_3 solutions.

drug much greater than 25 μ M made the ghosts leaky, but the effective concentrations were in the range of those that inhibit calmodulin-dependent processes (Luthra, 1982).

DISCUSSION

Kaji (1986) reported a Cl-dependent K influx into human red blood cells that increased when the cells were swollen. The measurements were made using cells from peripheral blood that were not separated with respect to density (and, there-

fore, to age), and the maximal influx was small. Higher flux rates through the volume-sensitive pathway are found when the cells are separated by density and when the measurements are made in the least dense (youngest) fraction (Ellory et al., 1985; Canessa et al., 1987). Treatment of sheep and goat red cells (Lauf and Theg, 1980), and human red cells (Wiater and Dunham 1983; Lauf et al., 1984; Kaji and Kahn, 1985) with NEM increases Cl-dependent K fluxes in cells at normal cell volume. It seems likely that treatment with NEM increases the maximal turnover of the Cl-dependent fluxes present in untreated cells. Dunham and Logue (1986) demonstrated that the Cl-dependent K influx into ghosts prepared from native red cells by a bulk hemolysis procedure was greater than the influx measured with intact cells, and the influx into ghosts was clearly volume sensitive. Ha et al. (1987) prepared resealed ghosts by a gel-filtration method similar to ours and found a Cl-dependent K efflux that was not, however, volume sensitive. The Cl-dependent K efflux was quite large when the ghosts were prepared from the least dense cells, but very small in ghosts prepared from the densest cells. The maximal influx in our ghosts was about the same as the maximal efflux found by Ha et al. (1987) in ghosts prepared from the least dense cell fraction. We cannot account for the differences between our findings when we measured K influx into gel-filtered ghosts and the findings of Ha et al. (1987), who measured K efflux from gel-filtered ghosts, but since Cl-dependent influx into our ghosts was volume sensitive, our preparation was better suited to the goal of attempting to define the way in which ghost swelling activates Cl-dependent K fluxes.

The Cl-dependent K influx that we measured has the kinetic characteristics of the similar processes described above in that the relation between transport rate and external K concentration can be described by the Michaelis-Menten relation and the $K_{1/2}$ for external K is high. The maximal velocity of the Cl-dependent K influx into our gel-filtered ghosts is higher than that seen in other preparations obtained from unfractionated cells, but values for V_M similar to those we found were obtained when measurements were made using cells obtained from the least dense fraction of gradient-separated cells (Ellory et al., 1985; Brugnara and Tosteson, 1987; Canessa et al., 1987). Dunham and Logue (1986) also found high values for volume-sensitive K influx in resealed human ghosts; they found that the increase was prevented if dithiothreitol is present during ghosting. It is possible that ghosting removes some restriction of the volume-sensitive influx that develops as the cells mature. Whatever the reason for the high capacity of the volume-sensitive transport system in ghosts, its characteristics are the same as the characteristics of the system in the least dense fraction of intact cells, and there is no reason to believe that it is not the same process.

The results shown in Table II show that the magnitude of the Cl-dependent K influx into ghosts containing K or Rb as the principal intracellular cation is somewhat greater than the influx into ghosts containing mostly choline; therefore, part of the influx may be a K-K or K-Rb exchange. However, the greater part of the influx is indifferent to the nature of the intracellular cation and, therefore, it is not likely to be an exchange. Swelling-activated Cl-dependent K efflux, along with osmotically obligated cell water, results in loss of cell volume in human red cells (Brugnara and Tosteson, 1987) and sheep reticulocytes (Lauf and Bauer, 1987) and,

therefore, must be a net K loss. The results in Table II also show that the transporter that mediates the Cl-dependent K influx is not absolutely specific for K; Rb, Cs, and even Na and Li are able to compete with K for the transport sites.

The way in which Cl stimulates K fluxes remains unclear. It seems unlikely that Cl is electrically coupled to K influx in ghosts since permeability of human red cells to NO_3 is comparable to their permeability to Cl, and, at any rate, the flux rates of K that we have measured are much less than the maximal flux rates of Cl or NO_3 . Lytle and McManus (1987) found that a Cl-dependent K flux in duck red cells is a K-Cl cotransport, and it would not be surprising if a similar mechanism were operative in our preparation. However, some caution is called for by the observation that replacement of Cl with NO_3 not only decreases K influx by decreasing Cl concentration, but that NO_3 is also an efficient inhibitor at constant Cl concentration. It is possible that NO_3 competes with Cl for binding sites on a K-Cl cotransporter, and that K- NO_3 cotransport does not occur. However, Adorante and Cala (1987) have shown that, in *Amphiuma* red cells, NEM stimulates a K-H exchange, and that stimulation by NEM is Cl dependent. It is likely that in *Amphiuma* red cells Cl is required at some step in the sequence through which NEM induces the K flux rather than for the flux itself, and a similar requirement for Cl, at some step in the process through which the signal of increased cell volume is transmitted to the K transporter, may explain the Cl-dependence of these fluxes instead of, or in addition to, a requirement for Cl of K-Cl cotransport.

There have been previous indications that volume-sensitive, Cl-dependent K fluxes require ATP. Hall and Ellory (1986), who metabolically depleted intact human red cells by a method known to reduce intracellular ATP concentrations to a few micromolar, found that such maneuvers markedly reduced volume-sensitive K influx. On the other hand, Lauf (1983) found that metabolic depletion by a method that reduced intracellular ATP concentrations to 50 or 60 μM , reduced NEM-stimulated but not baseline Cl-dependent K fluxes. Since their ATP concentrations were far above those which we have found to maximally stimulate Cl-dependent fluxes in our ghosts, the metabolic sensitivity of NEM-stimulated fluxes may be related to the disappearance of some metabolite other than ATP. Recently Ha et al. (1987) found that Cl-dependent K efflux from gel-filtered ghosts, which is not volume sensitive, depends on the presence of ATP, and the ATP concentration that was half the maximally effective value corresponds to the values we found.

We have found that the volume-sensitive Cl-dependent K influx in gel-filtered ghosts requires ATP at a low concentration (half maximal stimulation occurs at $<20 \mu\text{M}$ ATP). We also found that the proximate substrate is MgATP, that nonhydrolyzable ATP analogues cannot substitute for ATP, and that the influx is inhibited by vanadate, albeit at high concentrations. Taken together, the results indicate that ATP is used either as a substrate for an ATPase or as a substrate for the phosphorylation of some membrane component. The Mg^{2+} -dependent ATPase present in red cell membranes (ouabain resistant and Ca^{2+} independent) has a $K_{1/2}$ for MgATP of 0.5–0.6 mM and is half maximally inhibited by 1–2 μM vanadate (not shown), so that, if an ATPase is involved in the volume-sensitive influx, it is not the major Mg^{2+} -dependent ATPase. Since vanadate inhibits phosphoprotein phosphatases (Swarup et al., 1982), the observation that low concentrations of vanadate increase the Cl-

dependent K influx could be explained if ATP is used to phosphorylate some membrane component necessary for the influx, and dephosphorylation of the component is inhibited by vanadate at a low concentration. Vanadate increases the incorporation of ^{32}P from $\gamma[^{32}\text{P}]\text{ATP}$ into spectrin, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate of human red cell membranes (Patel and Fairbanks, 1986) so that there is precedent for such a mechanism. If phosphorylation of a membrane component is involved, it is probably not carried out by a cAMP-dependent or calmodulin-dependent protein kinase, by protein kinase C, or by phosphatidylinositol kinase.

Clearly, further work will be necessary to determine the exact relation between ATP utilization and the magnitude of the volume-sensitive K fluxes. Since the response of the Cl-dependent K influx to change in ghost volume is rapid, the temporal relation between the increase in K influx in swollen ghosts and the provision of ATP will be of particular interest; if utilization of ATP is in some way involved in the transmission of the signal of ghost swelling to the K transporter, either by phosphorylation of the transporter itself or of some other membrane component, the response of the Cl-dependent K influx to provision of ATP should also be rapid. Since very low concentrations of ATP are enough to support the Cl-dependent influx, it may be possible to detect membrane components phosphorylated during ghost swelling.

We have found that the Cl-dependent K influx is inhibited by Mg^{2+} , by neomycin, and by trifluoperazine. It was previously shown that Mg^{2+} inhibits Cl-dependent K fluxes in sheep red blood cells (Fujise and Lauf, 1987), and that in human red cells Mg^{2+} inhibits Cl-dependent K fluxes (Brugnara and Tosteson, 1987), but the effects of neomycin and trifluoperazine on these fluxes have not been examined. We examined the effect of neomycin because of its known effect on phosphatidylinositol metabolism, but we did not obtain independent evidence that metabolism of phosphatidylinositol phosphates modified the volume-sensitive K fluxes. Neomycin, at about the concentration (0.5 mM) that half maximally inhibited the Cl-dependent K influx, has been shown to have a number of effects in human red cells; it inhibits the echinocyte to discocyte shape transformation induced by ATP (Quist and Reece, 1980), decreases the lateral mobility of intrinsic membrane proteins (Schindler et al., 1980), and, of course, it modifies phosphatidylinositol phosphate metabolism (Lang et al., 1977; Quist and Reece, 1980). Trifluoperazine was used because it is known to inhibit calmodulin-dependent processes, although we were not able to detect an effect of Ca^{2+} removal on the volume-sensitive fluxes. The three inhibitors may lower the influx by different mechanisms, and pursuit of the characteristics of inhibition may yield some information about how the signal of cell swelling is transmitted to the transporter. It is notable, however, that the three substances that we found to inhibit the volume-sensitive fluxes are positively charged molecules at neutral pH and may be expected to change the surface potential of the inside of the ghost membrane, Mg^{2+} and neomycin (which has 4.5 positive charges at pH 7.0 [McLaughlin and Whitaker, 1988]) by binding to headgroups of negatively charged phospholipids, and trifluoperazine by hydrophobically binding to the phospholipid monolayer. It is possible that interaction between the phospholipid membrane and

the cytoskeleton is influenced by the surface charge of the membrane and is involved in the detection of cell swelling.

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