J. Membrane Biol. 98, 117–124 (1987)

The Journal of
Membrane Biology

Effect of Membrane Potential on Furosemide-Inhibitable Sodium Influxes in Human Red Blood Cells

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Summary. Furosemide-inhibitable Na influx (a measure of Na/K/Cl cotransport) was determined as a function of membrane potential in human red blood cells. The membrane potential was varied from −42 to +118 mV using valinomycin and gradients of K. The furosemide-inhibitable, unidirectional Na influx was independent of membrane potential over the entire range of potentials. The change in flux per mV, 0.443 μmol/(liter cells · hr · mV), was not significantly different from zero. The mean flux was 153 ± 16 μmol/(liter cells · hr) (± SEM, $n = 71$). The ouabain and furosemide-resistant influxes of Na and K were also measured as functions of membrane potential using either valinomycin and K or a chloride-free, tartrate flux medium to vary membrane potential. The unidirectional Na influx decreased slightly as the membrane potential was increased from negative potentials to about +10 mV. At higher membrane potentials Na influx rose dramatically with potential. This increase was not reversible and was also observed with K influx.

Key Words Na/K/Cl cotransport · furosemide · valinomycin · erythrocytes · DIDS

Introduction

Cotransport of Na and K in human red cells was proposed by Wiley and Cooper (1974) from observations that Na influx was stimulated by extracellular K and likewise, that the influx of K was stimulated by extracellular Na. This Na/K cotransport process was completely inhibited by the diuretic furosemide. The chloride dependence of this cotransport was established later (Chipperfield, 1980; Dunham, Stewart & Ellory, 1980). For reviews of the Na/K/Cl cotransport system *see* Ellory et al. (1982) and Saier and Boyden (1984).

The stoichiometry of the Na/K/Cl cotransport has been studied in a number of cells. In rabbit kidney cells (Greger & Schlatter, 1981), human red blood cells (Anatra et al., 1987[1]), duck red blood cells (Haas, Schmidt & McManus, 1982), ascites tumor cells (Geck et al., 1980), and cultured Madin-Darby canine kidney cells (McRoberts et al., 1982), the stoichiometry was found to be 1 Na/1 K/2 Cl. A different, but still electroneutral, stoichiometry has been reported in ferret red blood cells (Hall & Ellory, 1985) and squid axon (Russell, 1983): 2 Na/1 K/3 Cl. There has been no suggestion in these systems of variable stoichiometry. In contrast, recent reports suggest that Na/K/Cl cotransport in human red blood cells has a variable Na/K stoichiometry under different ionic conditions, between 2 Na/1 K and 1 Na/2 K, but that the physiologic ratio is 2 Na/3 K (Brugnara et al., 1986; Canessa et al., 1986). The stoichiometric relationship of Cl to Na and K was not measured or inferred, so no conclusion about electrical neutrality was drawn.

Whereas electroneutrality of Na/K/Cl cotransport is implied from the apparent stoichiometries, several studies have addressed this property directly. In these studies, inhibition of Na/K/Cl cotransport has no effect on the membrane potential nor did alteration of the membrane potential have an effect on Na/K/Cl cotransport (Geck et al., 1980; Haas et al., 1982; McRoberts et al., 1982; Russell, 1983, 1984). In a related study Chipperfield and Shennan (1986) have studied the effect of changes in membrane potential on diuretic-inhibitable Na and K efflux in human red blood cells. Although they reported that these effluxes were insensitive to changes in the membrane potential, it is unclear if the fluxes were mediated through the Na/K/Cl cotransporter since this cotransport system does not appear to operate in the outward direction in these cells (Warnock et al., 1984; Anatra et al., 1987[1]).

The present studies were initiated to investigate the effect of changes in membrane potential on furosemide-inhibitable Na influx in human red blood cells, to determine if the Na/K/Cl cotransporter

[1] Anatra, M.A., Kracke, G.R., Dunham, P.B. 1987. Asymmetry of Na/K/Cl cotransport in human red blood cells. *Am. J. Physiol. (submitted)*

functions in an electroneutral fashion. These cells have provided a convenient system for characterizing the fluxes of Na and K by the Na/K/Cl cotransporter. However, the great difference in cation and anion permeabilities in these cells has precluded direct studies of the cotransport of Cl with Na and K. Our results show that furosemide-inhibitable Na fluxes are independent of membrane potential, demonstrating electroneutrality.

In the course of these studies, it was necessary to measure furosemide-resistant Na influx (furosemide-inhibitable fluxes were the difference between total and furosemide-resistant fluxes). There was a marked increase in Na conductance at +10 mV as the membrane potential was made more positive inside. However, the conductance increase, whatever its basis, was not reversible and was not selective for Na.

Materials and Methods

Cells

Blood was drawn into heparin from healthy adult donors and washed three times in an isotonic choline Cl buffer (150 mM choline Cl, 5 mM glucose, 10 mM Tris-PO_4, pH 7.4) by centrifugation and resuspension at 4°C.

Varying Intracellular Cation Composition

The Na and K concentrations were varied by using *p*CMBS and dithiothreitol as described by Sachs (1970). Choline served as the replacement ion. Intracellular Na and K concentrations were measured in cell lysates by flame photometry (Instrumentation Laboratories Model 143). Cellular concentrations are expressed as mmol/liter cells.

Net K Influxes

*p*CMBS-treated cells were washed three times in a medium containing (mM): 140 KCl, 10 NaCl, 10 K-HEPES, 5 glucose, pH 7.4, and suspended in the same solution at a 10% hematocrit. Cells were incubated for 15 min at 37°C with 50 μM ouabain and, where appropriate, 10 μM DIDS and 0.3 mM furosemide. The flux was started by the addition of valinomycin at a final concentration of 2 μM. Aliquots of the cell suspension were removed prior to initiation of the flux (zero time point) and at various times during the flux and centrifuged for 1 min in a microcentrifuge. Intracellular Na and K concentrations were measured as described above.

Unidirectional Na Influxes

These were measured in cells at 10% hematocrit in a solution containing (mM): 10 NaCl, 0.05 ouabain, 5 glucose, 10 K-HEPES, pH 7.4, 37°C, and KCl and choline Cl to make 140 mM total cation. When present, DIDS (10 μM final concentration) and furosemide (0.3 mM) were added to the cells at 37°C for 15 min prior to the flux, and were included in the flux medium. ^{22}Na was used as a tracer for Na and fluxes were calculated according to Sachs et al. (1974). Fluxes are expressed in mmol or μmol/(liter cells · hr), abbreviated as mmol or μmol/(liter · hr).

Undirectional Na Effluxes

Washed cells were incubated overnight at 4°C with ^{22}Na at a 45% hematocrit in the isotonic choline Cl buffer. The cells were washed three times in the above buffer and suspended at a 7% hematocrit in the flux media used for undirectional Na influxes. After a 10-min preincubation at 37°C, portions of the cell suspension were removed and centrifuged at zero, 10 and 20 min. The supernatant solutions and a portion of cells not centrifuged but lysed by detergent were counted in a gamma counter and fluxes were calculated according to Sachs et al. (1974).

Modification of Membrane Potential

The red cell membrane potential was modified by two methods. The first (employed in most experiments) used K gradients in the presence of valinomycin and DIDS (Hunter, 1977). In this method DIDS blocks the major Cl conductance channel so that in the presence of valinomycin the membrane potential is shifted from a Cl equilibrium potential close to a K equilibrium potential. The membrane potential (in mV) was assumed to be equal to the K equilibrium potential calculated from the Nernst equation, $E_K = 61 \log [K]_o/[K]_c$. $[K]_c$, in mmol/liter cell water, was determined by flame photometry. The accuracy of this calculation in these experiments depends on the stability of the K gradient during the assay. This question is addressed by experiments presented in the Results. Cell water was assumed to be 70% of cell volume. Although cell volume may vary in valinomycin-treated cells with an imposed K gradient, the movement of K is accompanied by an equivalent shrinkage (Knauf et al., 1977) and $[K]_c$, and therefore E_K, will not change with cell volume.

The second method for altering membrane potential (used in a few experiments) allowed the measurement of both Na and K fluxes in the cells. In this method the membrane potential was set by the chloride gradient in fresh cells incubated in a medium containing the impermeant anion, tartrate (Freedman & Hoffman, 1979). A disadvantage of this technique is the subsequent alkalinization of the cell interior. The flux media contained (mM): 50 NaK tartrate, 75 sucrose, 5 glucose, 0.3 furosemide, 0.05 ouabain, 10 HEPES, adjusted to pH 7.4 with an equimolar NaOH/KOH solution, and ^{22}Na or ^{86}Rb. The cells were washed three times in this solution minus the tracer before starting the flux. The membrane potential (in mV) was assumed to be equal to the chloride equilibrium potential, $E_{Cl} = 61 \log [Cl]_c/[Cl]_o$. $[Cl]_c$, in mmol/liter cell water, was measured with an Aminco-Cotlove chloride titrator.

Statistics

A *t* statistic was calculated to test if the slope of the least-squares regression line in Fig. 1 was significantly different from other slopes. The value of *t* was then used in a one-tailed Student's distribution test (Spiegel, 1961).

Materials and Abbreviations

Choline Cl (Syntex Agribusiness, Springfield, Mo.) was purified by recrystallization from hot ethanol. Furosemide, a gift from Hoechst-Roussel Pharmaceuticals (Sommerville, N.J.), was prepared in 100-mM stock solutions in water by titration with Tris base to pH 7.4. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), adenine, inosine, *p*CMBS (*p*-chloromercuribenzene sulfonic acid), ouabain, and valinomycin were purchased from Sigma (St. Louis, Mo.). A 1 mM valinomycin stock solution in 95% ethanol was prepared immediately before use. DTT (dithiothreitol) was purchased from Eastman Kodak Co. (Rochester, N.Y.). DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate) was purchased from Calbiochem (La Jolla, Calif.) and was dissolved in isotonic choline Cl buffer immediately before use. ^{22}Na and ^{86}Rb were purchased from New England Nuclear (Boston, Mass.).

$[K]_c$ and $[K]_o$ are the intracellular and extracellular concentrations of K. K_c and K_o refer to the intracellular and extracellular K without reference to concentration. Corresponding symbols are used for Na and Cl.

Results

Dependence of Furosemide-Inhibitable Na Influxes on Membrane Potential

Figure 1 shows unidirectional Na influx measured as a function of cell membrane potential, assumed to be equal to the K equilibrium potential, set by varying the K gradient in the presence of valinomycin. The membrane potential ranged 160 mV, from −42 mV (inside negative) to +118 mV. The medium contained 10 mM Na in order to maximize the ratio of Cl-dependent to Cl-independent Na influx. Chloride-dependent Na influx has an apparent K_m for Na of 8 mM; Cl-independent Na influx increases linearly with extracellular Na and becomes greater than the chloride-dependent flux at Na concentrations above 10 mM (Dunham et al., 1980). $[Na]_c$ was also 10 mmol/liter cells, so there was no Na concentration gradient. Furosemide-inhibitable Na influx was found to be independent of membrane potential. The data were fitted to a straight line; a bell-shaped curve may also have fit but given the error in the measurements, there was no compelling reason not to use a straight line. The change in flux per mV, 0.443 μmol/(liter · hr · mV), did not differ significantly from zero ($P > 0.25$, one-tailed Student's *t*-distribution test). The mean furosemide-inhibitable Na influx over the entire voltage range was 153 ± 16 μmol/(liter · hr) (± SEM, $n = 71$). If the furosemide-inhibitable unidirectional Na influx represented transport of Na as a cation through a conductance pathway, changing membrane potential by 160 mV from −42 to +118 mV would have reduced Na influx 37-fold (Goldman-Hodgkin-Katz equation for unidirectional flux; Hille, 1984). The slope of the regression line was significantly different ($P < 0.05$), and of opposite sign, from the chord slope of the predicted flux over this range, −0.930 μmol/(liter · hr · mV), assuming the mean flux of 153 μmol/(liter · hr) at −42 mV.

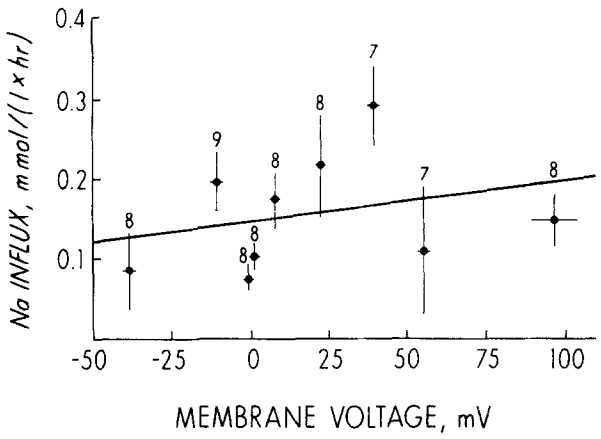

Fig. 1. Effect of membrane potential on furosemide-inhibitable Na influx in *p*CMBS-treated and fresh human red cells. Cells contained 10 mM NaCl and various concentrations of KCl and choline Cl. Influx of Na was measured at 37°C in media containing, in mM, 25, 75, or 140 KCl, 10 NaCl, 10 K-HEPES, 5 glucose, 0.05 ouabain, ±0.3 furosemide, 0.01 DIDS, and 2 μM valinomycin, pH 7.4. Choline Cl replaced KCl where appropriate. The membrane potential was calculated as described in Materials and Methods. The data are the results of 25 experiments with blood from 13 donors, 1 to 3 experiments on each. The mean and SEM of Na influxes and voltages for groups of 7 to 9 different experiments are shown by the points and vertical and horizontal error bars, respectively. The number of experiments included in each mean is indicated. The line was calculated from a least-squares fit of the data to the equation $Y = 0.000443X + 0.145$. The correlation coefficient is 0.25

The accuracy of the membrane potential calculation depends in part on the stability of the K gradient during flux experiments. Figure 2 shows the results of an experiment designed to determine how much $[K]_c$ changed in the presence of valinomycin during the flux measurements. *p*CMBS-treated cells with a $[K]_c$ of 9.4 mmol/liter cells were incubated in a medium containing 140 mM K and net K influx was measured. This initial K gradient was equal to or greater than those in all of the unidirectional flux experiments in Fig. 1. Under conditions similar to those in the unidirectional flux experiments in Fig. 1. Under conditions similar to those in the unidirectional flux assay (15 min at 37°C with ouabain, DIDS, and valinomycin), $[K]_c$ increased by 20 mM, and the membrane potential decreased from +72 to +41 mV. This error is acceptable since its effect on the relationship between furosemide-inhibitable Na

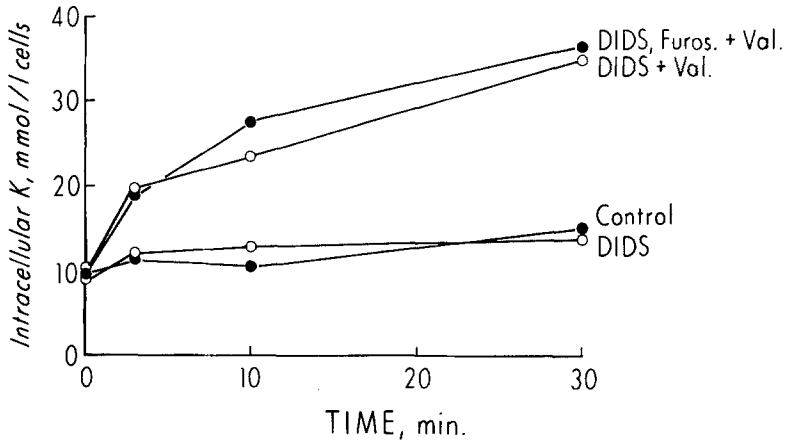

Fig. 2. Net K influx in *p*CMBS-treated human cells. Cells containing 11.0 ± 2.2 (SEM) mmol Na and 9.4 ± 0.3 mmol K per liter cells were incubated at 37°C in media containing, in mM, 140 KCl, 10 NaCl, 5 glucose, 0.05 ouabain, 10 K-HEPES, pH 7.4. Net influx was started by the addition of 2 μM valinomycin (final concentration) and samples of cells were removed at times indicated on the abscissa for analysis of K. Where present, inhibitors were used at final concentrations of 0.3 mM for furosemide and 10 μM for DIDS

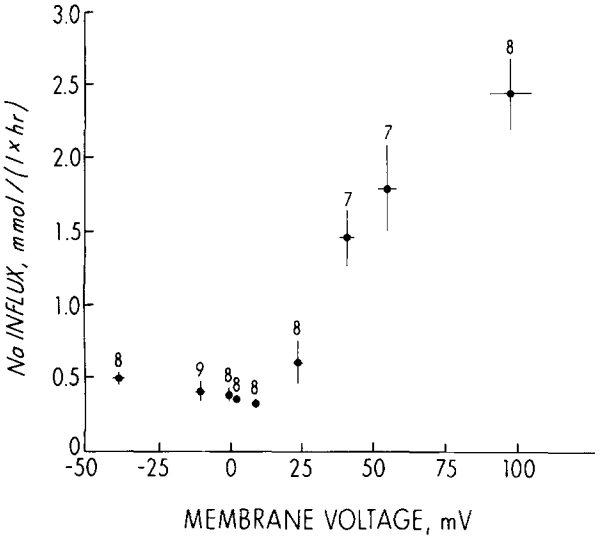

Fig. 3. Effect of membrane potential on furosemide-resistant Na influx in *p*CMBS-treated and fresh cells. Experimental details are described in the legend to Fig. 1, with the exception that all flux media contained 0.3 mM furosemide

influx and membrane potential is minor, as shown in the following examples.

In the experiments in Figs. 1 and 3, only the initial $[K]_c$ was measured and the membrane potential was calculated from this value. The magnitude of the likely errors in this calculation compared to the calculation using an average $[K]_c$ value during the assay is greater at the positive end of the voltage range where the K gradients are largest. For example at the positive voltage extreme (low $[K]_c$ cells in a 140-mM K flux medium), the likely error in calculation would be about 15 mV. At the negative end of the voltage range (fresh cells in a 25-mM K medium), the error would be about 2 mV. Correction of these errors would increase the slope of the relationship between Na influx and membrane voltage in Fig. 1 by 22% to 0.542 μmol/(liter · cells · hr · mV). However, the slope of the corrected line would still not be significantly different from zero ($p > 0.25$).

If a sizable fraction of furosemide-inhibitable Na influx is mediated by Na/Na exchange, then much of the measured Na influx might be electroneutral whether or not Na/K/Cl cotransport is electroneutral. Therefore, it was necessary to measure the contribution of Na/Na exchange to the furosemide-inhibitable Na influxes shown in Fig. 1. Table 1 shows the results of experiments in which unidirectional Na influxes and effluxes were measured as a function of the *trans* Na concentration. Total Na influx at $[Na]_o$ = 10 mM was insensitive to $[Na]_c$ over a range of 1 to 29 mmol/liter cells. Furosemide-inhibitable Na influx under the same conditions appeared to decrease somewhat as $[Na]_c$ increased, although the difference does not appear to be significant. Total and furosemide-inhibitable Na influxes at $[Na]_o$ = 40 mM were greater than at $[Na]_o$ = 10 mM as expected, but again were not sensitive to $[Na]_c$. Both total and furosemide-inhibitable Na effluxes at $[Na]_c$ of 9 and 30 mmol/liter cells were independent of $[Na]_o$ in a range of 0 to 40 mM. Therefore, it seems unlikely that Na/Na exchange contributes significantly to the furosemide-inhibitable Na influx in the experiments in Fig. 1, and it is reasonable to conclude that the unidirectional fluxes measured in these experiments represent net fluxes through the Na/K/Cl cotransporter.

Alteration of the membrane potential by the valinomycin technique necessitated the use of various $[K]_c$. Therefore, the effect of $[K]_c$ on the Na influx was studied. In the absence of valinomycin, K_c inhibited Na influx. In the presence of valinomycin, however, the effect was minimal either with a potential difference or with none ($[K]_c = [K]_o$). The

Table 1. Na/Na exchange, total and furosemide-inhibitable, in human red blood cells: *Trans* effects of Na on unidirectional Na influx and efflux[a]

$[Na]_c$	Influx, μmol/(liter · hr)			
	$[Na]_o$ = 10		$[Na]_o$ = 40	
	Total	Furos-Inh.	Total	Furos.-Inh.
1	648 ± 182	241 ± 111	1675 ± 325	385 ± 185
9	665 ± 84	168 ± 52	2065 ± 45	480 ± 100
29	775 ± 4	72 ± 49	2640 ± 70	430 ± 80

$[Na]_o$	Efflux, μmol/(liter · hr)			
	$[Na]_c$ = 9		$[Na]_c$ = 30	
	Total	Furos-Inh.	Total	Furos.-Inh.
0	682 ± 54	109 ± 80	2015 ± 25	0 ± 230
10	756 ± 16	168 ± 65	2260 ± 100	215 ± 105
40	728 ± 112	204 ± 106	2100 ± 75	0 ± 170

[a] Fluxes are the means of two separate experiments and the errors are half the total range. Furosemide-inhibitable fluxes (Furos.-Inh.) are the difference between total fluxes and fluxes measured in the presence of furosemide. Influx and efflux were measured simultaneously. Fluxes were measured under experimental conditions identical to those in Fig. 1 except for $[Na]_c$ and $[Na]_o$. Units for $[Na]_o$ are mM and $[Na]_c$ are mmol/liter cells.

explanation for the difference in results is not clear. However, since valinomycin was used in all of the experiments on voltage dependence of Na influx, a correction for an effect of $[K]_c$ was unnecessary.

Dependence of Furosemide-Resistant Na Influx on Membrane Potential

In the course of these experiments it was necessary to measure furosemide-resistant Na influx as a function of membrane potential. According to the Goldman-Hodgkin-Katz equation for undirectional flux (Hille, 1984), Na influx would be expected to decrease 54% from −38 to +8 mV. Figure 3 shows that Na influx in furosemide-treated cells decreased only 33% over this range. This less than expected decrease in Na influx can be explained by either an increase in Na conductance or a constant Na conductance together with a component of Na influx, perhaps Na/Na exchange, which is voltage-insensitive. A striking threshold for Na conductance was observed above 8 mV. Although predicted to decrease 50% from +8 to +40 mV, Na influx actually increased threefold over this range.

If the increased Na influx is due to a conductance increase, undirectional efflux should also increase. Therefore, unidirectional Na effluxes were measured in experiments like those in Fig. 3 with voltage varied over 130 mV (*data not shown*). As E_m was made more positive inside, a large increase in efflux was observed at +8 mV, confirming that a large increase in conductance occurs. If this increase in Na conductance, which occurs with depolarization, bears any resemblance to voltage-dependent conductances in nerve and muscle membranes, it should be reversible and specific.

Table 2. Reversibility and ion specificity of membrane potential-dependent changes in furosemide-resistant influxes[a]

Condition	Influx, mmol/(liter · hr)				
	Valinomycin		Tartrate		
	mV	Na influx	mV	Na influx	K influx
Control	3.4	0.76	−8.0[b]	1.65	0.95
Potential applied	73.1	2.11	87.8	2.38	1.98
Potential reversed	5.3	9.71	−8.0[b]	2.34	2.62

[a] Unidirectional influxes were measured and membrane potentials altered as described in Materials and Methods. Two different methods were used to vary membrane potential (valinomycin and tartrate). The flux media in the valinomycin experiments contained (mM): 0.3 furosemide, 0.01 DIDS, 0.05 ouabain, and 2 μM valinomycin. The flux media in the tartrate experiment contained (mM): 0.3 furosemide, and 0.05 ouabain. The valinomycin experiment is included in Fig. 3; the tartrate experiment is not. Similar results were obtained in one other experiment with valinomycin and two other experiments with tartrate.
[b] Values assumed for fresh cells (Knauf, 1985).

Reversibility of Voltage-Dependent Fluxes

To determine if the voltage-dependent increase in Na influx is reversible, we restored the valinomycin-induced membrane potential to its original value by two methods. In the first, cells containing low $[K]_c$ were transferred from a medium with valinomycin and high K (positive membrane potential) to one with valinomycin and low K. Average K calculated from initial and final $[K]_c$ was used in calculations of membrane potential. Table 2 shows that Na influx increased as E_m was changed from 3 to 73 mV. Upon return to the lower voltage, Na influx increased further. The increase observed upon return to lower voltage is what would be predicted from the change in membrane potential, assuming a constant conductance.

The second method of restoring the membrane potential was to incubate low $[K]_c$ cells for 1 hr at 37°C in the high-K flux media in the presence of valinomycin in order to dissipate the K gradient.

Furosemide-resistant Na influx increased at positive potentials and then doubled as the potential was returned to a control value (*not shown*), results similar to those in the valinomycin experiments in Table 2.

Ion Specificity of Voltage-Dependent Fluxes

K fluxes were determined in order to investigate if the voltage-dependent increase in furosemide-resistant influx is specific for Na. However, the use of the K ionophore in the above experiments obviated the simultaneous measurement of K and Na fluxes. This problem was circumvented by setting the membrane potential using the Cl gradient in cells incubated in a solution containing the impermeant anion tartrate.

Table 2 shows the results of an experiment in which Na and K unidirectional influxes were measured in cells suspended in a 50-mM Na, K-tartrate medium, identical in ionic strength and osmolarity to the Cl-containing medium of the control cells. Furosemide-resistant influxes of Na as well as K increased as the potential was made more positive. However, the increase in these fluxes was not reversible with restoration of voltage. The voltage-dependent increase in furosemide-resistant influx thus appears to be a permeability change in the membrane that is nonspecific and irreversible. The large potential difference, inside positive, may make the membrane unstable in an irreversible fashion. The sharp threshold is striking, but uninterpretable at present.

Discussion

The results presented here show that furosemide-inhibitable Na influx in human red blood cells is electroneutral. Insensitivity of transport to membrane potential was observed over a voltage range of −40 to +120 mV, achieved using valinomycin and DIDS and varying K gradients. The mean Na influx over this range was 153 ± 16 μmol/(liter · hr).

Electroneutral furosemide-inhibitable fluxes might be expected if this transport was entirely by 1 for 1 exchange of Na even if net fluxes through the same pathway are sensitive to voltage changes. However, most of the furosemide-inhibitable Na influx is probably not obligatory exchange. In experiments which measured Na influx and efflux simultaneously under conditions identical to the membrane potential experiments, Na/Na exchange did not contribute to furosemide-inhibitable Na influx because furosemide-inhibitable Na influx was not significantly activated by increasing $[Na]_c$ and, likewise, furosemide-inhibitable Na efflux was insensitive to changes in $[Na]_o$ (Table 1).

Since Na/K/Cl cotransport is electroneutral, it must transfer an equal number of anions and cations. If our proposed stoichiometry for cations, 1 : 1, is correct (Anatra et al., 1987[2]), then the overall stoichiometry is 1 Na/1 K/2 Cl, the same stoichiometry as reported for many systems. Our estimate of cation stoichiometry was made from measurements of net, mutually dependent Na and K influxes, but at a single set of high external Na and K concentrations, 50 mM each.

Measurements of electroneutrality of Na/K/Cl cotransport have been made in many cell types. Geck et al. (1980) depolarized Ehrlich ascites tumor cells either by inhibiting the electrogenic Na pump with ouabain or by adding glycine to dissipate the Na gradient. They found no change in furosemide-inhibitable transport of Na, K, Cl, or H_2O. Similarly furosemide-inhibitable K fluxes had no effect on the tetraphenylphosphonium distribution ratio, a measure of membrane potential. Haas et al. (1982) used valinomycin to study furosemide-inhibitable net Na movements over a voltage range of −80 to −10 mV. Net Na loss or gain was independent of the presence or absence of valinomycin. Russell (1983) found in squid axon that furosemide or bumetanide treatment hyperpolarized the resting membrane potential by less than 1 mV, and concluded that cotransport was electroneutral. Similarly, a 30-mV depolarization of the squid axon resting membrane potential had no effect on bumetanide-inhibitable Cl influx (Russell, 1984). In cultured MDCK cell monolayers, McRoberts et al. (1982) found that the presence or absence of valinomycin had no effect on ^{22}Na uptake in the presence of K and Cl. Having ruled out the possibility that H^+ or OH^- may be moving to maintain electroneutrality, they concluded that Na/K/Cl cotransport is electrically silent.

Chipperfield and Shennan (1986) have recently shown in human red blood cells that Na and K effluxes inhibited by piretanide, a loop diuretic similar to furosemide, are not significantly changed by either hyperpolarizing the membrane potential to −25 mV (by increasing external pH) or by reversing the membrane potential to positive values (not specified) in a Cl-free, gluconate-containing medium. However, these diuretic-inhibitable effluxes may not be through the Na/K/Cl cotransporter since this cotransport apparently does not operate in the out-

[2] *See* footnote 1, p. 117.

ward direction in human red blood cells (Warnock et al., 1984; Anatra et al., 1987[3]).

The Na and K influxes that persist in the presence of ouabain and furosemide are not a simple function of voltage. For example, Na influx decreases as the membrane potential is increased from negative values to about +8 mV. A further increase in potential caused a large increase in the unidirectional influx. This increase was not reversed when voltage was restored nor was it specific for Na. This behavior is not the same as excitable cell, voltage-gated Na and K channels.

Red blood cell volume is expected to change under some of the experimental conditions where membrane potential was varied using valinomycin and K gradients. This change would be greatest at the positive end of the membrane potential range where the K gradient is largest. Here the 20-mM increase in intracellular KCl concentration would be expected to increase the red cell volume by about 13%. Volume changes could be expected to affect the results in two ways. Calculation of the membrane potential using $[K]_c$ would be affected if $[K]_c$ changed with volume. However, since the movement of K under these conditions is isotonic with water, $[K]_c$ and therefore, E_K will not change with volume. When some cells change volume, fluxes are triggered which enable the cells to return to their original volume (Grinstein et al., 1984). However, in the case of human red blood cells it has been shown that Na/K/Cl cotransport is insensitive to volume changes (Warnock et al., 1984; Anatra et al., 1987[3]). Therefore, the furosemide-inhibitable Na fluxes measured in the present experiments would not be expected to change with volume.

The stoichiometries of Na/K/Cl cotransport reported so far for both vertebrate and invertebrate cells are such that the overall process is electroneutral. It is tempting to speculate why this property of the cotransporter is so widespread. One of the functions of the Na/K/Cl cotransporter may be to regulate cell volume (though apparently not in red cells). It would be advantageous for cells to have an electroneutral volume-regulating mechanism so that alterations in membrane potential would not affect cell volume and likewise so that the volume-regulating mechanism does not alter voltage when it is operating. This is especially true for excitable cells like neurons and muscle cells, and may also be important for epithelial cells.

We have shown that furosemide-inhibitable, unidirectional Na influx in human red blood cells is electrically neutral over a wide range of membrane potentials. Thus Na/K/Cl cotransport in human red cells shares a property with this pathway in all other cell types where it has been studied.

This work was supported by grants from the USPHS, NIH (AM-28290, AM-33640).

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[3] *See* footnote 1, p. 117.

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Received 28 April 1987