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Effect of Metabolic Depletion on the Furosemide-Sensitive Na and K Fluxes in Human Red Cells

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Summary. We report in this paper the effect of metabolic depletion on several modes of furosemide-sensitive (FS) Na and K transport in human red blood cells. The reduction of ATP content below 100 μmol/liter cells produced a marked decrease in the maximal activation (V_{max}) of the outward, FS transport of Na and K into choline medium in the presence of ouabain (0.1 mM) and 1 mM $MgCl_2$. The $K_{0.5}$ for internal Na to activate the FS Na efflux was not altered by metabolic depletion. However, metabolic depletion markedly decreased the K_i for external K (K_o) to inhibit the FS Na efflux into choline medium (from 25 to 11 mM). Repletion of ATP content by incubation of cells in a substrate-rich medium recovered control levels of V_{max} of the FS Na and K fluxes and of K_i for external K to inhibit FS Na efflux. The V_{max} of FS Na and K influxes was also markedly decreased when the ATP content dropped below 100 μmol/liter cells. This was mainly due to a decrease in the inward-coupled transport of K and Na (Na_o-stimulated K influx and the K_o-stimulated Na influx). The FS K_i/K_o exchange pathway of the Na-K cotransport, estimated from the FS K influx from choline-20 mM K_o medium into cells containing 22 mmol Na/liter cells, was also reduced by starvation. Starvation did not inhibit the FS Na_i/Na_o exchange pathway, estimated as FS Na influx from a medium containing 130 mM NaCl into cells containing 22 mmol Na/liter cells. The unidirectional FS ^{22}Na efflux and influx were also measured in control and starved cells containing 22 mmol Na/liter cells, incubated in a Na medium (130 mM) at varying external K (0 to 20 mM). In substrate-fed cells, incubated in the absence of external K, FS Na efflux was larger than Na influx. This FS net Na extrusion (400 to 500 μmol/liter cells · hr) decreased when external K was increased, approaching zero around 15 mM K_o. In starved cells the net Na extrusion was markedly decreased and it approached zero at lower K_o than in substrate-fed cells. Our results indicate that the FS Na and K fluxes, and their major component, the gradient driven Na-K-Cl cotransport system, are dependent on the metabolic integrity of the cells.

Key Words furosemide sensitive · Na and K fluxes · cotransport · human red cells · starvation · metabolism · transport

Introduction

Several studies in human red cells have characterized a Na-K-Cl cotransport system which can be inhibited by furosemide or bumetanide. Wiley and Cooper [44] reported that *cis*-stimulated Na and K influx were inhibited by furosemide. In subsequent studies [24] the kinetic parameters of furosemide-sensitive (FS) Na and K effluxes as well as their chloride dependence [16, 20] were estimated.

In recent years we have studied the equilibrium properties and the modes of operation of the FS Na and K fluxes in human red blood cells [7–9, 12–14]. We found that when cells with Na and K content similar to that of fresh cells were incubated in the presence of external Na (Na_o = 130 mM), there was a net FS Na and K extrusion, which increased with intracellular Na (Na_i). Increasing external K (K_o) stimulated FS Na influx and decreased the net FS Na extrusion. In conditions similar to those occurring *in vivo* (Na_i = 10 mM, K_i = 130 mM, Na_o = 130 mM, K_o = 4 mM, Cl_i/Cl_o = 0.7) net extrusion of Na occurred through the FS pathway [9, 14]. The concentration of K_o at which FS Na and K efflux and influx become equal (zero net flux condition or equilibrium point) increased when Na_i was increased. Both net FS Na and K fluxes approached zero at similar internal and external Na and K concentrations. At the equilibrium position of the FS fluxes, the stoichiometry of the unidirectional fluxes was 2 Na : 3 K [9, 14]. These results confirmed early findings by Hoffman and Kregenow [28] suggesting the presence of a ouabain-resistant second Na pump in human red cells.

We have also studied the modes of operation of the FS Na and K fluxes [7, 13]. Six different modes of Na and K transport were found to be inhibited by furosemide in the presence of ouabain: inward- and outward-coupled transport of Na and K, K_i/K_o and Na_i/Na_o exchange, and uncoupled Na and K efflux. The stoichiometric ratio of FS outward cotransport of Na and K into Na-free (choline substitution) medium varied according to the ratio of Na to K cell content, and with the presence of external K. We proposed a minimal model for a reaction scheme of

FS Na and K transport, to account for several types of coupling interactions between Na and K such as *cis*-stimulation, *trans*-inhibition or *trans*-stimulation and for variable stoichiometry of the FS fluxes.

The present paper deals with the metabolic dependence of several modes of the FS Na and K fluxes. Previous observations in human red cells [3, 25, 28, 43] have suggested that ouabain-resistant fluxes can be decreased by metabolic depletion. The present experiments were designed to evaluate the metabolic dependence of the net FS Na extrusion and of the different modes of Na and K transport inhibitable by furosemide in human red cells. We show in this paper that when the cell ATP content is decreased below 100 μmol/liter cells, there is a marked reduction of the FS net Na extrusion, of the outward- and inward-coupled transport of Na and K, and of the K_i/K_o exchange mediated by the Na-K-Cl cotransport system.

A preliminary report of this work has been published elsewhere [17].

Materials and Methods

All the experiments described in this paper were performed on the red cells of at least three normotensive males.

Blood was drawn into heparinized tubes and centrifuged at 1750 × *g* for 10 min to separate plasma and buffy coat. Red cells were washed four times with 10 volumes of cold (4°C) choline washing solution containing (mM): 150 choline chloride, 1 $MgCl_2$, 10 MOPS-Tris (pH 7.4 at 4°C). An aliquot of cells was then suspended in an equal volume of choline washing solution and from this cell suspension determinations of hematocrit, cell Na (1/50 dilution in 0.02% Acationox®, American Scientific Products, McGaw Park, Ill.), cell K (1/500 dilution) and hemoglobin (optical density at 540 nm) were carried out.

Procedures to Achieve Metabolic Depletion and Repletion

To reduce ATP levels, red cells were incubated at 10% hematocrit in a solution containing (mM): 150 NaCl, 4 KCl, 1 $MgCl_2$, 1 EGTA, 20 MOPS-Tris (pH 7.4 at 37°C) and penicillin G, 100 IU/ml (metabolic starvation). The cells were incubated at 37°C in a shaking bath and the incubation medium was renewed after 6 hr. Aliquots of the cell suspension were then removed at different time intervals (7 to 15 hr) and washed twice with an ice-cold choline washing solution. Subsequently, the cells were either processed for metabolic repletion or loaded with Na by the nystatin procedure. EGTA was added to minimize the Ca-dependent membrane breakdown taking place in metabolic-starved cells as described by Lutz et al. [31], Motais et al. [33] and Palek et al. [35] and to prevent volume changes caused by activation of the Ca-dependent K channel [30].

Metabolic repletion was achieved by incubating depleted cells for 3 hr at 37°C (10% hematocrit) in a medium containing (mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 1 EGTA, 5 adenine, 2 inosine, 10 glucose, 10 MOPS-Tris (pH 7.4 at 37°C), 2.5 Na phosphate buffer [43]. The cells were then washed twice with ice-cold choline washing solution.

Cation Loading of Red Cells

Cation content was varied by using the ionophore nystatin [11].

Two ml of washed packed cells were added to 10 ml of cold nystatin loading solution (NLS) and incubated at 4°C for 20 min. The NLS contained (mM): 140 of (NaCl + KCl), 52 sucrose, 10 MOPS-Tris (pH 7.4 at 4°C), and nystatin (40 μg/ml). To vary the cellular Na content, the Na concentration in the NLS was calculated from: Cell Na content = Na_o concentration (mM) × 0.65.

The cold suspension was centrifuged for 5 min at 5000 × *g*, and the cell pellet was suspended at 10% hematocrit for 5 min at 37°C in the nystatin washing solution (NWS). The NWS had the same composition as the NLS solution, with the pH adjusted to 7.4 at 37°C and 0.1% albumin. No substrate was added to the depleted cells. Glucose (10 mM) and K-phosphate buffer (1 mM, pH 7.4) were added to the NWS used for control and ATP-repleted cells. The cell suspension was then washed three times with NWS at 37°C.

To perform radioactive cation loading, the red cells incubated in the NLS were centrifuged to remove the supernatant. The cells were then suspended at 60 to 70% hematocrit in the same cold loading solution, without nystatin, containing 10 μCi ^{22}Na or 60 μCi ^{86}Rb. After a 20-min incubation at 4°C with adequate shaking, the suspension was warmed up to 37°C for 5 min and the red cells were then washed four times with warm NWS.

External cations were removed with five washes in ice-cold choline washing solution. After the final wash, the cells were suspended in equal volumes of washing solution for the determination of hematocrit, hemoglobin and cell electrolytes and specific activity.

The cell volume was estimated by measuring the hemoglobin/liter of fresh and loaded cells. The volume of nystatin-loaded cells was 99% ± 2 of the initial cell volume. The ATP content was not modified by the loading procedure in control, depleted or repleted cells. The rate constant of Na and K efflux in the presence of ouabain and furosemide was similar in control and nystatin-treated cells.

Protocol for Measurement of Na and K Fluxes

a) Unidirectional Tracer Efflux

One ml of cell suspension (30% Hct) was added to 10 ml of efflux medium. The media contained (mM): 150 to 130 choline chloride and 0 to 20 KCl, or 130 NaCl, 20 to 0 choline chloride and 0 to 20 KCl. All of the media contained (mM): 10 MOPS-Tris (pH 7.4 at 37°C), 1 EGTA, and 0.1 ouabain. Glucose (10 mM) was added to the media containing the control or metabolically repleted cells. 5.5 ml of the cold flux suspension were distributed into 5 tubes; 5 μl of furosemide in 1 M Tris-base was added to the remaining media which was distributed in five tubes. The flux suspension was incubated at 37°C and duplicates were removed at 5 min and triplicates at 65 min. These incubation times were chosen because control experiments indicated that the effluxes were linear up to 75 min at this hematocrit. The reaction was stopped by cooling at 4°C and after centrifugation, 0.8 ml of the supernatant were removed for counting in a gamma counter (Packard Instru-

ments). An aliquot of the initial cell suspension (diluted 1/50) was counted for the determination of the specific activity of the isotope-loaded cells.

b) Unidirectional Tracer Influx

Ten μCi of ^{22}Na or 60 μCi of ^{86}Rb (Amersham. Arlington Heights, Ill.) were added to 10 ml of medium and the initial specific activity was determined. Packed red cells (0.6 to 0.8 ml) were then added to the cold media and the cell suspension processed as described for the efflux measurement. Samples were taken at 5 and 25 min for ^{86}Rb influx, and 5 and 65 min for Na influx. Control experiments indicated that the influxes were linear under these experimental conditions. The supernatant was discarded and the cells were washed three times with 4 ml of cold Na washing solution containing (mM): 150 NaCl, 1 $MgCl_2$, 10 MOPS-Tris (pH 7.4 at 4°C). The cell pellet was lysed with 1 ml of 0.02% Acationox® and centrifuged for 10 min at 5000 × g to precipitate membrane fragments. 0.8 ml of lysate were counted in a gamma counter. A 1 : 50 dilution in double-distilled water of the lysate was used for spectrophotometrical determination of hemoglobin and cation content.

c) Na, K and Li Effluxes

To measure the ouabain-sensitive component of the Na efflux from Na-loaded cells, the red cells were incubated at 1% Hct in an efflux medium containing 140 mM choline chloride and 10 mM KCl, with and without 0.1 mM ouabain. The efflux times were 5 and 25 min.

To measure the furosemide-sensitive component of Na and K effluxes from Na-loaded cells, red cells were incubated at 2% Hct in efflux media containing 150 mM choline chloride, 0.1 mM ouabain, with and without 1 mM furosemide. The efflux times were 5 and 65 min. All the media contained (mM): 1 $MgCl_2$, 10 MOPS-Tris (pH 7.4 at 37°C) and 1 EGTA.

The Na-stimulated Li efflux was determined in cells loaded with Li (7 to 9 mmol/liter cells) using nystatin. The NLS contained (mM): 10 LiCl, 130 KCl, 10 MOPS-Tris (pH 7.4 at 4°C) + 52 sucrose. Li efflux was measured in Mg-sucrose and Na media. The Mg-sucrose media contained (mM): 75 $MgCl_2$, 85 sucrose, 10 MOPS-Tris (pH 7.4 at 37°C) and 0.1 ouabain. The Na media contained (mM): 150 NaCl, 10 MOPS-Tris, 1 $MgCl_2$ and 0.1 ouabain. The hematocrit of the flux suspension was 3% and the incubation times 5 and 65 min. The Na-stimulated Li efflux was calculated as the difference between Li efflux into Na and Mg-sucrose media [10].

The Na, K and Li concentrations of the supernatant were determined in a Perkin-Elmer atomic absorption spectrophotometer (Model 5000) using standards with the same composition as the flux media.

Determination of Cellular ATP and Glutathione Content

ATP content was determined using the luciferin-luciferase method [6]. The chemiluminescence was measured either in a beta counter or in a cell tester (Lumac Systems A.G.). Glutathione levels were determined as described by Beutler et al. [4].

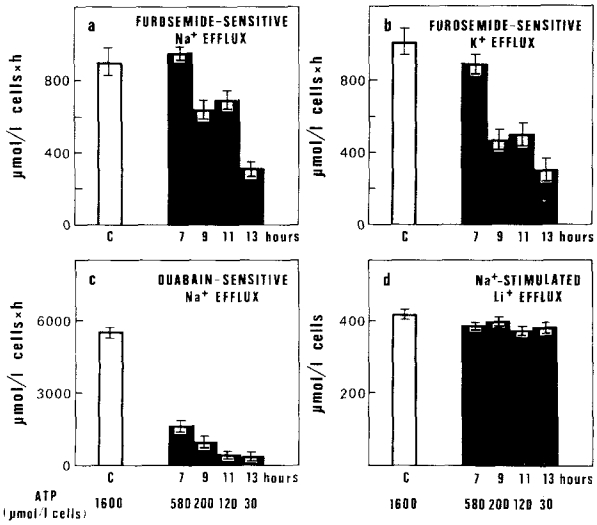

Fig. 1. Effect of metabolic depletion on the furosemide-sensitive Na and K effluxes, the ouabain-sensitive Na efflux and the Na-stimulated Li efflux. Starvation was achieved by incubating the cells in a substrate-free media containing (mM): 150 NaCl, 4 KCl, 1 $MgCl_2$, 20 MOPS-Tris (pH 7.4 at 37°C) and penicillin G, 100 U/ml. Aliquots were taken at different time intervals and the cells were loaded using the ionophore nystatin to contain 22 mmol Na and 70 mmol K/liter cells. The fluxes in Fig. 1*a* and *b* were measured in choline medium containing (mM): 150 choline chloride, 1 $MgCl_2$, 1 EGTA, 10 MOPS-Tris, 0.1 ouabain and 1 furosemide. Ouabain-sensitive Na efflux was measured into 140 mM choline chloride, 10 mM KCl medium. Li efflux was measured into 75 mM $MgCl_2$-85 mM sucrose and 140 mM NaCl, 1 mM $MgCl_2$ media. Subject: G.D. This is one of three experiments in different subjects. (C□) control cells; (■) starved cells and starvation time. The bars represent the standard error of the fluxes

Results

Effect of Metabolic Starvation on Outward Furosemide-Sensitive Na and K Fluxes

In our previous work [7, 13] we found that the FS component of Na and K effluxes into Na-free medium (choline substitution) represents outward cotransport of Na and K, because it is also chloride-dependent and *cis*-stimulated by internal Na or K. Figure 1 shows the time-course of metabolic starvation and its effect on the ouabain-sensitive Na efflux and on the furosemide-sensitive (FS) Na and K effluxes from cells containing 22 mmol Na/liter cells and 70 mmol K/liter cells. It can be seen that the FS Na and K effluxes were markedly decreased after 11 to 13 hr when ATP levels fell below 100 μmol/liter cells. The incubation time required for 80% inhibition of the Na-K cotransport varied in the three subjects between 13 and 17 hr. It can also be seen that the ouabain-sensitive Na efflux fell signifi-

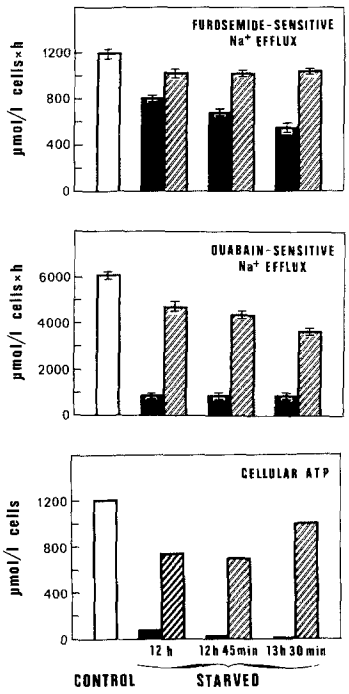

Fig. 2. Effect of metabolic depletion and repletion on the furosemide-sensitive Na efflux and the ouabain-sensitive Na efflux. Starvation was achieved by incubating the cells for different time intervals in a substrate-free media. Repletion was achieved by incubating starved cells in a substrate-rich media containing (mM): 150 NaCl, 4 KCl, 10 MOPS-Tris, 1 $MgCl_2$, 1 EGTA, 2 inosine, 5 adenine, 10 glucose, and 2.5 phosphate buffer. After 12 and 13 hr starvation, ATP content was 75 and 2.5 μmol/liter cells, respectively. Subject: G.D. This in one of three experiments in different subjects. (□) control cells; (■) starved cells; (▨) repleted cells. The bars represent the standard error of the fluxes

cantly during the first 7 hr of incubation when the cell ATP levels dropped from 1600 to 580 μmol/liter cells. Note in Fig. 1*d* that the Na-stimulated Li efflux (Na/Li exchange) was not affected by metabolic starvation, as previously reported by Pandey et al. [38].

Recovery of Furosemide-Sensitive Na and K Fluxes in Starved Cells

The effect of metabolic starvation on the FS Na efflux could be reversed by reincubating the cells in a substrate-rich medium. The ATP content was increased to 1000 μmol/liter cells by incubation in a medium containing adenine, inosine, glucose and phosphate [43]. Figure 2 shows the time-course effects of depletion and repletion on the ouabain-sensitive and FS Na efflux into choline medium. Control experiments were carried out by incubating fresh cells for similar periods in substrate-supplemented medium containing EGTA. The FS Na efflux could be restored to levels not significantly different from control, upon repletion of ATP-depleted cells. After 15 to 17 hr of depletion, we observed an inconsistent recovery of the FS Na efflux although cell ATP levels were completely recovered. The time of starvation which precluded return to normal values of the FS Na efflux upon repletion also varied slightly in these individuals. Glutathione levels fell by 50% during metabolic depletion (from 1.3 ± 0.2 to 0.6 ± 0.2 mmol/liter cells) and the addition of dithiothreitol (1 mM) did not improve the recovery of Na-K cotransport. We found no relationship be-

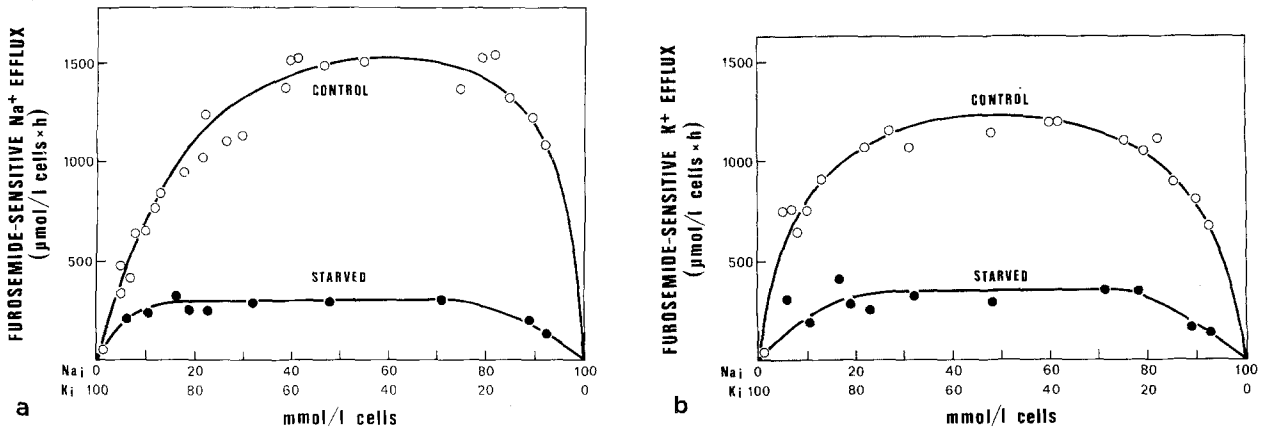

Fig. 3. The furosemide-sensitive Na (Fig. 3*a*) and K (Fig. 3*b*) efflux into choline media as a function of internal cation concentration. Starvation was achieved by incubating cells in a substrate-free media for 14 hr as described in Fig. 1. Cation content was then modified by using the ionophore nystatin. Cell ATP content of starved cells was 7 μmol/liter cells. Subject: A.K. Similar results were obtained in another two subjects

tween glutathione cell content and recovery of the FS Na and K effluxes.

Effect of Starvation on the Kinetic Properties and Stoichiometry of Outward Furosemide-Sensitive Transport of Na and K

Figure 3 shows the activation of FS Na and K effluxes as a function of internal Na and K concentrations in control and starved cells. It can be seen that the maximal velocity of the FS Na and K efflux markedly decreased in starved cells. However, metabolic starvation did not alter the $K_{0.5}$ for internal Na to stimulate FS Na efflux [7.5 ± 1.5 in starved and 8.7 ± 1.1 in control cells, subject: G.D.; $K_{0.5}$ calculated with the Hill plot according to Garay et al. [26]], when there was a 50% inhibition of the V_{max} (*data not shown*). However, when the inhibition of the maximal rate of FS Na and K effluxes was higher than 70%, it became difficult to determine changes in $K_{0.5}$ for internal Na, given the low values of FS fluxes (Fig. 3).

It can also be seen in Fig. 3, that the stoichiometry of outward FS Na and K fluxes is changed by starvation. In substrate-fed cells the ratio of FS Na/K efflux depends on the ratio of cell Na/K contents; the ratio of FS Na/K fluxes is 1 when cell Na/K ratio is close to one. This stoichiometric ratio is higher than 1 when cell Na/K is higher than 1, and is less than 1 when cell Na/K is less than 1 [7]. In starved cells, the ratio of FS Na to K efflux was 1 : 1 and constant over a wide range on internal Na/K ratios (Figs. 3*a* and *b*).

It has been shown that external K inhibits FS Na efflux into Na-free media with a K_i of 20 to 25 mM [7, 25, 40]. Figure 4 shows the effect of external K on the FS Na efflux from metabolically depleted cells, with different ATP contents. The K_i for external K to inhibit furosemide-sensitive Na efflux, calculated from the Dixon plot, decreased from 25.6 ± 0.7 mM in control to 11 ± 1.2 mM in starved cells. This change in K_i for K occurred when the outward FS Na efflux was 70 to 80% inhibited by starvation. Since this finding may indicate that metabolic depletion may have elicited membrane degradative processes, we investigated the reversibility of this effect. When starved cells were metabolically repleted by incubation for 3 hr in glucose-adenosine medium, the K_i for K_o-inhibition of FS Na efflux was restored to control levels. In this experiment, the K_i for K inhibition was 25 ± 2 mM in control cells, 5 ± 1 in starved cells and 24 ± 1.6 in starved and ATP-repleted cells.

The furosemide-resistant Na and K effluxes into choline media were compared in starved and control cells containing 22 mmol Na/liter cells and 70 mmol K/liter cells. The furosemide-resistant Na efflux (mmol/liter cells · hr ± SD; $n = 5$) was 0.57 ± 0.045 in control and 0.51 ± 0.09 in starved cells. The furosemide-resistant K efflux was 1.275 ± 0.15 in glucose-fed cells and 1.42 ± 0.14 in starved cells. Neither of these changes were significant. The same was true when furosemide-resistant Na and K ef-

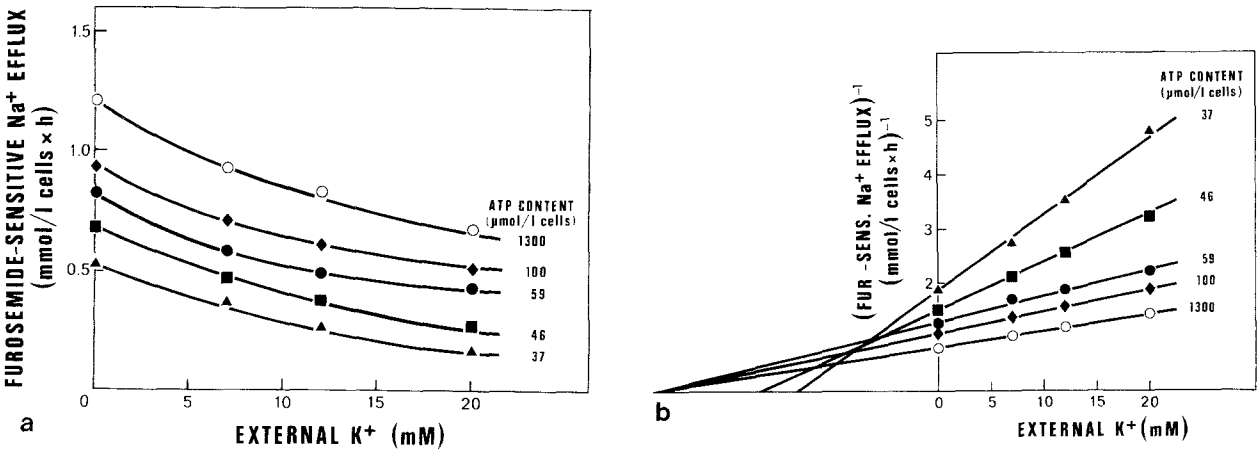

Fig. 4. (*a*) Effect of external K on the furosemide-sensitive Na efflux into choline media at different ATP contents. The red cell cation content was 22 mmol Na and 70 mmol K/liter cells. Starvation was achieved by incubating the cells in a substrate-free media for 13, 14, 15 and 16 hr. (*b*) Dixon plot of the inhibition of the furosemide-sensitive Na efflux by external K of Fig. 4*a*. The K_i values in mM ± SD were 11 ± 1.2, 16.2 ± 0.5, 25 ± 2, 22.3 ± 2.2, and 25.6 ± 0.7 for cells containing 37, 46, 59, 100 and 1,300 μmol ATP/liter cells, respectively. Subject: G.D. Similar results were obtained in two other subjects

Table 1. Effect of metabolic depletion on the furosemide-sensitive K_i/K_o and Na_i/Na_o exchange[a]

A. K_i/K_o Exchange Subject	Control			Starved				
	Ouabain	Ouabain and furosemide	FS	Ouabain	Ouabain and furosemide	FS	Starvation time (hr)	ATP (μmol/liter cells)
	K influx (μmol/liter cells · hr)							
G.D.	190	40	150	210	40	170	15	46
A.K.	600	170	430	320	70	250	17	7
C.B.	600	200	400	520	210	310	13	80
C.B.	600	200	400	420	200	220	15	60
C.B.	600	200	400	315	255	60	17	13
B. Na_i/Na_o Exchange Subject	Control			Starved				
	Ouabain	Ouabain and furosemide	FS	Ouabain	Ouabain and furosemide	FS	Starvation time (hr)	ATP (μmol/liter cells)
	Na influx (μmol/liter cells · hr)							
G.D.	1070	890	180	820	650	170	15	46
C.B.	970	840	130	1330	1200	130	13	80
C.B.	900	750	150	1070	925	145	13	60
C.B.	900	750	150	955	895	60	17	13
A.K.	1000	850	150	875	705	170	17	7

[a] The Na and K fluxes were measured in cells containing 22 mmol/liter cells of Na. In the experiments presented in section A, the medium contained 130 mM choline chloride and 20 mM KCl and ^{86}Rb as tracer. In section B the medium contained 130 mM NaCl and ^{22}Na as tracer. All the media contained (mM) 10 MOPS-Tris, pH 7.4 at 37°C and 0.1 ouabain, with and without 1 mM furosemide.

fluxes were measured from cells having different Na and K contents (cell Na varied from 0 to 60 mmol/liter cells and K varied reciprocally from 95 to 35 mmol/liter cells) (*data not shown*).

Effect of Starvation on the K_i-K_o Exchange Pathway of the Na-K Cotransport

We have previously shown that the Na-K-Cl cotransport system has a FS K_i/K_o exchange pathway [7, 13]. The evidence for that was provided by the stimulation by internal Na *and* K of FS K influx from Na-free medium, and by the stimulation of FS K influx ($K_{0.5}$ = 20 mM) and efflux in Na-free medium by external K. The dependence of this FS K influx on chloride, and on both internal Na and K contents, provided evidence for a K_i/K_o exchange pathway of the Na-K-Cl cotransport system. We have estimated the magnitude of the K_o/K_i exchange in metabolically depleted cells measuring the FS K influx at 20 mM K_o from a sodium-free medium (choline substitution). Table 1 shows that metabolic depletion markedly reduced the FS K influx from choline medium in two of the three subjects studied.

Effect of Starvation on the Furosemide-Sensitive Na_i/Na_o Exchange

We have previously shown that internal Na stimulates a FS Na influx from Na medium, in the absence of external K [9, 14]. This influx measurement is a good estimation of the Na_o/Na_i exchange pathway. Table 1 shows that FS Na influx from a K-free medium into metabolically depleted cells loaded with 22 mmol of Na/liter cells was not significantly decreased in starved cells of two subjects, but it was reduced in one subject (C.B.) after 17 hr of starvation.

Effect of Starvation on the Furosemide-Sensitive Inward Transport of Na and K

FS Na and K influxes from Na-containing media comprise different modes of transport: inward-coupled transport of Na and K (that can be estimated,

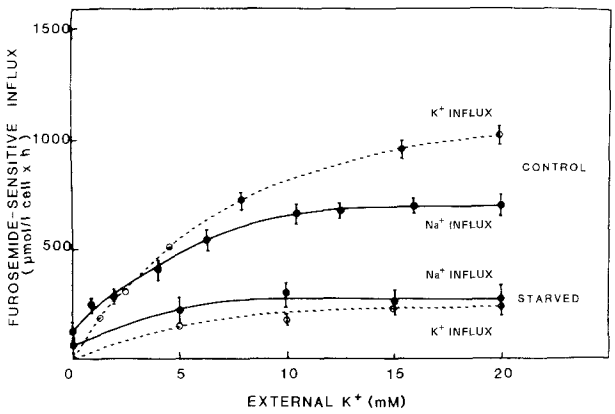

Fig. 5. Furosemide-sensitive Na and K influxes (± standard error of the fluxes) as a function of external K concentration. The red cells contained 22 mmol Na and 70 mmol K/liter cells. The flux media contained (mM): 130 NaCl, 20 to 0 choline chloride and 0 to 20 KCl, 1 $MgCl_2$, 10 MOPS-Tris, pH 7.4 at 37°C, 0.1 ouabain, with and without 1 mM furosemide. Subject: C.B. Similar results were obtained in two other experiments. The ATP content was 1,900 and 60 μmol/liter cells in control and starved cells, respectively

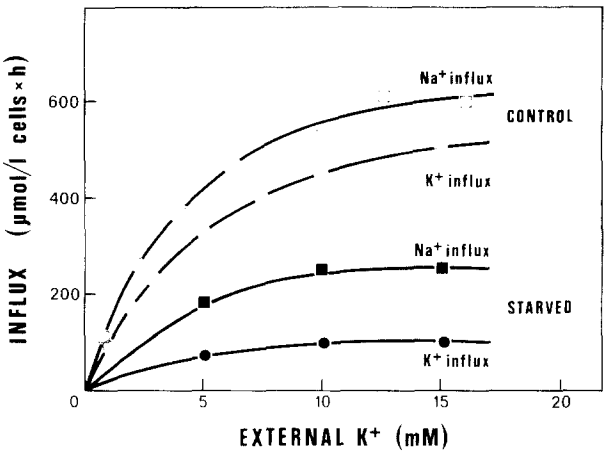

Fig. 6. Effect of metabolic starvation on the *cis*-stimulated Na and K influxes (± standard error of the fluxes). The *cis*-stimulation by external K of the ouabain-resistant Na influx was calculated by subtracting from the Na influx measured in the presence of NaCl (130 mM) and KCl (0 to 20 mM), the value of the Na influx measured from 130 mM NaCl medium in the absence of external K. The *cis*-stimulation by external Na of the ouabain-resistant K influx was calculated as the difference between K influx from a medium containing 130 mM NaCl and a medium containing 130 mM choline chloride, with external K varied from zero to 20 mM. Experimental conditions as in Fig. 5. Subject: C.B. The ATP content was 1,900 and 60 μmol/liter cells in control and starved cells, respectively. Similar results were obtained in the red cells of two other subjects

without using furosemide, from the *cis*-stimulation by Na_o of the ouabain-resistant K influx and from the *cis*-stimulation by K_o of the ouabain-resistant Na influx), a small Na_i/Na_o [9] and a larger K_i/K_o exchange [7].

Figure 5 shows the effect of metabolic depletion on the FS Na and K influxes from Na medium as a function of external K concentration. It can be seen that starvation produced a marked decrease in the maximal rate of the FS Na and K influxes. This reduction was due to the inhibition of inward cotransport of Na and K, because the *cis*-stimulated influxes (ouabain-resistant K_o-stimulated Na influx and Na_o-stimulated K influx, estimation of the coupled inward transport of Na and K), were markedly decreased by starvation (Fig. 6). The K_m for K_o to stimulate FS Na influx (5 mM) and FS K influx (4 mM) was not changed by starvation.

The stoichiometric ratio of FS Na/K influx in glucose-fed cells varies with the external K concentration, because the contribution of the two different pathways for inward FS K movement varies when external K is increased [13]. The affinity for K_o of the K_i/K_o exchange pathway ($K_{0.5}$ = 20 mM) is significantly lower than the one of the inward cotransport of Na and K ($K_{0.5}$ = 4 to 5 mM) [7, 9, 13, 14]. At 20 mM K_o, there is equilibrium between inward and outward cotransport and the stoichiometry of the FS fluxes is 2 Na : 3 K [9, 14]. It can be seen in Fig. 5 that the stoichiometric ratio of FS Na and K influxes in starved cells is 1 Na : 1 K.

Effect on Metabolic Depletion on the Net Na Extrusion and on the Equilibrium Position of the Furosemide-Sensitive Na Fluxes

We have previously shown that in the absence of external K, the FS Na efflux into 140 mM NaCl medium is larger than the influx [9, 14] and therefore there is net Na extrusion against an electrochemical gradient. It can be seen in Fig. 7, that in control cells there is maximal net FS Na extrusion of Na in the absence of external K; when external K is increased, the FS net Na extrusion is reduced and the Na fluxes approach equilibrium around 12 to 15 mM K_o.

In starved cells the FS net Na extrusion at K_o = 0 was markedly reduced (from 400–500 to 100–200 μmol/liter cells · hr) and it was abolished at lower K_o values than control (Fig. 7). In the starved cells, the FS Na efflux was 70% reduced and was not stimulated by K_o. In contrast, the FS Na influx was reduced by metabolic depletion, but it was still slightly stimulated by K_o.

We have also previously reported that the equilibrium position for FS Na influx and efflux (efflux = influx) depends on internal Na and external K

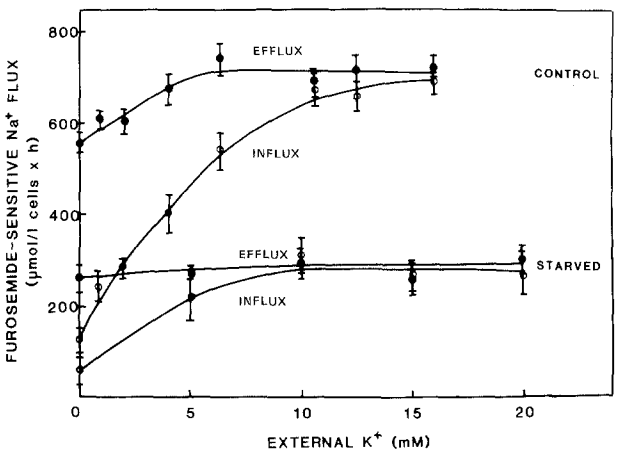

Fig. 7. Simultaneous measurement of the furosemide-sensitive Na efflux and Na influx (± standard error of the fluxes) as a function of external K concentration in control and starved cells. The red cells contained 22 mmol/liter cells of Na. The flux media contained (mM): 130 NaCl, 0 to 20 KCl and 20 to 0 choline chloride, respectively, 1 $MgCl_2$, 10 MOPS-Tris, pH 7.4 at 37°C, 0.1 ouabain, with and without 1 mM furosemide. Subject: C.B. Similar results were obtained in two other experiments. Starvation time = 17 hr. ATP content of the starved cells was 58 μmol/liter cells

concentrations [9, 14]. At physiological Na content (10 mmol/liter cells), 10 mM external K are required to bring FS Na influx equal to FS Na efflux. When cell Na is increased, more external K is needed to bring the FS Na influx equal to the FS Na efflux. At 22 mmol/liter cells of Na content, 14 to 18 mM external K are required to equilibrate outward and inward transport of Na and K.

It can be seen in Fig. 7 that metabolic depletion shifted the equilibrium position of the FS Na fluxes. Depleted cells required less external K than glucose-fed cells to achieve zero net FS Na movement (5 to 6 mM instead of 12 to 15 mM). These observations indicate that metabolism is in some way involved in the determination of the equilibrium point of this gradient-driven system.

Discussion

Furosemide-Sensitive Outward- and Inward-Coupled Transport of Na and K are Dependent on Cellular Metabolism

The FS Na and K effluxes are significantly reduced after 12 to 17 hr of starvation in a substrate-free medium (red cell ATP below 100 μmol/liter cells), while the Na-stimulated Li efflux (Na/Li countertransport), as previously reported by Pandey et al. [38] is not (Fig. 1). As reported by Hoffman and Kregenow [28] and Dunham [19], the Na-K pump is more sensitive to a decrease in ATP, being markedly reduced after 6 hr of starvation. Metabolic starvation reduces the maximal activation of the outward Na-K cotransport without altering the $K_{0.5}$ for internal Na to activate FS Na and K effluxes. The inhibition by starvation of the FS Na efflux could be reversed upon repletion of ATP to normal levels (Fig. 2), suggesting that the effect of metabolic depletion is due to a decrease in the turnover rate of the transporters rather than to a reduction in the number of transporters. It should be noted, however, that the recovery of the Na-K cotransport was inconsistent after 15 to 17 hr of starvation, suggesting that after this period there may be irreversible reduction in the number of transport sites. The furosemide-resistant Na and K fluxes were not affected by metabolic depletion, in contrast to the increase observed when metabolic inhibitors are used to deplete the red cells [1].

In our experimental design, the use of the nystatin-loading procedure to change cell Na and K content *after* metabolic depletion, avoided the shifts in water, cation and chloride content due to the changes in the concentration of the glycolytic intermediates [39].

In glucose-fed cell, external K inhibits FS Na efflux into Na-free medium with a K_i of 20 to 25 mM [7, 13, 40]. In the present experiments, we have found that metabolic depletion increased the affinity for external K to inhibit Na efflux (Fig. 4). The fall in cellular ATP seems to promote a shift from a low-affinity to a high-affinity site for external K to trap the loaded carrier in the external site. This result could suggest the presence in the Na-K-Cl cotransport system of an ATP-dependent, low-affinity, binding site for K_o.

The effect of metabolic starvation on the inward cotransport of Na and K was assessed by studying the FS Na and K influxes and the *cis*-stimulation of the ouabain-resistant influxes (of Na influx by K, and of K influx by Na). Metabolic starvation inhibited both the V_{max} of *cis*-stimulated Na and K influxes (Fig. 6) as well as the furosemide-sensitive Na and K influx (Fig. 5), but did not change the K_m for K_o to stimulate Na and K influx. Notably, at 15 to 20 mM K_o, the stoichiometric ratio of FS K : Na influx was 1 : 1 in starved cells, compared to 3 : 2 in control cells (Fig. 5).

Table 2. Metabolic dependence of ouabain-resistant (OR) and furosemide-sensitive (FS) Na and K fluxes

Authors	Cell type	Procedure of metabolic starvation	Observation
Hoffman and Kregenow [28]	Human red cells	22-hr starvation	Decrease in ethacrinic acid-sensitive Na efflux and OR influx
Beauge and Adragna [3]	Human red cells	Iodoacetamide poisoning	Decrease in the OR Rb influx
Beauge [2]	Human red cells	Iodoacetamide poisoning	Decrease in OR Na fluxes
Dunn [21]	Human red cells	22-hr starvation	Decrease in the FS Na efflux
Sachs [43]	Human red cells	24-hr starvation	Decrease of K_o effect on ouabain-insensitive Na efflux and influx
Lauf et al. [29]	Human red cells	Deoxyglucose for 2 hr	No change in the FS Rb influx
Russell [42]	Squid axons	Dialysis	Decrease in FS K and Cl influx
Rindler et al. [41]	MDCK cell line	Iodoacetamide plus oligomycin	Decrease in FS Na and K influx
Palfrey [36]	Turkey erythrocytes	Starvation	Decrease in cAMP or hypertonic stimulation of Rb influx

Net Furosemide-Sensitive Na Extrusion is Inhibited by ATP Depletion

There is net FS Na efflux from cells containing 20 mmol Na/liter cells into 130 mM Na medium [9, 14]. The FS net Na extrusion is chloride dependent and inhibited by increasing amounts of external K [9]. While in substrate-fed cells there is still substantial net Na extrusion at 4 to 6 mM K_o, in metabolically depleted cells FS Na efflux and influx were markedly reduced and the net FS Na efflux became zero around 6 mM K_o (Fig. 7). Thus, starvation markedly reduced the concentration of external K required to bring inward and outward FS Na fluxes to equilibrium. These results provide experimental evidence that the second Na pump initially proposed by Hoffman and Kregenow [28], is the gradient-driven Na-K-Cl cotransport system, in the sense that this pathway is metabolically dependent. Further studies by Sachs [43], Beauge [2] and Flatman and Lew [22] confirmed that there is net uphill Na extrusion in the presence of ouabain. Several authors have also investigated the effect of metabolic depletion on ouabain-resistant Na and K movements. As summarized in Table 2, several authors have observed a decrease in ouabain-resistant fluxes when ATP depletion was achieved by using either metabolic inhibitors or starvation. In many of these studies the time-course of starvation and recovery, the different components of the furosemide-sensitive Na and K fluxes, and the effect on the furosemide-resistant fluxes were not studied. Sachs [43] reported that in starved cells the stimulation of the ouabain-resistant Na influx by external K was greatly reduced. However, he also reported an increase of ouabain-resistant and furosemide-sensitive Na efflux from depleted cells. Lauf et al. [29] reported that the FS K influx did not decrease when the ATP level was not reduced below 100 μmol/liter cells (incubation in deoxyglucose).

Metabolic Depletion Inhibits the Furosemide-Sensitive K_i/K_o but not the Na_i/Na_o Exchange

We have previously shown [7], that the FS K influx from Na-free media (choline substitution) takes place through a K_i/K_o exchange pathway of the Na-K cotransport and not through a net K-Cl cotransport system, because it requires the presence of both internal Na and K, and chloride. This pathway also seems to be metabolically dependent, because starvation reduced the FS K influx from choline media when ATP fell below 100 μmol/liter cells (Table 1). This metabolic dependence of the FS K influx differs from that of the NEM-stimulated KCl cotransport system of human red cells, which is inhibited at ATP levels higher than 100 μmol/liter cells [29]. We have previously observed [7, 13] that in cells with normal ATP content, the FS Na efflux was inhibited and the FS K influx stimulated when external K was increased in a Na-free medium. This effect is due to the inhibition by external K of the outward cotransport of Na and K and the stimulation promoted by external K of the K_i/K_o exchange pathway. The $K_{0.5}$ for external K to inhibit FS Na efflux and stimulate FS K influx in choline media were also similar (20 to 25 mM). Metabolic starvation decreased the K_i for external K to inhibit FS Na efflux (from 25 to 11 mM), and inhibited the inward transport of K through K_i/K_o exchange

pathway. One could speculate that ATP might be involved in the conversion from a high-affinity to a low-affinity state of the binding site for K.

Another FS pathway of Na transport is the Na_i/Na_o exchange, that can be estimated from the stimulation by internal Na of a FS Na influx in the absence of external K. This pathway is bumetanide-insensitive, DIDS-sensitive and stimulated by substitution of chloride by nitrate [9]. This FS Na_i/Na_o exchange can be operationally separated from the phloretin-sensitive pathway, which is not stimulated by replacement of chloride by nitrate [11]. We have suggested that this FS Na movement takes place through the anion exchange pathway, rather than through the Na-K cotransport system. As shown in Table 1, metabolic depletion did not significantly reduce the FS Na_i/Na_o exchange pathway, providing therefore further evidence that this FS inward transport of Na is not a mode of operation of the Na-K-Cl cotransport.

Role of Metabolism in the Gradient-Driven Na-K-Cl Cotransport System

The present study shows that the movement of Na and K through the furosemide-sensitive Na-K-Cl cotransport system depends not only on the chemical gradients for Na, K and Cl, but also requires the presence of ATP and/or of the cell metabolic integrity. Thus, ATP seems to be necessary for the system to perform net Na transport against an electrochemical gradient or downhill movement of both Na and K. Since starvation did not change the diffusional leak pathways for Na and K, the impairment in the operation of the cotransport system is not caused by increased dissipation of the Na and K gradients. Another "gradient-driven" transport system, the Na/Ca exchange of heart sarcolemma and squid axons, is also modulated by ATP [15, 18]. It seems, therefore, that the Na-K-Cl cotransport is not the only gradient-driven transport system that is dependent on cellular metabolism. While transport systems able to generate concentration gradients across the cell membrane (such as the Na-K pump and the Ca pump) are coupled to ATPase reactions with low affinity for ATP, the Na-K-Cl cotransport appears to be dependent on high-affinity binding sites for ATP. It was necessary to reduce ATP levels below 100 μmol/liter cells to reduce the FS Na and K movement through the Na-K-Cl cotransport system. These findings are in agreement with the lack of effect of furosemide on the ATP content and lactic acid production in Antymicin-treated Ehrlich ascites tumor cells observed by Geck et al. [27]. It is possible that this property of the Na-K-Cl cotransport system may allow its operation under conditions of relative metabolic depletion, such as in anoxia, in which the rate of active cation pumping can be markedly inhibited by low ATP levels.

Our findings raise the question of how metabolic substrates might be involved in the coupling of Na, K and Cl gradients through the Na-K-Cl cotransport system. Certainly, the effect of starvation does not provide direct evidence that ATP is the only substrate involved in the turnover rate of this transport system. A distinct possibility exists that ATP is an allosteric modulator of the Na-K-Cl cotransport system.

It has been shown in avian red cells that the Na-K-Cl cotransport can be activated by agonists of its beta-adrenergic receptor and by cyclic AMP [32, 37]. The simulation of this transport system by cyclic AMP could not be elicited in starved cells [36]. In other cells, however, such as vascular endothelial cells [5], the Na-K cotransport is activated by bradykinin and is inhibited by cyclic AMP. An inhibition of the Na-K cotransport by cyclic AMP has been observed in human red cells [23], in smooth muscle cells [34] and in the MDCK cell line [41]. Although more work is needed to clarify the role of cell metabolism, it can be suggested that ATP, directly or indirectly, is involved in the transport of Na and K through the Na-K-Cl cotransport system.

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