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The $\alpha 1$ Na^+-K^+ Pump of the Dahl Salt-Sensitive Rat Exhibits Altered Na^+ Modulation of K^+ Transport in Red Blood Cells

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Abstract. The properties of the $\alpha 1$ Na^+-K^+ pump were compared in Dahl salt-sensitive (DS) and salt-resistant (DR) strains by measuring ouabain-sensitive fluxes (mmol/liter cell × hr = FU, Mean ± SE) in red blood cells (RBCs) and varying internal ($_i$) and external ($_o$) Na^+ and K^+ concentrations. Kinetic parameters of several modes of operation, i.e., Na^+/K^+, K^+/K^+, Na^+/Na^+ exchanges, were characterized and analyzed for curve-fitting using the Enzfitter computer program.

In unidirectional flux studies (n = 12 rats of each strain) into fresh cells incubated in 140 mM Na^+ + 5 mM K^+, ouabain-sensitive K^+ influx was substantially lower in the DS than in DR RBCs, while ouabain-sensitive Na^+ efflux and Na_i were similar in both strains. Thus, the coupling ratio between unidirectional $Na^+ : K^+$ fluxes was significantly higher in DS than in DR cells at similar RBC Na^+ content. In the presence of 140 mM Na_o, activation of ouabain-sensitive K^+ influx by K_o had a lower K_m and V_{max} in DS as estimated by the Garay equation (N = 2.70 ± 0.33, K_m 0.74 ± 0.09 mM; V_{max} 2.87 ± 0.09 FU) than in DR rats (N = 1.23 ± 0.36, K_m 2.31 ± 0.16 mM; V_{max} 5.70 ± 0.52 FU). However, the two kinetic parameters were similar following Na_o removal. The activation of ouabain-sensitive K^+ influx by Na_i had significantly lower V_{max} in DS (9.3 ± 0.4 FU) than in DR (14.5 ± 0.6 FU) RBCs but similar K_m. These data suggest that the low K^+ influx in DS cells is caused by a defect in modulation by Na_o and Na_i.

Na^+ efflux showed no differences in Na_i activation or *trans* effects by Na_o and K_o, thus accounting for the different $Na^+ : K^+$ coupling ratio in the Dahl strains. Further evidence for the differences in the coupling of ouabain-sensitive fluxes was found in studies of net Na^+ and K^+ fluxes, where the net ouabain-sensitive Na^+ losses showed similar magnitudes in the two Dahl strains while the net ouabain-sensitive K^+ gains were significantly greater in the DR than the DS RBCs.

Ouabain-sensitive Na^+ influx and K^+ efflux were also measured in these rat RBCs. The inhibition of ouabain-sensitive Na^+ influx by K_o was fully competitive for the DS but not for the DR pumps. Thus, for DR pumps, K_o could activate higher K^+ influx in DR pumps without a complete inhibition of ouabain-sensitive Na^+ influx. This behavior is consistent with K_o interaction with distinct Na^+ and K^+ transport sites. In addition, the inhibition of K^+ efflux by Na_i was different between Dahl strains. Ouabain-sensitive K^+ efflux at Na_i level of 4.6 mmol/liter cell, was significantly higher in DS (3.86 ± 0.67 FU) than in DR (0.86 ± 0.14 FU) due to a threefold higher K_{50} for Na_i-inhibition (9.66 ± 0.41 *vs.* 3.09 ± 0.11 mmol/liter cell. This finding indicates that Na^+ modulation of K^+ transport is altered at both sides of the membrane.

The dissociation of Na^+ modulatory sites of K^+ transport from Na^+ transport sites observed in RBCs of Dahl strains suggests that K^+ transport by the Na^+-K^+ pump is controlled by Na^+ allosteric sites different from the Na^+ transport sites. The alterations in K^+ transport may be related to the amino acid substitution (Leu/Gln_{276}) reported for the cDNA of the $\alpha 1$ subunit of the Na^+-K^+ pump in the DS strain or to post-translational modifications during RBC maturation.

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Key words: Na-K pump fluxes — Dahl rats — Red blood cells — K^+ transport — Ouabain-sensitive fluxes

Introduction

In recent years significant progress has been made in our knowledge of the molecular structure of the Na^+-K^+ pump, that couples the transport of Na^+ and K^+ to ATP hydrolysis. Two subunits, α and β, in a 1 : 1 molar ratio comprise the functional enzyme. Several isoforms of these subunits are encoded by separate genes with different tissue-specific and developmental expression [20], but the isoform-specific functional characteristics and physiologic roles remain to be elucidated. Investigation by various methods has provided insight into α and β subunit membrane topology, tertiary and quaternary structure, enzyme kinetics, and various conformational states as well as structural domains involved in ATP binding and phosphorylation, ouabain binding, and relative ouabain resistance [17, 20, 22, 25, 28]. The delineation of structural domains involved in ion transport, such as cation binding, transport, and modulation sites, has been elusive. A revealing, although fortuitous, mode of study of structure-function relationships is the identification of naturally occurring genetic mutations that alter discrete functional properties of the Na^+-K^+ pump.

Recently, alleles of the gene encoding the $\alpha 1$ subunit of the Na^+-K^+ pump isoform were identified in Dahl salt-resistant (DR) and Dahl salt-sensitive (DS) rats [15]. Sequencing of the cDNA for these two alleles identified a leucine substitution of glutamine at position 276 for the allele of the DS rat. In microinjection expression experiments performed in Na-loaded *Xenopus laevis* oocytes, this Leu/Gln_{276} substitution resulted in decreased ^{86}Rb influx. This region has been proposed to be involved in the conformational transition necessary for ion transport [18, 19]. These findings led us to study the kinetic properties of the several transport modes and the stoichiometry of the $\alpha 1$ Na^+-K^+ pump present in red blood cells (RBC) of both Dahl strains, to provide information about potential structure-function relationships. However, it is important to remark that rat RBC Na-K pump-mediated fluxes have not been characterized as thoroughly as those in human RBCs even though the structural differences in the gene encoding for the $\alpha 1$ isoform in both species are very well known [16, 20]. Therefore, we have approached the study of the functional operation of Na-K pump in RBC Dahl rats by an analysis of the kinetic behavior of unidirectional Na^+ and K^+ fluxes taking place in different transport modes, i.e., Na^+/Na^+, K^+/K^+, Na^+/K^+ exchanges, uncoupled and reversed modes [7–13, 34, 36]. In addition, we have also determined that Dahl RBCs express only the $\alpha 1$ Na-K pump isoform and the metabolic ATP/ADP $\times$ PO_4 ratio driving the transport reaction in these rat strains.

The results indicate, first, that rat RBCs Na-K pump-mediated fluxes differ from human RBCs in several ways; second, that the DS $\alpha 1$ Na^+-K^+ pump exhibits altered regulatory effects of internal ($_i$) and external ($_o$) Na^+ on K^+ transport but not on Na^+ transport demonstrating the presence of Na^+ modulatory sites for K^+ transport different from Na^+ transport sites. Third, RBCs of both rat strains also showed that K_o modulatory sites of Na^+ transport were different from K_o transport sites. Thus, Na^+ and K^+ transport by the Na^+-K^+ pump is controlled by allosteric Na^+ and K^+ sites different from the transport sites.

Materials and Methods

Rat Strains and Protocol

Fifty male DS and fifty male DR rats (Harlan Sprague Dawley, Madison, WI from the inbred strain of J. Rapp) were studied at 10–11 weeks of age. All rats were fed a low-salt diet (0.05% NaCl, #82049, Teklad, Madison, WI) with tap water to drink *ad libitum*, for one week before being sacrificed. Body weight for the two strains (231 ± 11 g) was not significantly different. Blood pressure was measured one week after acclimatization by the tail-cuff method and reported as the average of five consecutive determinations. The mean ± SE arterial blood pressure was not different between both strains: 113.7 ± 2.74 mm Hg for DR (n = 30 rats) and 120 ± 2.97 mm Hg for DS (n = 31 rats).

Western Blot Analysis of the $\alpha 1$ Na^+/K^+ Pump

Membranes were isolated from washed RBCs of DS and DR, and protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL) [14]. Two hundred μg of RBC membrane protein was size-fractionated in 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels, along with 5 μg of kidney and brain membranes as controls. The gel was then electrotransferred onto nitrocellulose and Western analysis was performed as described [5] with an $\alpha 1$-specific monoclonal antibody, MCK-1, and an $\alpha 2$-specific monoclonal antibody, MCB-2. To ascertain equality of DS and DR RBC membrane protein, a duplicate gel was stained with Coomassie blue.

Blood Sampling

Rats were anesthetized with Nembutal (intraperitoneal dose, 50 mg/Kg) and ~8 ml of blood was withdrawn from the abdominal aorta. Whole blood was collected in tubes with 15% EDTA

Table 1. Composition of the sodium loading solutions for varying cell Na^+ and K^+ content

A: Composition of the loading solutions, mM							
Na-Salicylate	0	20	50	70	90	135	135*
NaCl	140	120	90	70	60	5	5
B: Measured RBC cation content, mmol/liter cell							
Cellular Na^+	4.9	7.6	11.9	15.4	17.4	24.1	40.4
± SE	0.2	0.3	0.5	0.8	0.6	1.6	2.1
Cellular K^+	106.2	102	98.2	94.6	93.2	87.9	70.6
± SE	2.4	1.3	2.9	2.7	3.4	2.2	3.5

After 3 hr incubation; *after 5 hr incubation; $n = 8$.

(Monoject, Sherwood Medical, St. Louis, MO) on ice and centrifuged at 1,000 × *g* for 20 min at 4°C in a Sorval centrifuge (RT6000B, Dupont, Biomedical Products Div., Wilmington, DE). Plasma and buffy coat cells were carefully removed, and the remaining RBC pellet was washed three times with a magnesium washing solution (MgWS) containing (mM) 75 $MgCl_2$, 85 sucrose, 10 Tris-MOPS (morpholine pH 7.4 at 4°C) and suspended in an equal amount of MgWS. Aliquots of this suspension were used for measurements of hematocrit (Hct); hemoglobin (optical density at 540 nm); cellular Na^+ content lyzed in a 1 : 50 dilution of 0.02% Acationox (American Scientific Products, Boston, MA) in double-distilled water; and cellular K^+ content lyzed in a 1 : 500 dilution of 0.02% Acationox. Cellular Na^+ and K^+ contents were determined by atomic absorption spectrophotometry (model 3030 Perkin-Elmer, Norwalk, CT), with standards prepared with double-distilled water.

Modification of Intracellular Na^+ Concentration

Several loading procedures frequently used for human RBCs could not be used in rat RBCs for a variety of reasons. The nystatin-loading procedure requires ionophore removal at 37°C and in RBCs containing high Na^+ may lead to ATP depletion. PCMBS loading does not allow complete recovery of the rat RBC membrane permeability [31]. Phosphate (P_i) loading changes the intracellular levels [31], which can affect the modes of operation of the Na^+-K^+ pump [9]. The salicylate loading procedure [38] was chosen because it is carried out at low temperature and therefore avoids ATP depletion of rat RBCs containing high intracellular Na^+ and a high number of pumps. Packed RBCs were resuspended at a 20% Hct and incubated in a medium containing varying concentrations of NaCl and sodium-salicylate (Fisher Scientific, New York, NY) depending on the desired Na_i, to maintain a total of 140 mM Na^+ (*see* Table 1) as well as 10 mM Tris-MOPS (pH 7.4 at 4°C), and 1.0 mM $MgCl_2$. Na_i increased by about 0.045 mmol/liter cell for each 1.0 mM increase in sodium-salicylate after 1 hr of incubation at 4°C. Cells were then washed four times with MgWS. Cellular Na^+ and K^+ levels, Hct, and hemoglobin were determined as described above.

The salicylate loading procedure was also examined for reproducibility and comparability measuring simultaneously ion fluxes in fresh and Na-loaded RBCs. The effect of salicylate Na-loading on Na^+ fluxes was tested in paired experiments performed in the same rat. Ouabain-sensitive Na^+ efflux and K^+ influx were measured in fresh cells and in salicylate-loaded cells containing similar RBC Na^+ content (4.5 mmol/liter cell). Additionally, K^+ efflux was compared in fresh cells and at lower Na_i values (1.2 mmol/liter cell) by incubation of RBCs (at 20% Hct) for 2 hr in a solution containing (mM) 150 KCl, 10 Tris-MOPS (pH 7.4 at 37°C), 10 glucose, and 1 $MgCl_2$. In both conditions, fresh cells *vs.* salicylate-loaded cells, Na^+ efflux, K^+ efflux and K^+ influx gave identical values.

Measurements of Unidirectional K^+ Fluxes

K^+ influx was measured with use of ^{86}Rb (Amersham, Arlington Heights, IL, specific activity 5 mCi/mg) as a tracer. ^{86}Rb influx was measured in the absence and presence of 2.5 mM ouabain (Sigma, St. Louis, MO) in a medium containing (mM): 10 Tris-MOPS (pH 7.4 at 37°C), 1 $MgCl_2$, 10 glucose, 35 μCi/ml ^{86}Rb, and NaCl and/or KCl as indicated. The K^+ concentration of all the flux media was determined by atomic absorption spectrophotometry. Ion fluxes were started by adding a 50% red cell suspension to flux medium prewarmed to 37°C to a final 2% Hct. In the absence of ouabain, ^{86}Rb influx was linear for up to 11 min; therefore, duplicate samples were removed at 1, 3, and 6 min, placed in 1.5 ml Eppendorf tubes containing 0.4 ml butyl-phthalate and 0.7 ml MgWS, and centrifuged for 6 sec in a microcentrifuge (Allied Fisher Model 235). Longer centrifugation times made it more difficult to lyze the cells. In the presence of 2.5 mM ouabain, influx was linear for up to 20 min and duplicate samples were removed at 1 and 7 min. After centrifugation, the supernatant and oil were removed by vacuum aspiration, and the pellet was lyzed with 1 ml 0.02% Acationox and mixed well by vortex. This suspension was centrifuged to separate membranes, and a 150-μl aliquot was used to determine hemoglobin. Radioactivity of the remaining suspension was measured in a gamma counter (Model 1282 Compugamma, LKB Wallac, Gaithersburg, MD). K^+ influx (expressed as mmol/liter cell × hr) was determined with a linear regression analysis [21] (Enzfitter, Elsevier Biosoft, Cambridge, UK) of the cellular K^+ content, estimated from the measured specific activity *vs.* time. The program calculated the flux (slope) ± SE for the six samples; the correlation coefficients of the regression lines were always higher than 0.985.

For measurement of K^+ efflux, fresh cells were loaded with ^{86}Rb by incubation for 2 hr in a solution containing (mM): 100 NaCl, 85 sucrose, 10 glucose, 10 Tris-MOPS (pH 7.4 at 37°C), and 50 μCi/ml of ^{86}Rb at a 50% Hct. Cells were then washed four times with cold MgWS to remove external isotope. A 50% suspension of the cells was used for efflux measurements in a standard medium (Na + K) containing (mM): 140 NaCl, 5 KCl, 10 Tris-MOPS (pH 7.4 at 37°C), 1 $MgCl_2$, and 10 glucose with and without 2.5 ouabain. Flux measurements were started by the

addition of 0.2 ml of RBC suspension to 7 ml of efflux medium prewarmed to 37°C. Duplicate samples were removed at 1, 4, and 7 min, placed in a 1.5 ml Eppendorf tube containing 0.2 ml butyl-phthalate oil, and centrifuged at 6,000 × *g* for 6 sec. ^{86}Rb was measured in 0.8-ml aliquots of the supernatant in a gamma counter. The efflux expressed in mmol/liter cell × hr was calculated by converting the counts per minute (cpm) in the supernatant of the sample to mmol/liter cell from the following formula: 1/cell SA × $1/V_s$ × 1000 × F, where SA = Specific Activity in cpm per mmol, F = ml of flux media per ml of RBC, and V_s = the volume of supernatant counted. The Enzfitter program was used to determine the linear regression of the extracellular K^+ *vs.* time of the six samples. K^+ efflux was also determined in experiments where RBCs were Na-loaded by the sodium-salicylate procedure with 50 μCi/ml ^{86}Rb added to the loading solution. The flux media without ouabain were sampled at 1, 4, and 7 min and with ouabain at 1 and 10 min.

Measurements of Unidirectional Na^+ Fluxes

Na^+ influx measurements in fresh cells were performed as for K^+ influx by addition of 20 μCi/ml of ^{22}Na. For RBCs incubated in the absence of ouabain, duplicate samples were removed at 1, 8, and 20 min and, in the presence of ouabain, at 1 and 20 min. Na^+ influx was calculated as described for K^+ influx.

For Na^+ efflux measurements, fresh RBCs were loaded with ^{22}Na by incubation for 2 hr at 37°C in media containing (mM): 100 NaCl, 40 sucrose, 10 glucose, 10 Tris-MOPS, pH 7.4 at 37°C and 30 μCi/ml ^{22}Na at a 50% Hct. Cells were then washed four times with cold MgWS. Na^+ efflux was determined from duplicate samples removed at 1, 3, and 5 min and 1 and 7 min in the absence and presence of ouabain, respectively. Na^+ efflux was calculated using the Enzfitter software program as described for K^+ efflux. Na^+ efflux was also determined in experiments where RBCs were Na^+ loaded by the sodium-salicylate procedure with 20 μCi/ml ^{22}Na added to the loading solution. The flux media without ouabain were sampled at 1, 4, and 7 min and with ouabain at 1 and 7 min.

Measurements of Net Na^+ and K^+ Fluxes

Fresh cells were washed four times with cold MgWS and incubated for 1 hr at 37°C in media containing (mM): 140 NaCl, 5 KCl, 0.3 Na-phosphate, 10 Tris-MOPS (pH 7.4 at 37°C), 1 $MgCl_2$, 10 sucrose and 10 glucose with and without 2.5 ouabain at 5% Hct. Because the incubation time was longer than during measurements of unidirectional fluxes, 0.3 mM phosphate buffer was added to the media to maintain ATP levels [39]. Quadruplicate samples were removed at 0, 10, 20, 40 and 60 min, placed in chilled 1.5-ml Eppendorf tubes containing 0.4 ml butyl-phthalate and 0.7 ml MgWS, and centrifuged for 6 sec in a microcentrifuge. After this centrifugation, the supernatant and oil were removed by vacuum aspiration and the pellet was lyzed with 1 ml 0.02% Acationox and mixed well by vortex. This suspension was centrifuged to separate membranes, and a 150-μl aliquot was used to determine hemoglobin and cellular Na^+ and K^+ content as described above. For a given condition, RBC cation content in every rat (n = 20) was averaged at every time point and then the mean values obtained in four rats were averaged. Net Na^+ and K^+ fluxes were calculated from the slope (Mean ± SE, n = 20 for each curve) of the regression of RBC cation content *vs.* time using the Enzfitter software computer program.

Red Cell ATP, ADP and P_i Content

For these determinations, 300 μl of packed RBCs were precipitated with 300 μl of 12% trichloroacetic acid solution (TCA). After addition of TCA, samples were mixed well, incubated at 4°C, and centrifuged for 1 min. For ATP determinations, 0.5 ml of the supernatant was neutralized and used for spectrophotometric measurements of NADH oxidation in a phosphoglyceric acid, phosphoglycerate phosphokinase, and glyceraldehyde phosphate dehydrogenase suspension (Sigma, Kit #366-A). Aliquots of the neutralized TCA extract were also used to separate ATP from ADP by high-pressure liquid chromatography in a 25-cm C18 particle column (Supelco) DB deactivated for basic compounds. A 25-μl aliquot was used for P_i determination by the malachite green-phosphomolybdate complex color reaction [37].

Kinetic Analysis

Kinetic parameters for activation or for inhibition of Na-K pump-mediated fluxes were analyzed using the Enzfitter software computer program [21]. This program offers the possibility of performing the kinetic analysis for different equations i.e., Michaelis-Menten, Hill, and Garay and Garrahan [6] equations. Furthermore, this program can also fit the experimental data using a nonlinear regression program and thus avoid transformations such as Lineweaver-Burk, Hanes-Woolf, Eadie-Hofstee which change the error distribution [21]. Another advantage of this type of computer analysis of the pump-mediated fluxes is the inclusion of all fluxes measured in all the experiments performed in all rats.

The following equations were used:

(i) The Garay and Garrahan equation [6, Eq. (4)] was used for the analysis of Na_i activation of Na^+ and K^+ influx. In this equation, ion translocation is assumed to take place in those pump units which have attained a certain degree of occupation by Na^+ or K^+ ions, and Na^+ pump units do not interact.

$$v = \frac{V_{max}}{(1 + K_{Na}/[Na_i])^N}$$

where v is the transport rate in mmol/liter cell × hr, V_{max} is the maximal transport rate, K_{Na} is the apparent dissociation constant, N is the number of noninteracting pump sites, and $[Na_i]$ is the intracellular Na^+ content. These parameters were determined by plotting $[Na_i]/(v^{1/N})$ *vs.* $[Na_i]$. From the best fit for the linear regression, K_{Na} was estimated from the x intercept and the V_{max} from the slope. The same equation form was used for the analysis of K^+ influx *vs.* K_o. The Enzfitter program [21] was used to test for N values from 1 to 4 to determine the lowest error for the parameters; we also calculate the N values from the best fit for the equation. The concentration for half-maximal stimulation (K_m) was determined from the equation:

$$K_m = K_{Na}/(2^{1/N} - 1).$$

(ii) The logarithmic form of the Hill equation was used to determine K_m and Hill coefficient by plotting $y = \log[v/(V_{max} - v)]$ *vs.* $x = \log[S]$.:

$$\log \frac{v}{V_{max} - v} = n \log[S] - \log K'$$

where [S] is the concentration of substrate; n, the Hill coefficient; V_{max}, the maximal transport rate; and K', $[S_{50}]^n$; $[S_{50}]$, substrate concentration that yields 50% V_{max}, named K_m. The Enzfitter software computer program [21] was used to obtain the best fit for the linear regression, allowing estimates of the Hill coefficient from the slope and $K_m = [S_{50}]$ from the x intercept at $y = 0$.

(iii) The K_{50} of ouabain inhibition of K^+ influx, and the K_{50} of Na_i inhibition of K^+ efflux were calculated from a Dixon plot of $y = 1/v$ *vs.* $x = I$, where v is the transport rate and I, the concentration of the inhibitor. The K_{50} for inhibition were determined from the x intercept at $y = 0$.

Statistical Analysis

The statistical analysis for mean ± SD and SE as well as Student's t-test were performed using the Statgraphics software computer program (STSC, Rockville, MD). Ion fluxes were compared between strains using Student's t-test and intrastrain flux components were analyzed by paired t-test.

Results

Identification of Na^+-K^+ Pump α Subunit Isoform in Dahl RBCs

The Na^+-K^+ pump isoform of the α subunit present in rat RBCs of the DS and DR strains was identified by use of isoform-specific monoclonal antibodies (Fig. 1). Equal amounts of $\alpha 1$ protein were detected by Western blot analysis in DS and DR RBC membranes. The lack of reaction with monoclonal antibodies for the $\alpha 2$ Na^+-K^+ pump subunit indicated that only the $\alpha 1$ subunit was present in rat RBC. These results are consistent with those of previous studies in sheep RBCs that show the presence of the $\alpha 1$ isoform [3]. The presence of only $\alpha 1$ Na^+-K^+ pumps in equal amounts in DS and DR RBC validates the comparison of the kinetic characteristics of the $\alpha 1$ Na^+-K^+ pumps in RBCs of the Dahl strains.

Ouabain Inhibition of the Na^+-K^+ Pump in Dahl RBCs

Ion fluxes driven by the $\alpha 1$ Na^+-K^+ pump isoform are experimentally defined as the component inhibited by ouabain. Aliquots of 300 mM ouabain stock solution in DMSO were added at zero time to the ouabain-media and a similar aliquot of DMSO to the ouabain-free media. As previously explained in Materials and Methods, ouabain-resistant K^+ influx was measured during a 7 min incubation. The ouabain inhibition of K^+ influx was studied in fresh RBCs incubated in medium containing 140 mM Na^+ and 5 mM K^+ (Na+K). Seven different concentrations ranging between 0 and 5 mM ouabain were studied (*data not shown*). Ouabain-resistant K^+ influx exhibited similar and maximal inhibited values between 1.5 and 5 mM ouabain in both strains. The Dixon plot gave a K_{50} for inhibition of 53 ± 0.5 μM for DR and 54 ± 0.7 μM for DR rats ($n = 3$ rats for each strain). On the basis of these data, we concluded that the affinity for this inhibitor was similar in both strains and Na^+-K^+ pump-mediated fluxes were defined as those inhibited by 2.5 mM ouabain which is 50 times the K_{50}. Thus, the $\alpha 1$ Na^+-K^+ pump of these rodents has an approximately 100-fold lower sensitivity to ouabain than $\alpha 1$ Na^+-K^+ pumps from human, monkey, sheep, dogs and other sources [16].

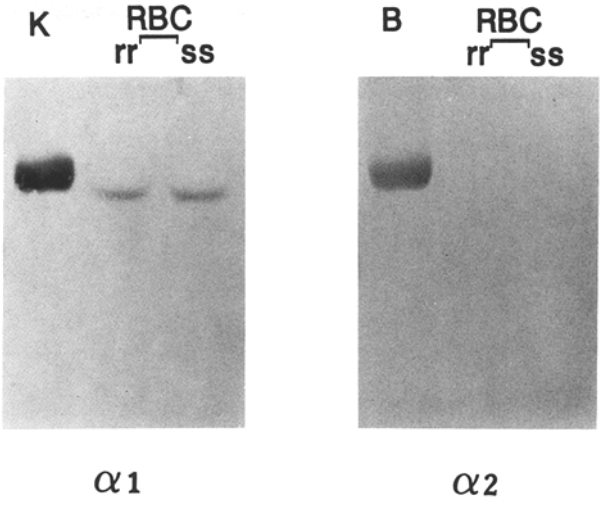

Fig. 1. Identification of the Na^+ pump isoforms in Dahl RBCs with use of isoform-specific monoclonal antibodies. RBC membranes (200 μg of membrane protein) were loaded on 10% SDS-PAGE gels, electrophoresed, and electroblotted onto nitrocellulose membranes along with control membranes: kidney (*K*), (+) control for alpha-1; and brain (*B*), (+) control for alpha-2. Nitrocellulose blots were then treated and immunoreacted with respective monoclonal antibodies and developed with use of the alkaline phosphatase-conjugate/indoxy phosphate/nitro blue tetrazolium method. As seen in the left panel, equal amounts of $\alpha 1$ protein are detected between DS (*ss*) and DR (*rr*) RBC membranes. In right panel, the (+) control lane *B*, 5 μg of brain membranes shows $\alpha 2$ protein, but not with the RBC membranes (200 μg of protein). Equality of RBC membranes loaded was ascertained by Coomassie blue staining of parallel lanes (*data not shown*).

ATP, ADP, and P_i Content of Dahl RBCs

Because the modes of operation of the Na^+-K^+ pump are strongly influenced by the [ATP/ADP × P_i] potential [9–13, 36], we determined the content

Table 2. ATP, ADP, and P_i content of Dahl rat RBCs

Experimental condition	RBC content (mmol/liter cell)	
	Fresh cells	Na^+-loaded cells
A. DR Strain		
ATP	0.78 ± 0.03	0.75 ± 0.02
ADP	0.100 ± 0.009	0.095 ± 0.001
P_i	0.195 ± 0.008	0.202 ± 0.007
B. DS Strain		
ATP	0.83 ± 0.08	0.81 ± 0.03
ADP	0.120 ± 0.006	0.095 ± 0.006
P_i	0.186 ± 0.009	0.210 ± 0.010

All values are mean ± SE; ATP, $n = 6$ experiments in 6 rats. ADP and P_i, $n = 3$ rats. Na^+-loaded cells contained 24 mmol/liter cell of Na_i.

of these metabolites in RBCs of both strains (Table 2). No significant differences in ATP, ADP, and P_i content were found between the RBCs of these two strains. Furthermore, ATP, ADP, and P_i levels in Na^+-loaded cells (24 mmol/liter cell) after 6 min incubation in the (Na + K) medium at 37°C exhibited similar content of these metabolites and did not differ from those of fresh RBCs.

Ouabain-Sensitive Na^+ Efflux and K^+ Influx in Dahl RBCs

Fresh DS and DR cells have similar intracellular Na^+ contents (4.6 ± 0.2 and 4.8 ± 0.1, respectively, $n = 36$ rats for each strain). However, the intracellular K^+ content was slightly but significantly lower in DS than in DR RBCs (104 ± 1.5 *vs.* 108 ± 1.2 mmol/liter cell, $n = 29$ rats for each strain, $P < 0.05$). RBCs of both strains had similar hemoglobin content per liter of cells.

Figure 2 shows that ouabain-sensitive Na^+ efflux has similar values in Dahl RBCs of both strains (12 rats for each strain). In contrast, ouabain-sensitive K^+ influx was significantly lower in DS cells than in DR cells. DS cells also showed lower K^+ influx than DR cells in the absence of ouabain (5.38 ± 0.39 FU *vs.* 6.89 ± 0.35 FU, $n = 12$ rats, $P < 0.01$) while the ouabain-resistant K^+ influx was not different (2.01 ± 1.17 *vs.* 2.05 ± 0.89 FU, mean ± SD, $n = 12$ rats) but varied more between rats.

Pump-Mediated Na^+ Efflux and Influx in Dahl RBCs

The similarities in ouabain-sensitive Na^+ efflux and differences in ouabain-sensitive K^+ influx giving disparate $Na^+ : K^+$ coupling ratios require a more detailed study of pump-mediated fluxes in Dahl RBCs. Na^+ efflux was further studied by investigating the effect of K_o and Na_o removal (Table 3). In the absence of Na_o and K_o, DR and DS cells showed similar ouabain-sensitive Na^+ efflux. In K-free media, the initial K^+ contamination was 5 μM and raised up to 15 μM in 6 min incubation at 2% Hct. Because this K_o concentration cannot activate Na^+/K^+ exchange, Na^+ efflux should be mediated by the uncoupled transport mode.

Na_o-stimulated, ouabain-sensitive Na^+ efflux was also similar in each strain, indicating that the Na^+/Na^+ exchange had similar values. Furthermore, the K_o-stimulated component of Na^+ efflux was not significantly different in DS and DR cells.

Simultaneous measurements of ouabain-sensitive Na^+ efflux and influx were performed in RBC aliquots of the same rat incubated in (Na + K) media (Fig. 3). Negligible amounts of ouabain-sensitive Na^+ influx were observed in DS cells, while DR cells exhibited high rates of ouabain-sensitive Na^+ influx in the presence 5 mM K_o. Because ouabain-resistant Na^+ influx was determined in both strains by a 10 min incubation in 2.5 mM ouabain, it is unlikely that the different magnitude of ouabain-sensitive fluxes observed can be accounted for by differences in ouabain sensitivity. This Na^+ influx was similar to Na^+ efflux into Na media but larger than Na_o-stimulated Na^+ efflux (Na^+/Na^+ exchange, Table 3). In RBCs of both strains, there is a net ouabain-sensitive Na^+ efflux. The smaller ouabain-sensitive Na^+ influx into DS RBCs resulted in a net Na^+ efflux larger in DS than in DR cells (3.0 *vs.* 1.5 FU, $P < 0.01$). As expected, the net ouabain-sensitive Na^+ fluxes were compensated by net ouabain-resistant Na^+ gain. In DS cells, net ouabain-sensitive Na^+ extrusion compensated a net ouabain-resistant net Na^+ gain of 2.7 ± 0.6 FU. In DR cells, the net ouabain-sensitive Na^+ extrusion compensated a net ouabain-resistant Na^+ gain of 1.4 ± 0.7 FU. Ouabain-resistant Na^+ efflux was similar in RBCs of both strains, but ouabain-resistant Na^+ influx was significantly higher in DS (4.9 ± 0.5 FU) than in DR cells (3.2 ± 0.5 FU) resulting in the different magnitudes of net ouabain-resistant Na^+ gain.

Pump-Mediated K^+ Efflux and Influx in Dahl RBCs

Measurements of K^+ efflux and influx were performed in (Na + K) media using ^{86}Rb in paired experiments with fresh RBCs (Fig. 4). In these paired experiments, ouabain-sensitive K^+ influx was significantly higher in DR than DS cells and similar to the unpaired data shown in Fig. 2. In addition, DR cells exhibited

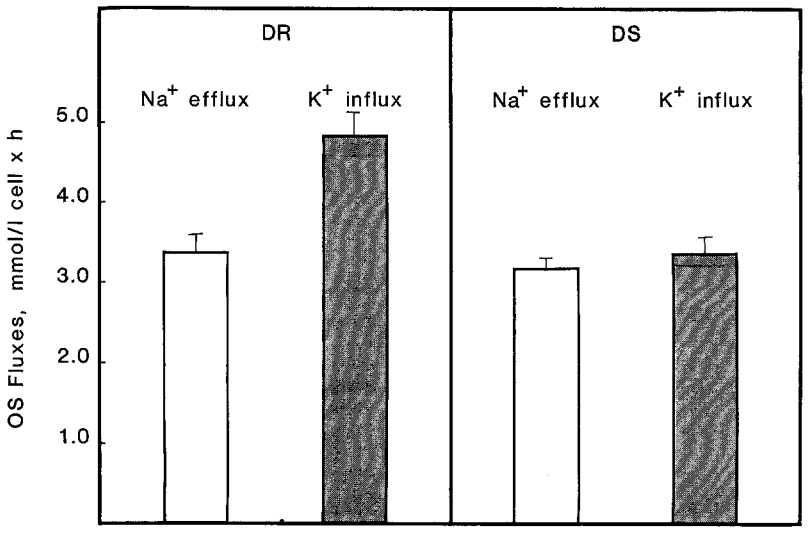

Fig. 2. Ouabain-sensitive (*OS*) Na^+ efflux and K^+ influx in fresh RBCs of the Dahl salt-sensitive (*DS*) and salt-resistant (*DR*) strains. The cell Na^+ and K^+ contents were 4.5 and 107 mmol/liter cell for DR and 4.31 and 105 mmol/liter cell for DS cells, respectively. The ouabain-sensitive K^+ influx was significantly lower ($P < 0.001$) in DS ($n = 12$ rats) than in DR cells ($n = 12$ rats). The (Na + K) flux media included 140 mM NaCl, 5 mM KCl, ±2.5 mM ouabain. Bars report the SEM.

Table 3. The effect of external Na^+ and K^+ on ouabain-sensitive Na^+ efflux in Dahl rat RBCs

Medium (mM)	Ouabain-sensitive Na^+ efflux (mmol/liter cell × hr)		Transport mode
	DR strain	DS strain	
75 $MgCl_2$	1.17 ± 0.29	1.32 ± 0.15	Uncoupled flux
140 NaCl	2.21 ± 0.47	2.65 ± 0.17	
Na_o-stimulated	1.04 ± 0.22*	1.33 ± 0.28*	Na/Na exchange
140 Na^+ + 5 K^+	2.96 ± 0.23	3.23 ± 0.20	
K_o-stimulated	0.75 ± 0.20**	0.58 ± 0.03**	Na/K exchange

All values are mean ± SE of three experiments performed in three rats. The K_o-stimulated and Na_o-stimulated components of Na^+ efflux were analyzed using a paired *t*-test. Fresh DR cells contained 4.1 ± 0.22 and fresh DS cells contained 4.23 ± 0.12 mmol/liter cell of Na^+.
For each Dahl strain, Na_o-stimulated Na^+ efflux (*) was significant at the $P < 0.02$ and the K_o-stimulated Na^+ efflux (**) was significant at the $P < 0.05$ level. K_o- and Na_o-stimulated fluxes were not significantly different between the strains (nonpaired *t*-test).

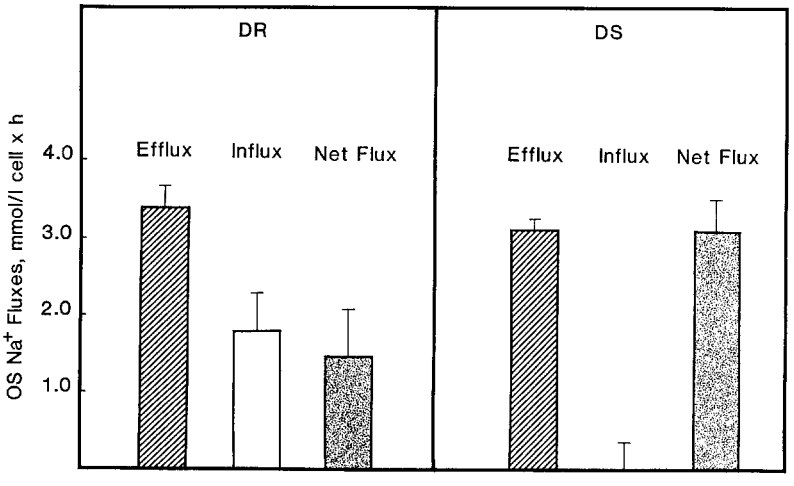

Fig. 3. Ouabain-sensitive (*OS*) Na^+ efflux and influx into fresh RBCs of Dahl rats. Na^+ efflux is similar in both strains, while Na^+ influx is significantly ($P < 0.01$, $n = 3$ rats) greater in the DR strain. Net Na^+ efflux is therefore significantly higher in the DS than in the DR strain ($P < 0.01$, $n = 3$ rats). The Na_i was 4 mmol/liter cell. The (Na + K) flux medium included 140 mM NaCl, 5 mM KCl, ±2.5 mM ouabain. Bars report the SEM.

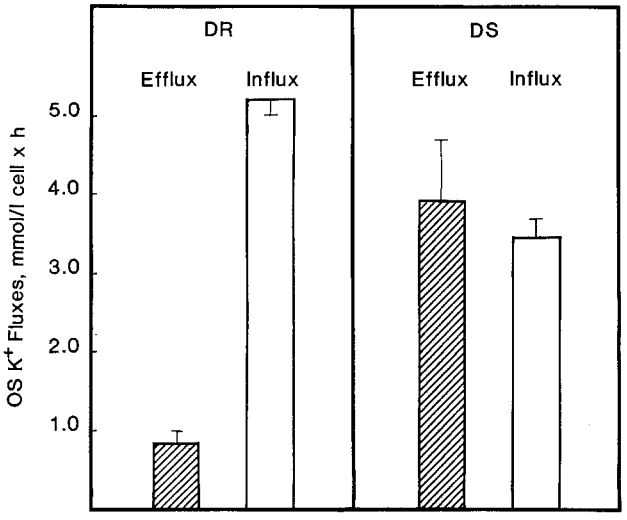

Fig. 4. Ouabain-sensitive (*OS*) K^+ efflux and influx of fresh RBCs of Dahl rats. Paired measurements of efflux and influx were performed in different aliquots of RBC of the same rat. K^+ efflux was significantly higher ($P < 0.001$) and K^+ influx significantly lower ($P < 0.001$) in DS ($n = 8$ rats) than in DR ($n = 7$ rats) strain. Na_i was 4 mmol/liter cell. The bars report SEM. The (Na + K) flux medium included 140 mM NaCl, 5 mM KCl, ±2.5 mM ouabain.

some ouabain-sensitive K^+ efflux, as expected for a low Na^+ content (4.5 mmol/liter cell). In contrast, ouabain-sensitive K^+ efflux in DS cells was fourfold higher than in DR cells. As previously shown in a large number of unpaired experiments, ouabain-resistant K^+ influx in paired experiments was similar in both strains (2.0 ± 0.52 FU, mean ± SD, $n = 3$ rats). In contrast, ouabain-resistant K^+ efflux was significantly higher in DR (7.0 ± 1 FU, $n = 3$ rats) than in DS cells (5.0 ± 1 FU, $n = 3$ rats). In DR cells, net ouabain-sensitive K^+ gain balanced high values of net ouabain-resistant K^+ lost (5.0 ± 1 FU). In DS cells, net ouabain-sensitive K^+ gain also balanced net ouabain-resistant K^+ lost (3.0 ± 1.1 FU) which was significantly lower than in DR cells.

The high rates of ^{86}Rb efflux into (Na + K) medium exhibited by DS cells appear to be driven by a K_i/K_o exchange, because in the absence of external K^+, ouabain-sensitive K^+ efflux was not significantly different than zero (0.67 ± 0.42 FU, $n = 3$ rats, P = NS). In contrast, K^+ removal markedly stimulated ouabain-sensitive K^+ efflux (3.08 ± 0.17 FU, $n = 3$ rats, ($P < 0.001$) in DR RBCs, an ion pathway that could be driven by a pump reversal mode [10].

Net Na^+ and K^+ Fluxes in Dahl RBCs

Because ^{86}Rb may not determine accurately K^+ efflux, calculation of net K^+ fluxes from the difference between unidirectional efflux and influx may not give their true magnitude. However, RBCs from both strains were exposed to the same tracer to estimate the unidirectional K^+ fluxes and marked differences in net fluxes were observed. To circumvent this problem, net Na^+ and K^+ fluxes were determined incubating RBCs for 60 min with and without ouabain in four rats for each strain (Figs. 5 and 6).

In the absence of ouabain, the mean Na^+ content of DR fresh cells (Fig. 5, right) was 4.7 ± 0.2 mmol/liter cell and after one hour incubation in ($Na^+ + K^+$) media decreased at a rate of −1.25 ± 0.32 FU ($n = 20$, $P < 0.001$). In the presence of ouabain, RBC Na^+ increased 1.83 ± 0.64 FU ($n = 20$, $P < 0.05$), thus yielding a net ouabain-sensitive Na^+ extrusion of 3.08 FU. In DS cells (Fig. 5, left), a similar value of net ouabain-sensitive Na^+ extrusion was obtained (3.05 FU). The ouabain-resistant cell Na^+ gain in one hour was also similar in DR and in DS cells (1.83 ± 0.6 *vs.* 1.65 ± 0.14 FU).

The mean K^+ content of DR cells (Fig. 6, right) incubated without ouabain was 108.2 ± 1.9 mmol/liter cell and decreased in one hour incubation at a rate of −4.92 ± 2.37 FU ($n = 20$, $P < 0.05$). In the presence of ouabain, cell K^+ decreased at a rate of −16.6 ± 1.63 FU ($n = 20$, $P < 0.0001$) which yielded a net ouabain-sensitive K^+ accumulation of 11.68 FU. In DS cells (Fig. 6, left), the initial cell K^+ (104 mmol/liter cell) remained nearly constant in the absence of ouabain (−3.37 ± 3.03 FU, $n = 20$, P = NS), and in its presence decreased at a rate of −5.3 ± 1.91 FU, ($n = 20$, $P < 0.01$). The net ouabain-sensitive K^+ gain was 1.93 mmol/liter cell. Thus, the ouabain-resistant cell K^+ lost was lower in DS (5.3 FU) than in DR cells (16 FU) and was compensated by different rates of K^+ pumping.

Activation of Ouabain-Sensitive K^+ Influx by External K^+ and Na^+

To investigate the mechanisms involved in the low rates of ouabain-sensitive K^+ influx observed in DS RBCs, we analyzed the K_o dependence of ouabain-sensitive K^+ influx keeping Na_o constant at 140 mM. As seen in Fig. 7, K^+ influx into DR cells showed a hyperbolic dependence on K_o and saturated between 5 and 8 mM K_o, reaching a maximal observed value of 4.75 FU. Kinetic analysis of these experiments was performed using the Garay and Garrahan [6] and Hill equations as described in Materials and Methods. The best fit yielded an $N = 1.23 \pm 0.36$ for the number of noninteracting sites, V_{max} 5.70 ± 0.52 FU and a K_m 2.31 ± 0.16 mM K^+ (Fig. 7, insert).

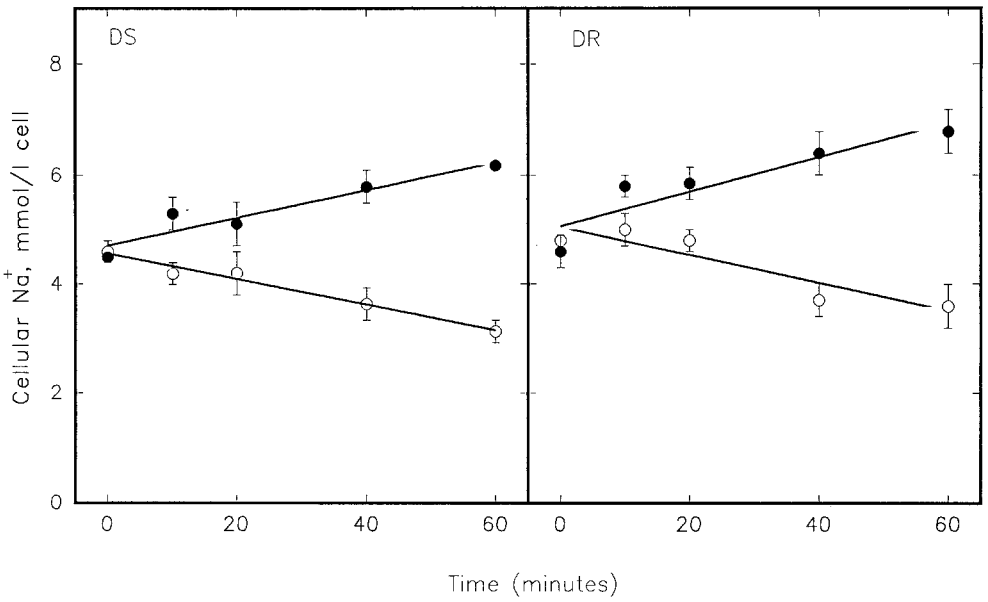

Fig. 5. Net Na^+ fluxes in DS (left panel) and DR fresh cells (right panel). RBCs were incubated for one hour at 37°C with (-●-●-●-) and without (-O-O-O-) 2.5 mM ouabain in (Na+K) media containing 140 mM NaCl, 5 mM KCl and 0.3 mM phosphoric acid, pH 7.4. In every rat, the RBC Na^+ content was determined in quadruplicate samples at the five time points (n = 20). The mean Na_i values obtained in every rat were then averaged for the four rats to plot the mean ± SE at each time point. The bars indicate the SEM except when smaller than the symbol. Net Na^+ fluxes were determined from the slope of a linear regression of RBC Na^+ content *vs.* time for the five time points (n = 20). In DR fresh cells (right) incubated in the absence of ouabain, RBC Na^+ decreased at a rate of −1.25 ± 0.32 FU (n = 20, $P < 0.001$) and in the presence of ouabain, increased at a rate of 1.83 ± 0.64 FU (n = 20, $P < 0.05$), thus yielding a net ouabain-sensitive Na^+ extrusion of 3.08 FU. In DS cells (left) incubated in the absence of ouabain, RBC Na^+ decreased at a rate of −1.4 ± 0.11 FU (n = 20, $P < 0.001$) and in the presence of ouabain, increased at a rate of 1.65 ± 0.14 FU (n = 20, $P < 0.001$), thus yielding a net ouabain-sensitive Na^+ extrusion of 3.05 FU.

A Hill plot of the experimental data yielded V_{max}, 4.75 ± 0.28 FU; K_m, 1.99 ± 0.20 mM; Hill coefficient, 0.96 ± 0.09.

In contrast, the K_o dependence of ouabain-sensitive K^+ influx in DS RBCs (Fig. 7) saturated at a lower K_o (2 mM), reaching a maximal observed value of only 2.5 FU. The best fit for the Garay equation gave a N = 2.70 ± 0.33, V_{max} 2.87 ± 0.09 and a K_m 0.74 ± 0.09 mM K^+ (Fig. 7, insert). A Hill plot of the experimental data gave a V_{max}, 2.58 ± 0.09 FU; K_m, 0.63 ± 0.23 mM; Hill coefficient, 1.9 ± 0.20. These parameters indicate that more than one site is interacting to generate the K^+-activation curve of K^+ influx in DS cells. Thus, in the presence of external Na^+, the K_o activation of ouabain-sensitive K^+ influx into DS cells was characterized by a lower V_{max} and a higher affinity than that of DR cells.

When external Na^+ was replaced by 75 mM $MgCl_2$ and 85 mM sucrose, a marked change in the K^+ influx was observed (Fig. 8). Ouabain-sensitive K^+ influx increased sharply between 0 and 0.5 mM K_o and was saturated at lower K^+ concentrations in both strains. The observed and the calculated V_{max} of K^+ influx was slightly higher in DS than in DR cells but not significantly different. A Garay plot of the data for DR cells yielded N = 1.18 ± 0.42, V_{max} 1.41 ± 0.14 FU and K_m 0.21 ± 0.08 mM K^+. For DS cells N = 1.35 ± 0.09, V_{max} 1.65 ± 0.09 FU and K_m 0.23 ± 0.05 mM K^+. The V_{max} and the K_m were not significantly different between both strains. Thus, Na_o stimulated K^+ influx 4-fold in DR but only 1.7-fold in DS cells (Table 4). Furthermore, the K_m for K_o increased 11-fold in DR cells and only 3-fold in DS cells. These results clearly indicate that the Na^+-K^+ pump in DS and DR cells exhibits marked differences in the modulation of K^+ influx by external Na^+. The increase in K^+ influx induced by a Na_o of 140 mM cannot be accounted for by a raise in cell Na^+ content, since the 6 min incubation of RBCs in 140 mM Na^+ media increased Na_i 0.4 mmol/liter cell. Furthermore, the smaller effect of Na_o on K^+ observed in DS cells cannot be accounted for by ouabain-resistant Na^+ influx which was significantly higher in DS than in DR cells (4.9 *vs.* 3.2 FU). It is also unlikely that in the absence of Na_o, the K_{50} for ouabain inhibition should have decreased from 50 μM to 1 mM to explain the reduction in K^+ influx.

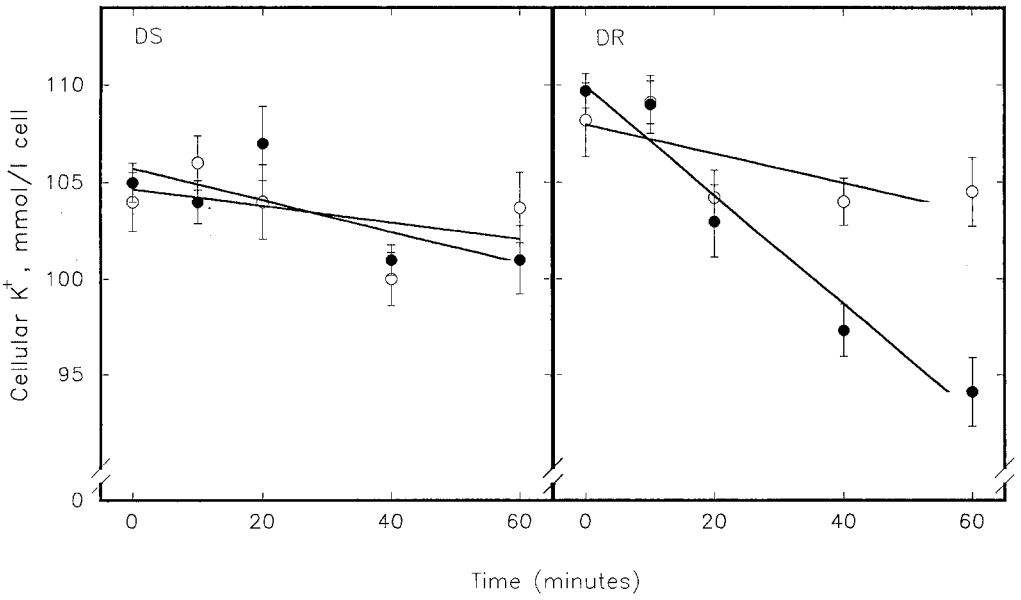

Fig. 6. Net K^+ fluxes in DS (left panel) and DR cells (right panel). RBCs were incubated for one hour at 37°C in (Na + K) media with (-●-●-●-) and without (-○-○-○-) 2.5 mM ouabain. The (Na + K) flux media contained 140 mM NaCl, 5 mM KCl and 0.3 mM phosphoric acid, pH 7.4. In every rat, the RBC K^+ content was determined in quadruplicate samples at the five time points (n = 20). The mean Na_i values obtained in every rat were then averaged for the four rats to plot the mean ± SE at each time point. The bars indicate the SEM except when smaller than the symbol. Net K^+ fluxes were determined from the slope of a linear regression of RBC K^+ content *vs.* time for the 5 time points (n = 20). In DR fresh cells (right) incubated in the absence of ouabain, RBC K^+ decreased at a rate of −4.92 ± 2.37 FU (n = 20, $P < 0.05$) and in the presence of ouabain, decreased at a rate of −16.6 ± 1.63 FU (n = 20, $P < 0.0001$), thus yielding a net ouabain-sensitive K^+ accumulation of 11.68 FU. In DS cells (left) incubated in the absence of ouabain, K^+ did not change significantly (−3.37 ± 3.03 FU; n = 20, P = NS) and in the presence of ouabain, decreased at a rate of −5.3 ± 1.91 FU (n = 20, $P < 0.001$), thus yielding a net ouabain-sensitive K^+ accumulation of 1.93 FU.

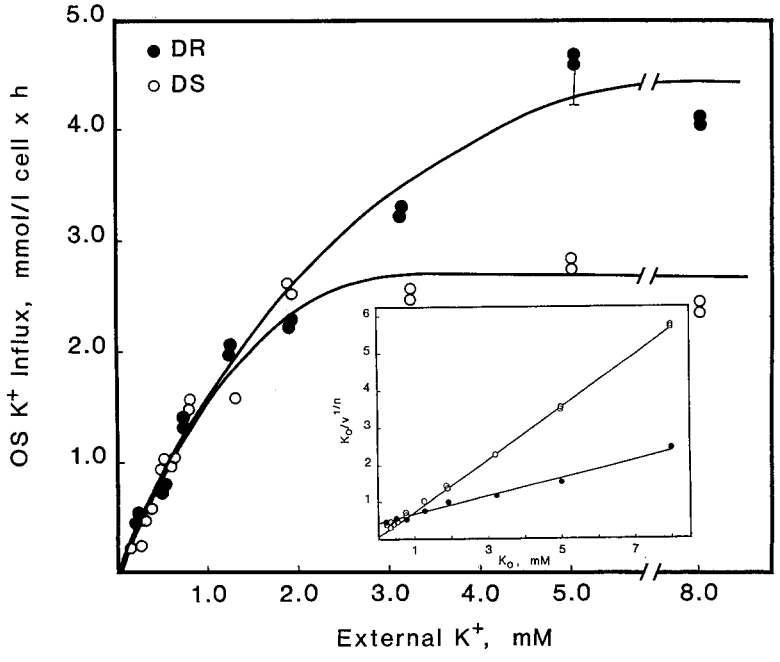

Fig. 7. Activation of ouabain-sensitive (*OS*) K^+ influx by external K^+ in DR and DS fresh cells in the presence of external Na^+. The experimental points for DR cells (n = 16) were obtained in duplicate in three experiments in three rats. The insert shows a plot of the data according to the Garay equation [6]. The best fit yielded an N = 1.23 ± 0.3 for the number of noninteracting sites, V_{max} 5.7 ± 0.52 FU and a K_m 2.31 ± 0.16 mM K^+. The flux media included 140 mM NaCl and choline 0–10 mM to compensate for K^+ variation. Na_i was 4.6 mmol/liter cell.

The experimental points for DS cells (n = 19) were obtained in duplicate in three experiments in three rats. The insert shows a plot of the data according to the Garay equation. The best fit yielded a N = 2.70 ± 0.33, V_{max} 2.87 ± 0.09 and a K_m 0.74 ± 0.09 mM K^+. The V_{max} was significantly lower in DS than in DR cells ($P < 0.0001$) as well as the K_m ($P < 0.00001$).

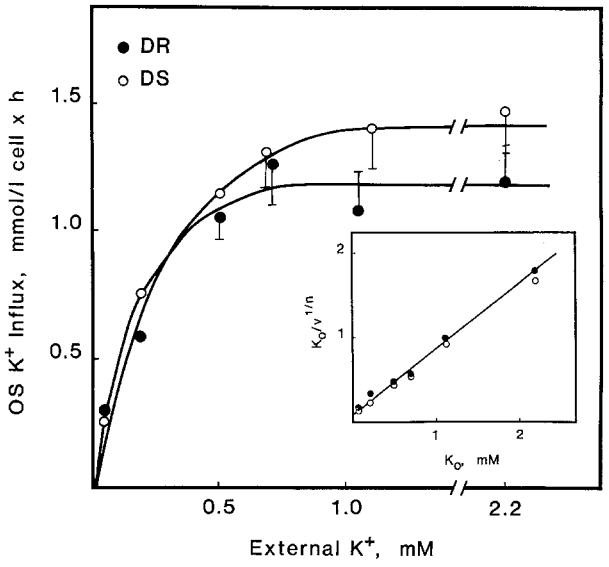

Fig. 8. Activation of ouabain-sensitive (*OS*) K^+ influx by external K^+ in DS and DR fresh cells in the absence of external Na^+. The flux medium included 75 mM $MgCl_2$, 85 mM sucrose, ±2.5 mM ouabain. Variations on the K^+ concentration were balanced by variations in the sucrose concentration. Na_i was 4 mmol/liter cell. Every experimental point is the mean value ± SE of three experiments performed in duplicate in three rats. The experimental points (n = 12) were obtained in duplicate in three experiments in three rats for each strain. The insert shows a Garay plot of the experimental data. For DR cells N = 1.18 ± 0.42, V_{max} 1.4 ± 0.12 FU and K_m 1.65 ± 0.09. For DS cells N = 1.36 ± 0.09, V_{max} 1.59 ± 0.62 FU and K_m 0.23 ± 0.04 mM K^+.

Inhibition of Ouabain-Sensitive Na^+ Influx by External K^+

As shown in Fig. 9, the K_o inhibition of ouabain-sensitive Na^+ influx behaved differently in DS and DR cells. Between 3 and 5 mM K_o, ouabain-sensitive

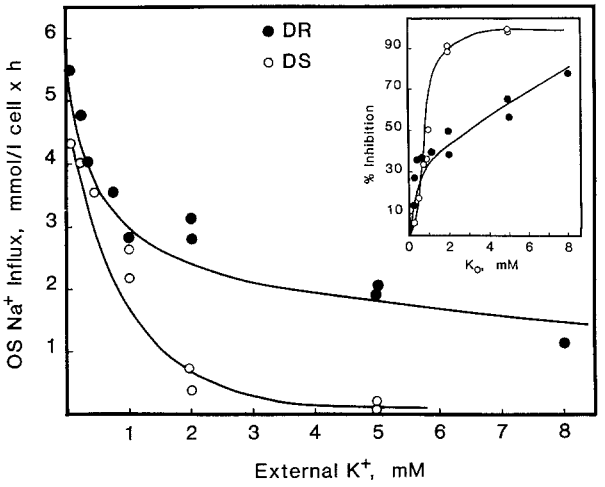

Fig. 9. Inhibition of ouabain-sensitive (*OS*) Na^+ influx by external K^+ in DR (-●-●-●-) and DS (-○-○-○-) fresh RBCs. Every experimental point is the mean ± SE of three experiments performed in duplicate in three rats. The insert shows the % inhibition of the Na^+ influx as a function of K_o. The K_o concentration for half-maximal inhibition was 3 mM for DR RBCs and 0.8 mM for DS RBCs. The influx medium contained 140 mM NaCl, ±2.5 mM ouabain. Na_i was 4 mmol/liter cell.

Table 4. Comparison of the kinetic parameters of the Na^+-K^+ pump-mediated fluxes in red cells of Dahl rats

Experimental condition	Dahl		
	Salt-resistant	Salt-sensitive	DS *vs.* DR
K^+ Influx, Na_o = 140 mM,			
V_{max} for K_o activation	5.70 FU	2.87 FU	reduced
K_m for K_o	2.31 mM	0.74 mM	reduced
K^+ Influx, Na_o = 0 mM,			
V_{max} for K_o activation	1.41 FU	1.65 FU	same
K_m for K_o	0.21 mM	0.23 mM	same
K^+ Influx*			
V_{max} for Na_i activation*	14.5 FU	9.3 FU	reduced
K_m for Na_i	8.7 mmol/liter cell	7.9 mmol/liter cell	same
K^+ Efflux*			
K_{50} for Na_i inhibition	3.09 mmol/liter cell	9.66 mmol/liter cell	increased
Na^+ Influx, Na_o = 140 mM,			
K_{50} for K_o inhibition	3.0 mM	0.8 mM	reduced
Na^+ Efflux*			
V_{max} for Na_i activation	30.7 FU	32.7 FU	same
K_m for Na_i	17.3 mmol/liter cell	19.9 mmol/liter cell	same

FU = mmol/liter cell × hr. (*) Flux measurements in (Na+K) media.

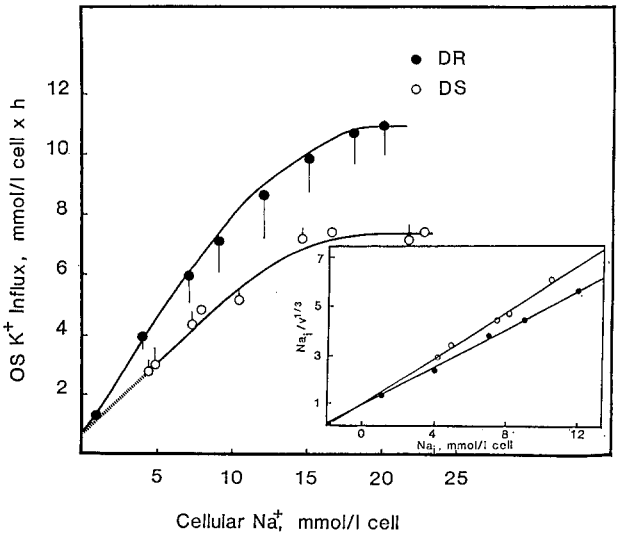

Fig. 10. Activation of ouabain-sensitive (*OS*) K^+ influx by intracellular Na^+ in Dahl rat RBCs. Every experimental point is the mean ± SE of five experiments performed in duplicates in five rats. The insert shows a plot of the data using the Garay equation [6] for the calculation of the kinetic parameters; the best fit was obtained for three noninteracting sites.

DS RBCs (-○-○-○-) exhibited a lower calculated V_{max} (9.3 ± 0.4 FU) than DR RBCs (-●-●-●-) (14.5 ± 0.6 FU). Both strains have similar K_m for Na_i (7.9 ± 0.7 and 8.7 ± 0.8 mmol/liter cell, for DS and DR, respectively). The (Na + K) flux medium included 140 mM NaCl, 5 mM KCl, ±2.5 mM ouabain.

Na^+ influx was completely inhibited in DS while substantial ouabain-sensitive Na^+ influx was exhibited even at 8 mM K_o in DR cells. Thus, K_o was not a perfect competitive inhibitor of Na^+ entry in DR pumps (Fig. 9, insert). Notice that ouabain-sensitive Na^+ influx at $K_o = 0$ was significantly higher than Na^+ efflux into Na^+ media (Table 3).

Activation of Ouabain-Sensitive K^+ Influx by Intracellular Na^+

Because cellular Na^+ also stimulates ouabain-sensitive K^+ influx, experiments were conducted to define this kinetic behavior in RBCs of both strains. Intracellular Na^+ was varied by the Na-salicylate loading procedure, and ouabain-sensitive K^+ influx was measured in (Na + K) medium. The elevation of Na_i content always increased K^+ influx more in DR than in DS cells (Fig. 10). Kinetic analysis of these experiments was performed using the Garay equation [6] as described in Materials and Methods (Fig. 10, insert). We tested for the number of sites and the best fit was obtained for three noninteracting sites. DS cells showed significantly lower calculated V_{max} of ouabain-sensitive K^+ influx than in DR strain but similar K_m for Na_i activation. The marked differences in ouabain-sensitive K^+ influx between both strains cannot be accounted for by changes in RBC Na^+ content since it did not change significantly during a 10 min incubation (Fig. 5).

Inhibition of Ouabain-Sensitive K^+ Efflux by Intracellular Na^+

Studies in human RBCs have shown that intracellular Na^+ not only activates Na^+ efflux and K^+ influx but inhibits ouabain-sensitive K^+ efflux [34, 36]. Ouabain-sensitive K^+ efflux was also studied as a function of intracellular Na^+ content in DR and DS RBCs incubated in (Na + K) medium. As shown in Fig. 11, elevation of intracellular Na^+ markedly inhibited ouabain-sensitive K^+ efflux in RBCs of both strains. In DR cells, K^+ efflux was completely inhibited at Na_i levels of 8 to 10 mmol/liter cell. However, DS cells exhibited a sizable ouabain-sensitive K^+ efflux (2.0 FU) even at Na_i as high as 15–20 mmol/liter cell. Dixon plot kinetic analysis (Fig. 11, insert) indicated that the K_{50} for Na_i inhibition of ouabain-sensitive K^+ efflux was significantly higher in DS than in DR cells. Because K^+ efflux into (Na + K) media could be driven by a K_o/K_i or K_i/Na_o exchange which are affected by intracellular Na^+, ouabain-sensitive K^+ efflux was calculated at Na_i levels close to zero. We found significantly higher values in DS (6.8 ± 0.4 FU) than in DR cells (4.5 ± 0.3 FU). These differences suggest that K_i/Na_o exchange is also increased in the DS rats.

Activation of Ouabain-Sensitive Na^+ Efflux by Intracellular Na^+

The dependence of Na^+ efflux on cellular Na^+ content was studied in RBCs loaded by the sodium-salicylate method. As shown in Fig. 12, ouabain-sensitive Na^+ efflux into (Na + K) medium increased upon the elevation of cellular Na^+ content (Na_i = 22 to 45 mmol Na/liter cell) and reached values of 22 FU. Kinetic analysis of the intracellular Na^+ activation of ouabain-sensitive Na^+ efflux was performed using the Garay equation [6] which gave the lowest errors. The calculated V_{max} and K_m had similar values for both strains.

Discussion

In the present study, we have examined Na^+ and K^+ transport driven by the $\alpha 1$ Na^+-K^+ pumps in RBCs of a large number of DS and DR rat strains. Table 4 summarizes the kinetic parameters of these

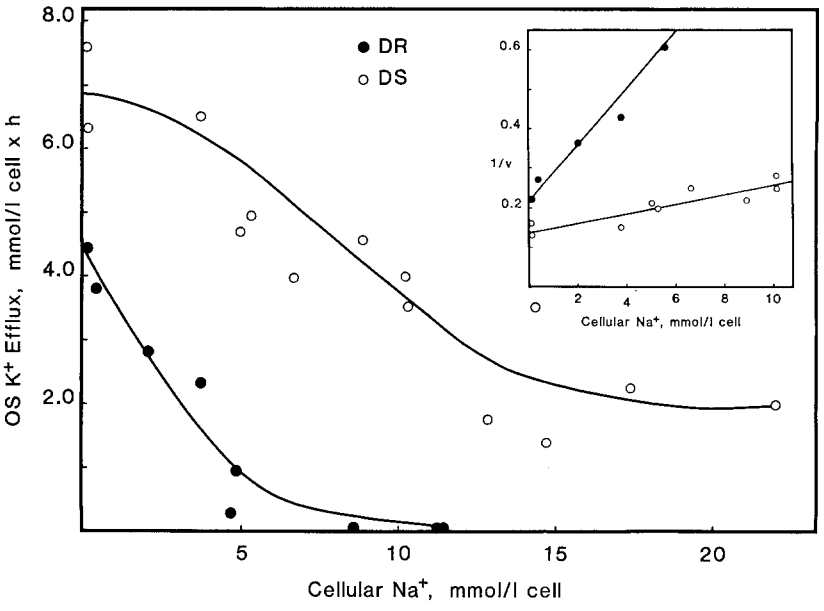

Fig. 11. Inhibition of ouabain-sensitive (*OS*) K^+ efflux by intracellular Na^+ in Dahl rat RBCs. Every point is the mean ± SE value of three experiments performed in duplicate in three rats. The insert shows a Dixon plot of the experimental data. The K_{50} for Na_i inhibition was 3.09 ± 0.11 mmol/liter cell for DR RBCs (-●-●-●-) and 9.66 ± 0.41 mmol/liter cell for DS RBCs (-○-○-○-). The (Na + K) flux medium included 140 mM NaCl, 5 mM KCl, ±2.5 mM ouabain.

two types of Na^+-K^+ pumps indicating the critical differences in the modulation of K^+ transport by internal and external Na^+ which lead to different Na : K coupling ratios.

Most of the kinetic studies of the Na^+-K^+ pump fluxes have been performed in human RBCs, and one tends to consider it as the "normal" pump behavior because few kinetic studies have been performed in rat RBCs. Studies in RBCs of the SHR and WKY rat strains did not show significant differences in the Na_i-activation kinetics of ouabain-sensitive Na^+ efflux [31], similar to our findings in the Dahl strains. Benos and Tosteson [1] have reported a coupling ratio of 1 : 1 for the scillaren-sensitive Na^+ efflux and K^+ influx in RBCs of the B_{10}. A/SgSnAo mouse strain. Thus, this is the only study of rat RBC Na-K pump mediated fluxes which has characterized kinetic parameters of Na^+ and K^+ efflux and influx of several transport modes in well-defined genetic rat strains. The data demonstrate that the $Na^+ : K^+$ coupling ratio in the Dahl strains appears quite different whether unidirectional or net ouabain-sensitive fluxes are considered.

Furthermore, in this study of the pump we have used a computer program to perform curve-fitting to calculate the kinetic parameters for Na^+ and K^+ fluxes from a large number of data points obtained in a large number of rats of both strains. The quantitative analysis of the intra- and interstrain variations of pump-mediated fluxes provides, therefore, solid support for the validity of the kinetic differences reported for DS and DR rats.

Our findings indicate that Dahl RBCs differ from human RBCs in several ways: (i) Rat RBCs have a higher K_{50} for ouabain inhibition than human cells [20]. (ii) Rat RBCs have five times higher V_{max} of Na^+ efflux and K^+ influx than human RBCs. (iii) The K_m for Na_i for Na^+ efflux is higher for rat than for human cells. (iv) The mirror image of K_o activation of K^+ influx *vs.* inhibition of Na^+ influx observed in human does not hold for rat RBCs. (v) The K_m for Na_i to activate K^+ influx is higher in Dahl rats than in human RBCs. Furthermore, the amino acid composition of the $\alpha 1$ Na^+-K^+ pump differs across species in the Lys_{349} segment involved in ATP binding and the H_1-H_2 and H_3-H_4 segment involved in ouabain binding [16].

K^+ Influx is Decreased in the DS $\alpha 1$ Na^+-K^+ Pump

Kinetic analysis of the DS $\alpha 1$ Na^+-K^+ pump revealed a decreased V_{max} of unidirectional K^+ influx, which is not due to differences in the kinetics of ouabain inhibition, cellular Na^+ content, or [ATP/ADP × P_i] potential. This unidirectional K^+ influx has a lower transport rate for the DS $\alpha 1$ Na^+-K^+ pump arising from a lower activation by both Na_i and Na_o (Table 4). For Na_i activation, the blunted ouabain-sensitive K^+ influx had a similar K_m, but a reduced V_{max} in DS cells. In the presence of Na_o, the K_o activation of K^+ influx led to a lower V_{max} and K_m in DS than in DR cells. Furthermore, DS pumps exhibited a higher number of noninteracting sites, and higher affinity for K_o while the DR pump followed Michaelis-Menten kinetics (Table 4). These differences suggest abnormal modulation of K^+ influx by Na_o. In the absence of Na_o, the K_o activation of K^+ influx becomes similar in both Dahl rats, fur-

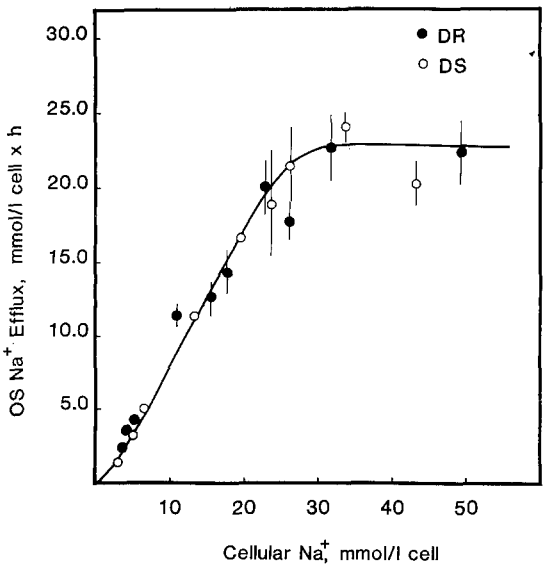

Fig. 12. Activation of ouabain-sensitive (*OS*) Na^+ efflux by intracellular Na^+ in Dahl rat RBCs. Every experimental point is the mean value of three experiments performed in duplicate. The experimental data were analyzed using the Garay equation [6] as described in Materials and Methods. The best fit was obtained for three noninteracting sites. For DS (-○-○-○-) RBCs the calculated V_{max} was 32.7 ± 3.5 FU and the K_m was 19.9 ± 0.9. For DR (-●-●-●-) RBCs, the V_{max} was 30.7 ± 2.4 FU and the K_m was 17.3 ± 0.7. The (Na + K) flux medium included 140 mM NaCl, 5 mM KCl, ±2.5 mM ouabain. The bars indicate the SE except when smaller than the symbol.

ther demonstrating the important differences in Na_o modulation of K^+ influx between the two strains. Na_o stimulation of the maximal K^+ influx is not observed in human RBCs [7]; this difference might be related to structural differences, but the underlying mechanisms remain to be elucidated.

Our results are not in agreement with those of Zicha and Duhm in DS and DR RBCs [39]. These authors studied 5-week-old rats maintained for 7 weeks in a 0.06% NaCl diet and having a significantly higher weight than our Dahl rats. Measurements of net ^{85}Rb influx at Na_i of 2, 4 and 6 mmol/liter cell in the absence of external Mg^{2+} showed no differences in the calculated V_{max} and K_m for ouabain-sensitive ^{85}Rb influx between the DS and DR strains. In addition, RBCs were preincubated for 2 hr with a 2.5 mM phosphate medium prior to the influx measurements in these experiments—a condition which can modify ATP and P_i content and affect the modes of operation of the Na^+-K^+ pump [10, 12]. As shown by Mercer and Dunham [24], phosphate can either stimulate (<0.3 mmol/liter cell) or inhibit (>0.5 mmol/liter cell) K^+ influx depending of its concentration. We have no other explanation for these conflicting findings.

It is important to mention that studies on kidney and RBC membranes of Dahl strains have indicated that ATPase activities of the Na^+-K^+ pump do not differ when assayed in media containing 120 mM Na^+, 10 mM K^+, 2 mM ATP and $MgCl_2$ [23, 26, 27, 30]. Under these experimental conditions, the substrate ATP is set at saturating levels, a condition probably not met by ion fluxes in intact Dahl RBCs and the sidedness of Na^+ and K^+ effects on ion transport are lost.

Ouabain-Sensitive K^+ Efflux is Enhanced in DS α1 Na^+-K^+ Pumps

Ouabain-sensitive K^+ efflux is markedly increased in DS α1 Na^+-K^+ pumps. In human RBCs ouabain-sensitive K^+ efflux is inhibited by intracellular Na^+ [10, 34, 36]. We hypothesized that the high rates of K^+ efflux observed in DS α1 Na^+-K^+ pumps might reflect abnormal modulation by intracellular Na^+. Indeed, the K_m for Na_i to inhibit ouabain-sensitive K^+ efflux into (Na+K) medium was significantly higher in DS than in DR α1 Na^+-K^+ pumps (Table 4). Moreover, the DS α1 Na^+-K^+ pump still exhibited substantial ouabain-sensitive K^+ efflux at high intracellular Na^+ concentrations (>15 mmol/liter cell), an indication that the *cis* Na^+ ion did not act as a fully competitive inhibitor. These different effects of Na_i on K^+ influx and efflux result in a different null point for net K^+ flux and a marked shift in the Na_i concentration required to achieve net K^+ influx. For example, only at saturating cellular Na^+ content (25–30 mmol/liter cell) with maximal Na^+ efflux can the DS α1 Na^+-K^+ pump reach a net K^+ influx (4.5 FU) equivalent to the net K^+ influx (4 FU) exhibited by the DR α1 Na^+-K^+ pump at baseline Na^+ content (4.2 mmol/liter cell).

Na^+ Efflux Is Not Altered in DS α1 Na^+-K^+ Pumps

In contrast to the marked difference in the *trans* effect of Na_i on K^+ influx between DS and DR Na^+-K^+ pumps, the *cis* effect of Na_i on Na^+ efflux was similar (Table 5). Investigation of the *trans* effect of Na_o and K_o on the outward Na^+ transport indicated that uncoupled Na^+ efflux and Na^+/Na^+ exchange components were equivalent in both Dahl strains (Table 3). These results strongly suggest that different intracellular Na^+ sites are involved in modulating the transport rates of the *cis* ions (Na^+ and K^+ efflux), as well as the transport rates of the *trans* ions (Na^+ and K^+ influx).

In DS cells, there was no ouabain-sensitive Na^+

influx at physiological external K^+ (4–5 mM), as seen in human RBCs. However, this component was nearly 2 FU at 4 mM K_o and remained elevated even at 8 mM K_o in DR cells (Fig. 9). These data indicate that K_o did not behave as a fully competitive inhibitor of Na^+ influx and exhibited a higher K_{50} for inhibition in DR cells.

Proposed Mechanisms Involved in the Different Kinetic Behavior of $\alpha 1$ Na^+-K^+ Pumps in DS Rats

The differences in the kinetic behavior of DS and DR $\alpha 1$ Na^+-K^+ pumps might be caused by alterations in primary structure, post-translational modifications during RBC maturation or in the metabolism driving the transport reactions.

A Leu/Gln_{276} substitution has been reported for DS $\alpha 1$ complementary DNA [15]. However, methodological issues regarding the primary amino acid sequence of DS and DR $\alpha 1$ Na^+-K^+ pumps remain to be settled [32, 35]. Na^+-K^+ pumps in RBCs can also be subjected to post-translational modifications during the process of reticulocyte maturation. For example, studies in sheep RBCs identified the HK and LK variants of the Na^+-K^+ pumps with different reactivity to antibodies during reticulocyte maturation [4]. Finally, the metabolic energy for driving the pump-mediated fluxes was demonstrated to have similar $ATP/ADP \times PO_4$ potential in both rat strains at low and high cell Na^+ content.

Studies in human RBCs show that the K_o activation of K^+ influx and K_o inhibition of Na^+ influx are mirror images [8]. This observation has been interpreted as Na^+ and K^+ competing for a common transport route. The uncoupling of K^+ and Na^+ transport in DS Na^+-K^+ pumps suggests Na^+ and K^+ transport might be transported across the pump protein through distinct routes. For example, K_o completely inhibited Na^+ influx in the DS Na^+-K^+ pump, but led to only a modest increase in K^+ influx. On the other hand, DR pumps exhibited incomplete inhibition of Na^+ influx by K_o but a large increase in K^+ influx. These results suggest that external K^+ sites involved in activation of K^+ influx may be different from those involved in the inhibition of Na^+ influx. Similarly, the dissociation of the intracellular Na^+ effects on Na^+ and K^+ efflux also suggests that K^+ and Na^+ transport are modulated by a different set of internal Na^+ sites. Earlier studies of the kinetic behavior of the human RBC Na-K pump also proposed intra- and extracellular modulatory sites different than transport sites [2, 11, 33]. Alternative interpretation of these results is that DR pumps transport $2K^+$ and $1Na^+$ inward, whereas in DS and human RBC pumps, only $2K^+$ are transported.

DS pumps may have abnormalities involving Na^+ regulation of the low-affinity ATP binding site that controls the K^+ deocclusion steps [13, 19, 29]. Since the mutant amino acid in DS pumps is close to the E_1-specific tryptic and chymotryptic site [15–17], we speculate that Leu/Gln_{276} substitution may induce a loss of K^+-ATP antagonism, which would be consistent with increased K^+ occlusion and the observed enhancement of K^+ efflux via the K_i/K_o exchange mode. In summary, our results provide strong experimental evidence that DS α Na^+-K^+ pumps exhibit abnormal kinetic behavior for K^+ but not for Na^+ transport which leads to a different coupling ratio for operation at steady-state RBC Na^+ content.

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