Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8575–8579, November 1990
Physiology/Pharmacology

Volume-sensitive K–Cl cotransport in inside-out vesicles made from erythrocyte membranes from sheep of low-K phenotype

(cell volume regulation/secondary active transport)

GEORGE R. KRACKE* AND PHILIP B. DUNHAM

Department of Biology, Syracuse University, Syracuse, NY 13244-1220

Communicated by Robert W. Berliner, July 18, 1990

ABSTRACT **Unidirectional K ion effluxes were measured from inside-out vesicles prepared from erythrocyte membranes from sheep of the low-K phenotype. Total K efflux was 150 nmol per mg of protein per hr in a Cl medium of 295 mosmol/kg (with the Na/K pump inhibited). Cl-dependent K efflux (determined with methanesulfonate replacing Cl) was 54 nmol/(mg·hr). Cl-dependent K efflux (K–Cl cotransport) increased to 77 nmol/(mg·hr) with osmotic swelling of ≈30% in 230-mosmol/kg medium and decreased to 13 nmol/(mg·hr) after shrinkage of ≈60% in 430-mosmol/kg medium. Osmotically induced changes in transport and vesicle volume were reversible. K–Cl cotransport was enhanced by ATP. Nonhydrolyzable ATP analogues failed to substitute for ATP, indicating that phosphorylation is involved. However, in the absence of added ATP there was significant K–Cl cotransport, suggesting that phosphorylation is not essential for function. The results provide clues about the nature of the signals detected by the sensor of cell volume changes and demonstrate that inside-out vesicles from sheep erythrocyte membranes provide an advantageous experimental system for investigation of the volume sensor.**

Effluxes of K and Cl from cells promote regulation of cell volume in response to swelling. In epithelia they play roles in transcellular movement of salt and water. The fluxes can be tightly coupled to one another with a 1:1 K/Cl stoichiometry mediated by a K–Cl cotransporter, prototypes being in *Necturus* gall bladder (1) and duck red cells (2, 3). In some cells, the fluxes can be through separate conductive K and Cl channels as in lymphocytes (4). Ehrlich ascites cells can exhibit conductive K and Cl channels as well as K–Cl cotransport (5).

The most detailed studies of K–Cl cotransport have been carried out in mammalian red cells, particularly from people and sheep (see ref. 6 for review). K–Cl cotransport has not been demonstrated directly in these cells owing to the enormous Cl permeabilities. Rather, K–Cl cotransport has been inferred from the dependence of ouabain-insensitive K fluxes on Cl (and Br) (7, 8), the pharmacological similarity to K–Cl cotransport in other cells (low and equal sensitivity to furosemide and bumetanide) (9), and a lack of requirement for Na. The latter two characteristics distinguish K–Cl cotransport from Na–K–Cl cotransport (see refs. 10 and 11 for reviews). Net uphill K efflux driven by a Cl gradient has been demonstrated in sheep and human red cells (12). For these reasons we consider Cl-dependent K transport in sheep red cells to be K–Cl cotransport (6, 11).

K–Cl cotransport is very sensitive to changes in cell volume. Osmotic swelling of less than 10% causes large increases in transport in cells from sheep (7) and people (13). Indeed in normal human erythrocytes, K–Cl cotransport is evident only in swollen cells. It is also present in young human cells (14) and in resealed membrane "ghosts" (15–17).

Fundamental unanswered questions about K–Cl cotransport and other volume-sensitive transport pathways are about the mechanisms by which changes in volume are detected and transduced into changes in transport. Though there is a paucity of direct evidence, two classes of signals that volume sensors of swelling detect can be envisioned: (*i*) a change in concentration of a critical solute; (*ii*) a mechanical change in the membrane and/or ancillary structures such as the cytoskeleton (18–20). K–Cl cotransport in resealed human erythrocyte ghosts is sensitive to osmotically induced changes in volume (15–17). Sachs (16) showed volume-sensitive Cl-dependent K influx in ghosts of varying volume but containing identical solutions. This observation tends to rule out a changing concentration of a cytoplasmic solute as a signal to the volume sensor.

Whatever the nature of the signal, the volume sensor is likely to reside at the cytoplasmic membrane surface. An advantageous experimental system for the study of the volume sensor would provide direct, continuous access to the cytoplasmic membrane surface and at the same time permit measurement of transmembrane fluxes. Inside-out vesicles (IOVs) made from erythrocyte membranes can provide such an experimental system. We chose erythrocytes from sheep of the low-K (LK) phenotype from which to make IOVs. These cells have relatively low Na/K pump fluxes and lack both Na–K–Cl cotransport (7) and Ca-activated K transport (21). Most of the K transport in these cells is through volume-sensitive K–Cl cotransport, making them a good source of IOVs for the study of this transport pathway, provided it is functional in the vesicles.

There have been reports of bumetanide-inhibitable Na transport in IOVs from rat erythrocytes (22) and volume-sensitive Na–K–Cl cotransport in membrane vesicles from rat kidney (23). Brush-border membranes from rabbit kidney exhibit K–Cl cotransport (24), but volume sensitivity was not tested. There is also K–Cl cotransport across membranes of rough endoplasmic reticulum of rat liver (25). We know of no studies of K–Cl cotransport in erythrocyte IOVs.

We have demonstrated K–Cl cotransport in IOVs made from sheep erythrocyte membranes. It was sensitive to osmotically induced changes in vesicle volume: swelling the vesicles enhanced the flux and shrinkage reduced it. Preliminary reports of some of these results have been published (6, 26).

MATERIALS AND METHODS

Materials. Choline chloride was a gift of Syntex Agribusiness (Springfield, MO). Methanesulfonate ($MeSO_3$) salt so-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: IOVs, inside-out vesicles; LK, low-potassium phenotype; ATP[β,γ-NH], adenosine 5′-[β,γ-imido]triphosphate; TnpATP, 2′(3′)-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate.

*Present address: Department of Anesthesiology, University of Missouri–Columbia, Columbia, MO 65212.

lutions were prepared by titrating methanesulfonic acid (Aldrich) to neutral pH with NaOH or KOH. Nystatin, strophanthidin, ATP, and Mops were from Sigma. Me_2SO was from Fisher. Dextran T-70 was from Pharmacia. ^{86}Rb was from NEN. Adenosine 5'-[β,γ-imido]triphosphate (ATP[β,γ-NH]) was from ICN; 2'(3')-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (Tnp-ATP) was from Molecular Probes; Adenosine 5'-[β,γ-methylene]triphosphate was from Boehringer Mannheim.

Cells. Sheep (Suffolk breed) were maintained at Vinzant Family Farms (Borodino, NY). Blood was drawn from LK sheep by venipuncture into heparin. The red cells were washed within 1 hr by centrifugation and resuspension three times in 150 mM choline chloride/10 mM Tris phosphate/5 mM glucose, pH 7.4, 295 mosmol/kg (Wescor osmometer, Salt Lake City). The seven LK sheep used were heterozygous for the LK allele (27).

Preparation of Vesicles. IOVs were prepared from these cells by a modification of earlier methods (28, 29). Washed, packed cells were lysed in 12 volumes of ice-cold 5 mM Tris phosphate buffer, pH 8.1, and centrifuged at 31,000 × g for 10 min (all centrifugations of membranes were at 31,000 × g). The membranes were washed twice in this medium, suspended in 0.5 mM Tris phosphate, pH 8.5 (TP-8.5), and incubated at 37°C for 40 min to cause vesiculation. The membranes were centrifuged, suspended in ice-cold TP-8.5, and fragmented by passage five times through a 1-inch, 27-gauge hypodermic needle. The resulting vesicle suspension was diluted with TP-8.5 and incubated 20 min at 37°C. Three milliliters of the suspension was layered over 3 ml of ice-cold dextran T-70 (4% wt/vol) in 0.5 mM Tris phosphate, pH 7.6, and centrifuged for 40 min. Vesicles were removed from the dextran interface, diluted to 80 ml with TP-8.5, and incubated on ice overnight.

The IOVs were treated with nystatin to achieve the desired intravesicular salt concentrations. Vesicles were suspended in TP-8.5 containing nystatin at 40 μg/ml (stock solution, 4 mg/ml in Me_2SO). After 5 min the IOVs were centrifuged 20 min and suspended in 100 mM NaCl/50 mM KCl/10 mM Tris Mops/1 mM $MgCl_2$, pH 7.4, 295 mosmol/kg, with nystatin at 40 μg/ml. The vesicles were incubated on ice for 20 min and centrifuged. Nystatin was removed by suspension of the pellets in the above solution (lacking nystatin; 37°C), incubation for 15 min, and centrifugation. The incubation and wash were repeated. The pellet was suspended in this washing solution at a protein concentration of 6–8 mg/ml. The vesicles were kept on ice before use for up to 3 days.

Vesicle Sidedness and Protein Content. The fraction of vesicles that were inside-out was determined by measuring the acetylcholinesterase activity of the vesicles before and after detergent (29). Typically, 80–90% of the vesicles were inside-out. Protein was determined by the bicinchoninic acid assay (30) using bovine serum albumin as a standard.

Relative Vesicle Volumes. Intravesicular volumes, expressed as μl of water per mg of protein, were measured as the difference in volumes of distribution of [^{3}H]water and of [^{14}C]sucrose, which is excluded from the vesicles (31). To a microcentrifuge tube were added 0.1 ml of vesicle suspension, containing 0.2 mg of protein, and 0.9 ml of the Cl medium described above, containing [^{3}H]water and [^{14}C]sucrose in a cpm ratio of 6, ^{3}H/^{14}C. The mixture was incubated at 25°C for 5 min and centrifuged for 20 min. A 0.4-ml sample of the supernatant was added to a scintillation vial and the remaining supernatant was removed by aspiration. The centrifuge tube tip containing the pellet was cut off and placed in another vial. Counts determined in a two-channel Beckman LS 7500 liquid scintillation counter were corrected for channel spillover. The volume of distribution of [^{3}H]water, V_H, in the vesicle pellets was calculated as $V_H = (C_p/C_s) \times 0.4$ ml, where C_p and C_s are the ^{3}H cpm in the pellet and supernatant samples, respectively. The volume of distribution of [^{14}C]sucrose, V_C, was calculated similarly. The intravascular volume in μl/mg is $(V_H - V_C)$/mg of membrane protein in the pellet.

K Efflux. Unidirectional K effluxes from IOVs were measured using ^{86}Rb, a good tracer for K in LK sheep red cells (7). Vesicles were incubated 3–18 hr at 4°C in 2 ml of 100 mM NaCl/50 mM KCl/1 mM $MgCl_2$/10 mM Tris Mops, pH 7.4, with 80 μCi of ^{86}RbCl (1 μCi = 37 kBq). The vesicles were washed twice by centrifugation in flux medium containing 77 mM Na, 38 mM K, 7.7 mM Tris Mops, 0.8 mM $Mg(NO_3)_2$, 115 mM either Cl or $MeSO_3$, and 20 μM strophanthidin (a permeant inhibitor of the Na/K pump) at pH 7.4. ATP was present at 1 mM except where noted. The medium also contained sucrose at 5, 60, or 180 mM to give osmolalities of 230, 295, or 430 mosmol/kg, respectively. The pellets (0.6 mg of protein) were suspended in 4.5 ml of flux medium and incubated 1.5 hr at 4°C. The ratio of volume of medium to volume of vesicles was >1000 for all flux measurements. To measure ^{86}Rb efflux, the vesicle suspension was incubated at 37°C, and 0.75-ml duplicate samples were removed at 5 and 35 min and centrifuged for 15 min. A sample of the supernatant solution was removed for γ counting; samples of the vesicle suspension were also taken for counting. Rate coefficients for effluxes were calculated from these counts (32). Effluxes are expressed as nmol of K per mg of protein per hr [nmol/(mg·hr)]; the amount of protein is proportional to the surface area of the vesicles. Fluxes were calculated from the expression $J = kcv$, where J is the efflux, k is the rate coefficient of efflux in hr^{-1}, c is the intravesicular K concentration in nmol/μl (= mM), and v is the measured relative vesicle volume in μl/mg of protein. It was necessary to know the K concentration in the vesicles at the time of the flux. The K concentrations in the vesicles immediately following the osmotic challenges were calculated (from K in the medium, 38 mM, and the assumed initial volume changes) to be 30 mM in swollen vesicles and 55 mM in shrunken vesicles. To estimate the changes during the 1.5-hr incubation at 4°C, the temperature dependence of the flux was determined from 37°C to 4°C. An anomalous dependence was observed: a minimum flux at ≈12°C, an increase in flux as temperature was reduced further, and nearly identical fluxes at 4° and 37°C (results not shown). The same anomalous temperature dependence for bumetanide- and ouabain-insensitive K influx was reported for human red cells (33). These results justify using the rate constants measured at 37°C to calculate the net K fluxes in swollen and shrunken vesicles during incubation at 4°C prior to measuring the tracer fluxes. The rate constants were 0.66 ± 0.05 hr^{-1} for swollen vesicles and 0.74 ± 0.06 hr^{-1} for shrunken vesicles (the rate constant for shrunken vesicles was higher because of the higher surface/volume ratio). The net fluxes were calculated by using the integrated forms of the first-order equations for influx and efflux (membrane potential of the vesicles was assumed to be zero). The K concentration was calculated to increase to 35 mM in 1.5 hr (92% equilibrated) in swollen vesicles and to decrease to 44 mM (86% equilibrated) in shrunken vesicles. These concentrations were used to calculate the fluxes in swollen and shrunken vesicles. Cl-dependent K effluxes were determined as the differences between fluxes in Cl media and in Cl-free media with $MeSO_3$ as the substitute anion. $MeSO_3$ was chosen because ouabain-resistant K fluxes in sheep erythrocytes are lower in $MeSO_3$ than in either NO_3, I, or Br (34).

In some experiments the IOVs were treated before the efflux assay with alkaline phosphatase to reduce endogenous phosphorylated protein (35). Alkaline phosphatase (type XXX-L powder, Sigma) was dissolved at 1 mg/ml in 100 mM NaCl/50 mM KCl/10 mM Tris Mops/1 mM $MgCl_2$, pH 7.4. A suspension of IOVs (containing ^{86}Rb) was brought to pH 7.9 with Tris base, and alkaline phosphatase was added at 35 μg/ml (34). After 30 min at 37°C, the IOVs were washed once

in the same solution except at pH 7.4 (and lacking alkaline phosphatase) and were assayed for ^{86}Rb efflux.

Statistics. A t statistic was calculated to test whether the slope in Fig. 1 of a least-squares regression line was significantly different from zero and was used in a one-tailed Student's distribution test (36). A randomization test for matched pairs (two-tailed) was used to determine the probabilities that the Cl-dependent K fluxes in Table 1 exceeded the Cl-independent fluxes (37).

RESULTS

Cl-Dependent K Efflux from IOVs as a Function of Relative Vesicle Volume. Table 1 shows K effluxes from IOVs suspended in Cl media and $MeSO_3$ media of 230, 295, and 430 mosmol/kg. Means are shown from 12 experiments. Also shown are the Cl-dependent fluxes at each osmolality, calculated from means of differences between fluxes in Cl and $MeSO_3$ media (not from differences between means). The Cl-dependent fluxes, presumably K–Cl cotransport, were highly significantly different from the Cl-independent fluxes at all three osmolalities (Table 1). Decreasing the osmolality from 295 to 230 mosmol/kg stimulated cotransport 1.4-fold and increasing it from 295 to 430 mosmol/kg inhibited the flux by a factor of 4. The Cl-independent flux was also sensitive to changes in osmolality, but much less so than the Cl-dependent flux. Similar results (not shown) were obtained with NO_3 as the Cl substitute.

Fig. 1 shows K–Cl cotransport from Table 1 plotted against relative vesicle volumes measured for vesicles equilibrated at each of the three osmolalities. K–Cl cotransport varied linearly with relative vesicle volume, with a 7-fold range over a 4-fold range of vesicle volumes. The estimate of relative volume of the vesicles in 295-mosmol/kg medium, 5.8 μl/mg of protein, is similar to values reported for IOVs from human erythrocytes (38, 39).

The relative effect of swelling in IOVs is less than in intact cells. Swelling of IOVs by about one-third stimulated Cl-dependent K flux ≈40%. A comparable swelling of intact cells stimulated K influx ≥5-fold (7). IOVs resemble resealed ghosts, in which a 2-fold volume difference was associated with a 2-fold difference in flux (16). On the other hand, transport in vesicles was much more sensitive to shrinkage than in intact cells (compare Fig. 1 to figure 1 in ref. 7).

Mg was routinely included in the flux media for vesicles because, with 1 mM ATP, K–Cl cotransport increased from 33 nmol/(mg·hr) in nominally Mg-free medium to 96 nmol/(mg·hr) with 1 mM Mg. Increasing Mg further to 4 mM neither stimulated the Cl-dependent flux further nor inhibited it. Mg inhibits K–Cl cotransport in untreated, intact sheep cells, but not in *N*-ethylamaleimide-treated cells (10). Low levels of Mg (<0.5 mM) in resealed human ghosts stimulate K–Cl cotransport, and higher concentrations inhibit it (16). Our results confirm the stimulation by Mg. To test for inhibition by Mg in IOVs it will be necessary to use concentrations below 1 mM, the concentration range in which inhibition was observed in ghosts.

Table 1. K effluxes from LK sheep red cell IOVs in Cl media and Cl-free media of osmolality 430, 295, and 230 mosmol/kg

	K efflux, nmol/(mg·hr)		
Principal anion	430	295	230
Cl	67 ± 6	150 ± 12	182 ± 15
$MeSO_3$	54 ± 6	96 ± 11	105 ± 13
(Cl-dependent)	13 ± 3	54 ± 10	77 ± 7

Shown are means ± SEMs (n = 12). Values for Cl-dependent fluxes are means of the differences, not differences between the means (which accounts for the lower errors for the Cl-dependent fluxes). The fluxes in Cl media were significantly greater than the fluxes in $MeSO_3$ media (P = 0.002, 430 mosmol/kg; P = 0.001, 295 mosmol/kg; P = 0.0005, 230 mosmol/kg). The sidedness of the vesicles was 85.0 ± 1.1% inside-out (n = 11).

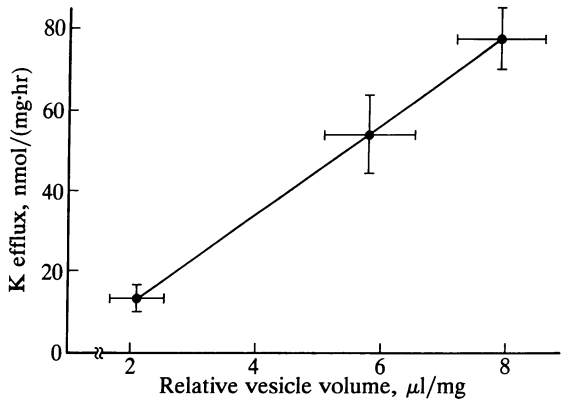

FIG. 1. Cl-dependent K effluxes from LK sheep erythrocyte IOVs as a function of relative vesicle volume. The Cl-dependent flux was calculated in each experiment from the mean of the differences of the effluxes between Cl and $MeSO_3$ media shown in Table 1. The symbols for fluxes show means ± SEMs from 12 experiments. Relative vesicle volumes, in μl of H_2O per mg of protein, were determined in 3 experiments for vesicles suspended in media of 430, 295, and 230 mosmol/kg. The slope of the line is 11.1 nmol/(μl·hr) (correlation coefficient, 0.74; intercept, −10.3) and was significantly different from zero ($P < 0.0001$).

Reversibility of Osmotically Induced Changes in Vesicle Volume and K Efflux. Vesicles were incubated in media of different osmolalities for 5 min at 37°C, and measurements were made of their volumes ("initial volumes" in Table 2). The vesicles in 230 mosmol/kg were swollen, and those in 430 mosmol/kg were shrunken, relative to those in 295 mosmol/kg. Then normal osmolality was restored, and volumes were measured again. These three aliquots of IOVs had the same relative volumes, demonstrating reversibility (Table 2).

If the vesicles had behaved as perfect osmometers, then the product of the vesicle volume and the osmolality of the medium would have been a constant (18). Predicted volumes at 430 and 230 mosmol/kg calculated from the volume at 295 mosmol/kg are given in Table 2. The vesicles swelled close to the predicted extent at 230 mosmol/kg; however, at 430 mosmol/kg they shrank more than predicted. This additional shrinkage was expected because the major solutes in the

Table 2. Reversibility of osmotically induced changes in relative vesicle volumes

	Relative vesicle volume, μl/mg		
	430	295	230
Initial volumes	2.10 ± 0.51	5.80 ± 0.82	7.90 ± 0.78
Volumes after return to 295 mosmol/kg	4.08 ± 1.12	4.94 ± 1.02	4.93 ± 1.02
Calculated initial volumes	3.98	(5.80)	7.43

The volumes are in μl of H_2O per mg of protein (means ± SEMs, n = 3). Vesicles were suspended in media of the three osmolalities (430, 295, and 230 mosmol/kg) containing [^{3}H]water and [^{14}C]sucrose for measurement of volumes (see *Materials and Methods*). These are presented as "initial volumes"; the same values are in Fig. 1. In the same experiments vesicles were suspended at the three osmolalities for 5 min at 37°C, then collected by centrifugation and resuspended in a medium of 295 mosmol/kg containing tracers for the volume measurements. Volumes of these vesicles, in this test of reversibility of volume changes, are presented as "volumes after return." Relative volumes were calculated for 230- and 430-mosmol/kg media from the volume measured at 295 mosmol/kg by assuming perfect osmometric behavior (see text).

vesicles, KCl and NaCl, will continue to redistribute after shrinkage owing to the impermeant sucrose.

To determine whether the osmotically induced changes in Cl-dependent K transport in the IOVs were reversible (as they are in intact cells; ref. 40), IOVs were first either shrunken or swollen in medium of 430 or 230 mosmol/kg and then returned to medium of normal osmolality. The K effluxes from these shrunken and swollen vesicles decreased and increased to extents similar to those shown in Table 1. The Cl-dependent K effluxes from these vesicles after their return to 295 mosmol/kg were 56, 45, and 65 nmol/(mg·hr) for vesicles preequilibrated at 430, 295, and 230 mosmol/kg, respectively. Therefore the osmotically induced changes in K–Cl cotransport are reversible.

Effects of ATP and ATP Analogues on K Efflux from IOVs. In LK sheep cells under isosmotic conditions, K influx is relatively independent of the metabolic state of the cells (7, 41, 42). However, in swollen or *N*-ethylmaleimide-treated cells, K influx is inhibited by metabolic depletion (7, 41, 43). Depletion of ATP was presumed, but not demonstrated, to be responsible. IOVs permit testing directly for a role of cytoplasmic ATP. To maximize an effect of added ATP, IOVs were preincubated at 37°C for 20 min to deplete bound ATP (28); the effect of added ATP was enhanced (results not shown). Table 3 shows the results of three experiments on K–Cl cotransport from IOVs as three different volumes with or without ATP. At each osmolality in all three experiments, ATP stimulated cotransport. The stimulation by ATP increased as vesicle volume increased; it was 12-fold higher at 230 mosmol/kg than at 430 mosmol/kg. There was significant K–Cl cotransport in the absence of added ATP at all three osmolalities, and swelling (430 → 230 mosmol/kg) stimulated the ATP-independent flux in all three experiments.

Experiments with nonhydrolyzable ATP analogues were carried out to investigate the mechanism of the stimulation of cotransport by ATP. IOVs were treated with alkaline phosphatase prior to flux measurements because this treatment caused an increase in the mean ATP-stimulated K–Cl cotransport from 12 to 22 nmol/(mg·hr) in swollen IOVs (n = 3). This result suggested that phosphorylation is necessary for stimulation of transport by ATP. The results of three additional experiments on phosphatase-treated vesicles are shown in Table 4. In each, ATP and one or two analogues were tested on swollen vesicles. Whereas ATP stimulated efflux 2- to 3-fold over the control value, ATP[β,γ-NH] and Tnp-ATP had little effect. Therefore, ATP apparently stimulates cotransport by phosphorylating something. In other experiments, we tested another nonhydrolyzable analogue, adenosine 5′-[β,γ-methylene]triphosphate. After treatment with hexokinase and glucose to hydrolyze contaminating ATP (28), this analogue also did not stimulate cotransport (results not shown).

Table 4. Effect of ATP and ATP analogues on Cl-dependent K efflux

	K efflux, nmol/(mg·hr)			
Principal anion	Control	ATP	ATP[β,γ-NH]	Tnp-ATP
Cl	98	124	94	74
$MeSO_3$	77	70	68	50
(Cl-dependent)	21 ± 6	54 ± 6	26 ± 8	24 ± 4

Control: no added nucleotides; all nucleotides were at 1 mM. Vesicles had been pretreated with alkaline phosphatase (see text). Vesicles were in 230-mosmol/kg medium. Shown are means ± SEMs (n = 3) or range/2 for Tnp-ATP (n = 2).

DISCUSSION

We have demonstrated Cl-dependent K transport (K–Cl cotransport) in IOVs made from membranes of erythrocytes from sheep of the LK phenotype. We have also shown that K–Cl cotransport in the IOVs is sensitive to osmotically induced changes in vesicle volume. Volume sensitivity in intact sheep red cells has long been known (7, 40), but volume sensitivity of K–Cl cotransport in membrane vesicles from erythrocytes (or other sources) had not been shown.

These results provide strong evidence against the signal to the volume sensor in IOVs being a change in concentration of a cytoplasmic solute. Thus, mechanical changes in the membrane should be considered as a signal in intact cells. However, a mechanical pressure is not likely to be responsible. Calculated steady-state pressures in human red cells after osmotic swelling are extremely small, less than 1 dyne/cm^2 for 40% swelling (44). Transient pressures following a hypotonic challenge will be greater but will persist for about a second (45) and are probably relatively small (46). The calculated pressure of imposed osmotic gradients, $\approx 3 \times 10^4$ dynes/cm^2 per mosm/kg, is realized only when cells cannot swell. If the pressures developed with swelling are insignificant, then other kinds of mechanical changes must be considered. The sensitivity of K–Cl cotransport to swelling in IOVs is in the same direction as that observed in intact cells (7), in that swelling stimulates in both. Therefore, since volume sensitivity is not dependent on membrane orientation, the signal to the volume sensor is not a change in membrane curvature. Changes in associations between elements of the cytoskeleton and the membrane should be considered. The signal might arise in a membrane-associated compartment bounded by the cytoskeleton (28), in which concentrations of solutes or binding sites change in response to changes in cytoskeleton–membrane interactions.

In any event, it seems likely that the cytoplasmic membrane surface is the locus of the volume sensor. If so, sheep red cell IOVs, with simultaneous volume-sensitive fluxes and access to the cytoplasmic membrane surface, permit direct investigation of the volume sensor.

The Cl-independent K fluxes in IOVs varied with vesicle volume, though to a considerably lesser extent than the Cl-dependent fluxes (Table 1). It is unclear whether this represents a separate volume-sensitive K-transport pathway or substitution of $MeSO_3$ for Cl as a substrate. Cl-independent K fluxes in LK sheep cells and resealed human ghosts show saturation kinetics (47) and volume sensitivity (16, 48); volume sensitivity of Cl-independent fluxes was

Table 3. ATP increases Cl-dependent K efflux from sheep erythrocyte IOVs in media of different osmolalities

	K efflux, nmol/(mg·hr)								
	430 mosmol/kg			295 mosmol/kg			230 mosmol/kg		
Exp.	Control	+ ATP	Increase	Control	+ ATP	Increase	Control	+ ATP	Increase
1	22.8	26.8	4.0	89.8	105.4	15.6	104	113	9.0
2	9.1	10.9	1.8	29.5	33.4	3.9	12.1	61.0	48.9
3	9.9	10.9	1.0	28.2	38.7	10.5	26.2	50.8	24.6
Mean			2.3 ± 0.9			10.0 ± 3.4			27.5 ± 11.6

Mean stimulated by ATP (1 mM) is shown ± SEM.

considerably larger in ghosts than in cells. Therefore swelling-activated Cl-independent K influx in IOVs is neither explained nor unexpected.

ATP stimulates K–Cl cotransport from vesicles. Since nonhydrolyzable analogues of ATP did not serve, phosphorylation must be involved. The same observation about K–Cl cotransport was made in resealed human ghosts (16). However, Cl-dependent K transport in untreated LK sheep erythrocytes is not only not inhibited by metabolic depletion, it may be stimulated (40, 42, 43). The explanation for these varying results is not clear and will be difficult to resolve in intact cells. A substantial portion of K–Cl cotransport in IOVs did not require added ATP. Sachs (16) observed this with resealed ghosts and attributed it to residual trapped ATP. This argument is difficult to apply to IOVs (though a membrane compartment of ATP is possible; ref. 28). Because a substantial fraction of cotransport proceeds in the absence of added ATP, and because metabolic depletion does not inhibit transport in untreated sheep cells of normal volume, we propose that it is not the transporter that is phosphorylated, but a regulator, perhaps a protein associated with, or identical to, the volume sensor (47). In this scheme, as swelling stimulates transport, sites are made available on the regulator for phosphorylation and further stimulation of transport. This suggestion is consistent with Table 3, where stimulation of efflux by ATP was shown to be enhanced in swollen vesicles.

It has been proposed that a phosphatase activates transport after swelling (49). This seems contradictory to our observations of stimulation of cotransport by ATP and inhibition of it by alkaline phosphatase. The apparent discrepancy may be resolved if this hypothetical protein phosphatase, perhaps the regulator, is activated by phosphorylation (49).

We are grateful for helpful suggestions from Drs. Robert Gunn, John Parker, John Sachs, and Richard Waugh. This work was supported by a grant from the National Institutes of Health (DK33640).

1. Reuss, L. (1983) *Nature (London)* **305,** 723–726.
2. Kregenow, F. M. (1981) *Annu. Rev. Physiol.* **43,** 493–505.
3. Haas, M. & McManus, T. J. (1985) *J. Gen. Physiol.* **85,** 649–667.
4. Grinstein, S., Rothstein, A., Sarkadi, B. & Gelfand, E. W. (1984) *Am. J. Physiol.* **246,** C204–C215.
5. Kramhøft, B., Lambert, I. H., Hoffmann, E. K. & Jørgensen, F. (1986) *Am. J. Physiol.* **256,** C369–C379.
6. Dunham, P. B. (1990) in *Regulation of Potassium Transport Across Biological Membranes*, eds. Reuss, L., Russell, J. M. & Szabo, G. (Univ. of Texas Press, Austin, TX), pp. 331–360.
7. Dunham, P. B. & Ellory, J. C. (1981) *J. Physiol. (London)* **318,** 511–530.
8. Lauf, P. K. (1983) *J. Membr. Biol.* **73,** 237–246.
9. Ellory, J. C., Dunham, P. B., Logue, P. J. & Stewart, G. W. (1982) *Philos. Trans. R. Soc. London Ser. B* **229,** 483–495.
10. Lauf, P. K. (1985) *J. Membr. Biol.* **88,** 1–13.
11. Parker, J. C. & Dunham, P. B. (1989) in *Red Blood Cell Membranes*, eds. Agre, P. & Parker, J. C. (Dekker, New York), pp. 507–561.
12. Brugnara, C., Van Ha, T. & Tosteson, D. C. (1989) *Am. J. Physiol.* **256,** C994–C1003.
13. Kaji, D. (1986) *J. Gen. Physiol.* **88,** 719–738.
14. Hall, A. C. & Ellory, J. C. (1986) *Biochim. Biophys. Acta* **858,** 317–320.
15. Dunham, P. B. & Logue, P. J. (1986) *Am. J. Physiol.* **250,** C578–C583.
16. Sachs, J. R. (1988) *J. Gen. Physiol.* **92,** 685–711.
17. O'Neill, W. C. (1989) *Am. J. Physiol.* **256,** C81–C88.
18. Siebens, A. W. (1985) in *The Kidney: Physiology and Pathophysiology*, eds. Seldin, D. W. & Giebisch, G. (Raven, New York), pp. 91–115.
19. Sachs, F. (1988) *CRC Crit. Rev. Biomed. Eng.* **16,** 141–169.
20. Hoffmann, E. K. & Simonsen, L. O. (1989) *Physiol. Rev.* **69,** 315–382.
21. Brown, A. M., Ellory, J. C., Young, J. D. & Lew, V. L. (1978) *Biochim. Biophys. Acta* **511,** 163–175.
22. Ferrari, P., Torielli, L., Ferrandi, M. & Bianchi, G. (1986) *Ann. N.Y. Acad. Sci.* **488,** 561–564.
23. Reeves, W. B., Dudley, M. A., Mehta, P. & Andreoli, T. E. (1988) *Am. J. Physiol.* **255,** F1138–F1144.
24. Eveloff, J. & Warnock, D. G. (1987) *Am. J. Physiol.* **252,** F883–F889.
25. Muallem, S., Schoenfeld, M., Pandol, S. & Sachs, G. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4433–4437.
26. Kracke, G. R. & Dunham, P. B. (1989) *J. Gen. Physiol.* **94,** 18a–19a (abstr.).
27. Dunham, P. B., Farquharson, B. E. & Bratcher, R. L. (1984) *Am. J. Physiol.* **247,** C120–C123.
28. Mercer, R. W. & Dunham, P. B. (1981) *J. Gen. Physiol.* **78,** 547–568.
29. Steck, T. L. & Kant, J. A. (1974) *Methods Enzymol.* **31,** 172–180.
30. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gardner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85.
31. Forbush, B., III (1982) *J. Biol. Chem.* **257,** 12678–12684.
32. Sachs, J. R., Ellory, J. C., Kropp, D. L., Dunham, P. B. & Hoffman, J. F. (1974) *J. Gen. Physiol.* **63,** 389–414.
33. Stewart, G. W., Ellory, J. C. & Klein, R. A. (1980) *Nature (London)* **286,** 403–404.
34. Fujise, H. & Lauf, P. K. (1987) *Am. J. Physiol.* **252,** C197–C204.
35. Smallwood, J. I., Gugi, B. & Rasmussen, H. (1988) *J. Biol. Chem.* **263,** 2195–2202.
36. Spiegel, M. R. (1961) *Theory and Problems of Statistics* (Shaum, New York), p. 349.
37. Siegel, S. (1956) *Nonparametric Statistics* (McGraw–Hill, New York), p. 312.
38. Rossi, J. P. F. C. & Schatzmann, H. J. (1982) *J. Physiol. (London)* **327,** 1–15.
39. Perrone, J. R. & Blostein, R. (1973) *Biochim. Biophys. Acta* **291,** 680–689.
40. Ellory, J. C. & Dunham, P. B. (1980) in *Membrane Transport in Erythrocytes*, Alfred Benzon Symposium 14, eds. Lassen, U. V., Ussing, H. H. & Wieth, J. O. (Munksgaard, Copenhagen), pp. 409–423.
41. Lauf, P. K. (1983) *Am. J. Physiol.* **245,** C445–C448.
42. Logue, P., Anderson, C., Kanik, C., Farquharson, B. & Dunham, P. (1983) *J. Gen. Physiol.* **81,** 861–885.
43. Lauf, P. K. (1984) *J. Membr. Biol.* **82,** 167–178.
44. Zarda, P. R., Chein, S. & Skalak, R. (1977) *J. Biomech.* **10,** 211–221.
45. Moronne, M. M., Mehlhorn, R. J., Miller, M. P., Ackerson, L. C. & Macey, R. I. (1990) *J. Membr. Biol.* **115,** 31–40.
46. Rand, R. P. & Burton, A. C. (1964) *Biophys. J.* **4,** 115–135.
47. Bergh, C., Kelley, S. J. & Dunham, P. B. (1990) *J. Membr. Biol.* **117,** 177–188.
48. Lauf, P. K. (1988) *Am. J. Physiol.* **255,** C331–C339.
49. Jennings, M. L. & Al-Rohil, N. (1990) *J. Gen. Physiol.* **95,** 1021–1040.