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Amiloride-Sensitive Na^+ Transport in Human Red Cells: Evidence for a Na/H Exchange System

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Summary. The role of transmembrane pH gradients on the ouabain, bumetanide and phloretin-resistant Na^+ transport was studied in human red cells. Proton equilibration through the Jacobs-Stewart cycle was inhibited by the use of DIDS (125 μM) and methazolamide (400 μM). Red cells with different internal pH (pH_i = 6.4, 7.0 and 7.8) were prepared and Na^+ influx was measured at different external pH (pH_o = 6.0, 7.0, 8.0). Na^+ influx into acid-loaded cells (pH_i = 6.4) markedly increased when pH_o was raised from 6.0 to 8.0. Amiloride, a well-known inhibitor of Na^+/H^+ exchange systems blocked about 60% of the H^+-induced Na^+ entry, while showing small inhibitory effects in the absence of pH gradients. When pH_o was kept at 8.0, the amiloride-sensitive Na^+ entry was abolished as pH_i was increased from 6.4 to 7.8. Moreover, measurements of H^+ efflux into lightly buffered media indicated that the imposition of an inward Na^+ gradient stimulated a net H^+ efflux which was sensitive to the amiloride analog 5-N-methyl-N-butyl-amiloride. Furthermore, in the absence of a chemical gradient for Na^+ ($Na_i^+ = Na_o^+$ = 15 mM, *Em* = +6.7 mV), an outward H^+ gradient (pH_i = 6.4, pH_o = 8.0) promoted a net amiloride-sensitive Na^+ uptake which was abolished at an external pH of 6.0. These findings are consistent with the presence of an amiloride-sensitive Na^+/H^+ exchange system in human red cells.

Key Words sodium transport · amiloride · Na^+/H^+ exchange · erythrocytes

Introduction

A Na^+/H^+ exchange has been described in the plasma membrane of a wide variety of mammalian cells (Aickin & Thomas, 1977; Moolenaar et al., 1981; Rindler & Saier, 1981; Pouyssegur et al., 1982; Aronson et al., 1983; Parker, 1983; Grinstein et al., 1984). The exchange system seems to be involved in pH_i homeostasis (Grinstein et al., 1984; L'Allemain et al., 1984; Piwnica-Worms et al., 1985), in the regulation of cellular volume (Cala, 1983; Grinstein et al., 1983; Parker, 1983) and in transepithelial ion transport (Aronson, 1983). The electroneutral Na^+/H^+ antiporter (Aronson, 1983; Grinstein et al., 1984) is inhibited by micromolar concentrations of amiloride, a pyrazine derivative (Benos, 1982). Recent work has implicated the Na^+/H^+ exchange in the mitogenic activation by serum, growth factors (Villereal, 1981; Rothenberg et al., 1983; Paris & Pouyssegur, 1984), and phorbol ester tumor promoters (Besterman & Cuatrecasas, 1984).

We have previously reported (Escobales & Canessa, 1984*a*,*b*), that human red cells possess an otherwise-silent, Ca^{2+}-activated and amiloride-sensitive Na^+ transport system. The amiloride-sensitive Na^+ pathway is insensitive to inhibitors of the Na^+-K^+ pump and Na^+-K^+ cotransport like ouabain and bumetanide, respectively. Moreover, the transport system is unaffected by phloretin and DIDS,[1] inhibitors of the red cell Na^+/Na^+ exchange and Cl^-/HCO_3^- countertransport, respectively. The activation of this pathway by Ca^{2+} seems to depend on the metabolic integrity of red cells. Thus, amiloride-sensitive sodium transport does not take place in ATP-depleted cells, ATP-repletion of ATP-depleted cells restored the amiloride-sensitive Na entry, and ATP enrichment enhanced the amiloride-inhibitable Na^+ uptake by about 100%.

The biochemical events linking Ca^{2+} with the activation of this amiloride-sensitive pathway are unknown at present. However, it is conceivable, that an increase in cellular H^+ concentration is one of the terminal steps in the sequelae of metabolic events turned on by Ca^{2+} that results in the activation of this transport system. A direct role of H^+ in the activation of this pathway was suggested by the finding that amiloride-sensitive Na transport was activated in ATP-rich cells which had a lower cellular pH (6.9) than fresh cells (7.2), without requiring

[1] *Abbreviations:* DIDS: 4,4′-Diisothiocyano-2,2′-disulfonic acid stilbene; EGTA: Ethylene glycol bis (β-aminoethylether)-N,N,N,′N-tetraacetic acid; MOPS: (3-N-morpholino-propanesulfonic acid); MES: (2-N-morpholino-ethanesulfonic acid).

any increase in cell Ca^{2+}. Furthermore, this basal amiloride-sensitive Na pathway was largely inhibited when the cellular pH was raised from 6.9 to 7.2.

In this paper, the role of transmembrane pH gradients in the activation of amiloride-sensitive Na^+ transport was examined by using red cells in which proton equilibration through the Jacobs-Stewart cycle (*see* Hladky & Rink, 1977) was inhibited by the use of DIDS and methazolamide. The results obtained indicate that human red cells possess an amiloride-sensitive Na : H antiport which can perform net Na^+ transport at the expense of a proton gradient.

Portions of this work have been published elsewhere (Escobales & Canessa, 1984*b*; 1985*a*).

Materials and Methods

NaCl, $MgCl_2$, and glucose were purchased from Fisher Scientific Company (Fairlawn, N.J.). Ouabain, MOPS, MES and DIDS were obtained from Sigma Chemical Co. (St. Louis, Mo.). KCl was from Mallinckrodt Inc. (St. Louis, Mo.). Amiloride and 5-N-methyl-N-butyl-amiloride were kindly provided by Dr. E. J. Cragoe from Merk, Sharp and Dohme (Rahway, N.J.). Bumetanide was obtained from Laboratoire LEO (Venouillet, France); phloretin from Nutritional Biochemicals Corp. (Cleveland, Ohio), and methazolamide was from Lederle Laboratories Div. (Pearl River, N.Y.).

Preparation of Red Blood Cells

Human red blood cells were drawn from three healthy male donors into heparin-containing tubes. The cells were spun down at 3,000 rpm for 15 min at 4°C in a Sorval centrifuge (RC 5B, Dupont Co., Biomedical Products Division, Wilmington, Del.). The plasma and buffy coat were removed by suction and the cells were then washed three times with a choline-washing solution (CHWS) containing (mM): 150 choline chloride, 1 $MgCl_2$, 10 Tris-MOPS (pH 7.4 at 4°C), and resuspended with CHWS to a 50% suspension. Aliquots of this suspension were used for determinations of hemoglobin (optical density at 540 nm), hematocrit, and cellular Na^+ and K^+ concentrations by using appropriate dilutions in double-distilled water containing 0.02% Acationox detergent (American Scientific Products, Boston, Mass.).

Internal pH Manipulation and Determination

In red cells, pH_i and pH_o rapidly equilibrate through the actions of carbonic anhydrase and the Cl^-/HCO_3^- exchange system, so that $[H^+]_i/[H^+]_o = [Cl^-]_o/[Cl^-]_i$ (*see* Hladky & Rink, 1977). Thus red cells of different pH_i (6, 7, 8) were obtained by preincubating washed cells (5% hematocrit) for 20 min in media containing (mM): 75 NaCl, 75 KCl, 0.15 $MgCl_2$, 0.1 ouabain, 10 glucose, 0.01 bumetanide and 10 Tris-MES (pH 6.0) or Tris-MOPS (pH 7.0 or 8.0 at 37°C). Since volume changes are known to occur subsequently to alterations in pH_i (acid and alkaline titration of red cells produce swelling and shrinkage, respectively), the osmolarities of the media were adjusted by the addition of 30 mM sucrose to the media with pH 6.0 (final osmolarity of 330 mOsm), and by reduction of the KCl content to 55 mM (final osmolarity, 280 mOsm) of media with pH = 8.0. All other solutions and media had osmolar concentrations between 295 and 305 mOsm. Following the 20-min preincubation at different pH (6, 7, 8), DIDS and methazolamide were added to a final concentration of 125 and 400 μM, respectively, and the cells were incubated for another 30 min. The cells were then spun down, washed once with their corresponding incubation media at room temperature and kept as packed cells (80% hematocrit) ready for use. Portions of each of these cells were washed 3 times with CHWS osmotically and pH adjusted as the incubation media described above, and resuspended to a 50% suspension for the determinations of hemoglobin, hematocrit and cellular Na^+ and K^+ concentrations. Water content of cells was measured from the dry-to-wet weight ratio.

The actual pH_i was determined by two different methods. Firstly, DIDS- and methazolamide-treated cells were packed at about 90% hematocrit and washed two times with 5 volumes of unbuffered media at 4°C. Tightly packed red cells were then lysed with the same volume of double-distilled water and the pH of the suspension determined. Secondly, due to the fact that in red cells $[H^+]_i/[H^+]_o = [Cl^-]_o/[Cl^-]_i$, pH_i can be estimated by measuring the chloride distribution ratio $[Cl^-]_i/[Cl^-]_o$. In this procedure the cells were pH equilibrated for 20 min after which ^{36}Cl (0.062 μCi/ml) was added and allowed to equilibrate for 30 min at 37°C. (No DIDS or methazolamide was added.) After equilibration $[Cl^-]_i/[Cl^-]_o$ was determined as described by Freedman and Hoffman (1979) and the pH_i estimated using the following equation:

$$pH_i = pH_o + \log[Cl^-]_i/[Cl^-]_o. \quad (1)$$

Since in the red cells the membrane potential (*Em*) is close to the Nernst potential for chloride (Lassen, 1977), *Em* was estimated from the chloride distribution ratio:

$$Em = RT/ZF \ln[Cl^-]_i/[Cl^-]_o. \quad (2)$$

Flux Media

The flux media used in the experiments (unless otherwise indicated) contained (mM): 75 NaCl, 75 KCl, 0.15 $MgCl_2$, 0.1 ouabain, 0.1 phloretin, 0.4 methazolamide, 0.01 bumetanide, 10 glucose and 10 Tris-MES/MOPS (pH 6.0, 7.0 and 8.0 at 37°C). These media were also osmotically adjusted so that cells with pH_i = 6.0, 7.0 and 8.0 were incubated, as indicated above, in hypertonic (330 mOsm), isotonic (300 mOsm) and hypotonic (280 mOsm) media, respectively, at different pH_o (6.0, 7.0 and 8.0).

In the H^+ efflux experiments K^+ and Na^+ media were used. The K media contained (mM): 75 KCl, 75 choline chloride, 0.15 $MgCl_2$, 0.1 ouabain, 0.01 bumetanide, 0.4 methazolamide, 0.1 phloretin, and 10 glucose. In the Na media, NaCl replaced choline chloride. To both media 0.25 mM Tris base was added to correct for the nonlinearity of the buffering capacity. The osmolarity of this media was adjusted to 330 by the addition of sucrose.

The cation composition of these media originated from the need to prevent changes in the volume and major ionic composition of the cells in the simultaneous presence of Ca^{2+} and ionophore A23187 (Lew & Brown, 1979). Ca^{++} and ionophore

were used in other aspects of this research (Escobales & Canessa, 1985*b*).

Amiloride was added to the media as required from a 1 M stock prepared in DMSO.

Measurement of Na Influx

Red blood cells were suspended to 6% hematocrit in 7 ml of the media described earlier (Flux Media section) containing ^{22}Na (1 μCi/ml). The suspension was then distributed in six 4-ml plastic tubes (Saarstedt Inc., Princeton, N.J.) in one-ml portions. This procedure was carried out at 4°C and usually took about 5 min. ^{22}Na influx was started by warming up the cell suspension at 37°C and was linear with time for at least 25 min. Therefore, at 5 and 25 min triplicate samples were taken out briefly placed in ice-cold water and centrifuged for 2 min at 4°C. The supernatant was discarded and the cells were washed three times with 75 mM KCl, 75 mM NaCl washing solution. The cell pellet was then hemolyzed with 0.02% Acationox and aliquots were removed for radioactivity and hemoglobin determinations. Fluxes were calculated from the slope of the regression line of Na^+ concentration in cells as a function of time and were expressed as mmol/liter cell · hr.

Preparation of Cells for Net Na^+ Transport

To determine net Na^+ movements, unidirectional ^{22}Na efflux and influx was measured in cells loaded to contain 15 mmol/liter cell water of internal Na^+.

Washed red cells were incubated (at 15% hematocrit) with Nystatin (40 μg/ml, Sigma Chemical Co.) for 20 min at 4°C in a medium containing (mM): 135 KCl, 15 NaCl, 40 sucrose, 10 Tris-MOPS (pH 7.4) with frequent shaking. After 20 min the cells were spun down and about 85% of the supernatant discarded. The sample was then split into two portions, one for Na efflux and the other for Na influx. To the cell sample used for the study of Na efflux, ^{22}Na (1 μCi/ml) was added and both cell samples were then incubated for an additional 20-min period at 4°C. Following this period of time the cell samples were transferred to a water bath at 37°C for 10 min after which the cells were washed five times with 5 volumes of a solution containing (mM): 135 KCl, 15 NaCl, 40 sucrose, 0.1 ouabain, 10 Tris MOPS (pH 7.4) 10 glucose 1 K-phosphate and albumin (1 mg/ml), at 37°C. The cells were then washed three times with CHWS (pH 7.4 at 4°C) and resuspended to 50% suspension for determinations of hemoglobin, hematocrit, Na^+, K^+ activity. Following this the cells were packed to about 80% hematocrit and resuspended to 5% hematocrit for acid loading (*see* pH_i manipulation).

Measurement of Net Transport

Na Efflux

Acid ^{22}Na-loaded cells were suspended at 4°C in 7 ml of the media described under the Flux Media section, except that NaCl and KCl were used at 15 and 130 mM, respectively. Following distribution of the cell suspension into 5-ml plastic tubes, Na efflux was started by warming up the cell suspension to 37°C. Since ^{22}Na efflux was linear with time for at least 25 min, at 5 and 25 min triplicate samples were taken out and centrifuged for 2 min at 4°C. Then the radioactivity in the supernatant was counted and the cell pellet was discarded. Na efflux was estimated from the appearance of ^{22}Na in the efflux media as a function of time and was expressed as mmol/liter cell · hr.

Na Influx

^{22}Na influx was determined in acid-loaded cells with 15 mmol/liter cell water of internal Na. The medium used was similar to that described under the Na Efflux section and the procedure used was described earlier (section on Measurement of Na Influx). Net Na transport was estimated from the difference between Na influx and efflux.

Determination of H^+ Efflux

Proton extrusion rates were measured as ΔpH_o. Acid-loaded cells (pH_i = 6.4) were washed three times at room temperature with 5 volumes of an unbuffered solution containing (mM): 75 KCl, 75 choline chloride, 45 sucrose, 0.15 $MgCl_2$, 0.1 ouabain and 10 glucose. Two milliliters of tightly packed cells were added to 8 ml of flux media (described earlier) in a water-jacketed chamber at 37°C under constant stirring, and allowed to equilibrate for 5 min. The pH was monitored with an Orion Research 611 Ionalyzer (Cambridge, Mass.) coupled to a chart recording system. After equilibration the pH_o was adjusted to pH 8.0 by addition of 0.1 N KOH, and pH_o was recorded for about 3 min. At the end of the experiment an aliquot of the suspension was taken and the hematocrit determined. The buffering capacity of the medium was determined by titrations with KOH or HCl. Acid extrusion rates were calculated from the slope of the curves (ΔpH_o/min), the buffering capacity of the medium, and the hematocrit of the suspension, and were expressed as mmol/liter cell · hr. In some experiments H^+ efflux was measured at constant pH_o by using a Radiometer pH meter (Type TTTI) coupled to a pH-stat titrator ABU 11 (Copenhagen, Denmark).

Cation Measurements

Cellular Na^+ and K^+ concentrations were measured by atomic absorption spectroscopy (Perkin Elmer, model 5000) with suitable standards prepared with double-distilled water. The cation content of cells was expressed per liter of original volume, as determined by relating the optical density (at 540 nm) of a sample (cell lysate) to that of a known volume of red cells.

Results

Characteristics of pH_i-Manipulated Cells

Table 1 shows some characteristics of pH_i-manipulated red cells. As can be observed following pH equilibration at pH_o = 6.0, 7.0 and 8.0, pH_i measurements in cell lysates from DIDS-treated red cells were 6.42 ± 0.04, 7.06 ± 0.07 and 7.80 ± 0.01,

Table 1. Characteristics of pH_i-manipulated red cells

Characteristics	pH of the loading medium		
	6.0	7.0	8.0
A) Internal pH_A	6.42 ± 0.04	7.06 ± 0.07	7.8 ± 0.01
Internal pH_B	6.11 ± 0.005	6.98 ± 0.01	7.8 ± 0.01
B) Cell Na^+	10.6 ± 1.24	11.00 ± 1.25	13.9 ± 2.9
Cell K^+ (mmol/liter cell)	94.6 ± 1.16	100.7 ± 0.76	94.8 ± 2.7
C) $[Cl^-]_i/[Cl^-]_o$	1.28 ± 0.02	0.96 ± 0.02	0.64 ± 0.01
D) Membrane potential (*mV*)	+6.7 ± 0.5	−1.06 ± 0.7	−12.0 ± 0.5
E) Water content (wt/wt)	0.65 ± 0.03	0.65 ± 0.005	0.65 ± 0.006

[a] The values are the means ± SE of three different experiments in three subjects. pH_i was measured by using cell lysates (pH_a) whereas pH_b represent pH_i calculated from the chloride distribution ratio ($pH_i = pH_o + \log[Cl^-]_i/[Cl^-]_o$). *Em* = membrane potential.

Table 2. Effect of pH gradients on Na^+ entry in human red cells

Conditions		pH of flux media		
		6.0	7.0	8.0
		(Na influx, mmol/liter cell · hr)		
pH_i 6.42	None	1.13 + 0.12	2.46 ± 0.21	5.50 ± 0.44
	Amil	0.98 ± 0.11	1.60 ± 0.11	2.86 ± 0.23
	As	0.15 ± 0.16	0.86 ± 0.23	2.64 ± 0.49
7.01	None	0.60 ± 0.10	0.90 ± 0.17	1.86 ± 0.25
	Amil	0.57 ± 0.10	0.90 ± 0.17	1.36 ± 0.25
	As	0.03 ± 0.14	—	0.50 ± 0.35
7.84	None	1.52 ± 0.59	1.51 ± 0.63	1.59 ± 0.46
	Amil	1.42 ± 0.60	1.49 ± 0.58	1.68 ± 0.50
	As	0.10 ± 0.84	0.02 ± 0.85	—

[a] The values are the means ± SE of three different experiments in three subjects. Na influx (Na_o^+ = 75 mM) was determined as described in Materials and Methods in the presence of (mM): 0.01 bumetanide, 0.1 phloretin and 0.4 methazolamide. Amiloride (Amil) was added at a final concentration of 1 mM. As = amiloride-sensitive fraction.

respectively. Similar values were obtained when pH_i was calculated from estimation of chloride distribution ratio ($pH_i = \log [Cl^-]_i/[Cl^-]_o + pH_o$) measured prior to the addition of DIDS. As expected, changes in the charge of impermeant anions (mainly hemoglobin) induced by acid and alkali titrations lead to alterations in the chloride distribution ratios (*see* Hladky & Rink, 1977). Thus, the chloride ratio increased, from control values of 0.71 ± 0.01 to 0.96 ± 0.02 and 1.28 ± 0.02 when pH_i was decreased from 7.2 ± 0.01 to 7.06 ± 0.07 and 6.42 ± 0.04, respectively, and decreased to 0.64 ± 0.01 when pH_i was increased to 7.80 ± 0.1. Since in DIDS-treated human red cells the conductive Cl^- permeability is still higher than that of Na^+ and K^+ (Knauf et al., 1977), the chloride distribution across the membrane can be used to estimate *Em* as calculated from the Nernst equation. Thus, *Em* varied from approximately +6.7 ± 9.5 to −12.0 ± 0.5 mV when pH_i was increased from 6.42 ± 0.06 to 7.84 ± 0.01, respectively. Under the experimental conditions employed, cellular Na^+, K^+, and water content were not significantly affected by the pH_i alteration.

It is relevant to point out that pH equilibration is remarkably slow in red cells which are DIDS- and methazolamide-treated. For instance, pH_i increased 0.2 unit in acid-loaded cells (pH_i = 6.42) after 25 min of incubation in media with pH = 8.0 at 37°C. The data thus suggest that $[Cl^-]_i/[Cl^-]_o$ ratios and hence *Em* were relatively constant during the time course of the experiments.

Effect of pH Gradients on Na Influx

The effect of pH gradients on the ouabain- and bumetanide-resistant Na^+ uptake in red cells is illustrated in Table 2. Na^+ entry into acid-loaded cells (pH_i = 6.4) markedly increased (from 1.13 ± 0.13 to 5.50 ± 0.44 mmol/liter cell · hr) when pH_o was raised from 6.0 to 8.0. Moreover, at constant pH_o (8.0), the stimulation of Na^+ uptake was abolished as pH_i was increased from 6.42 to 7.84. Na^+ influx was not significantly affected in the absence of pH gradients. These results are thus compatible with a Na^+/H^+ exchange on these membranes.

The data in Table 2 also show that Na^+ transport was not a linear function of H^+ gradients. Thus, at pH_o = 8, Na^+ entry was not affected by a pH_i decrease from 7.84 to 7.01 (1.59 ± 0.46 *vs.* 1.86 ± 0.25 mmol/liter cell · hr), but was markedly in-

Table 3. Effect of an outward H^+ gradient on amiloride-sensitive Na^+ entry

Conditions		Na^+ Transport		
		Influx	Efflux (mmol/liter cells · hr)	Net
$pH_o = 8.0$	None	2.18 ± 0.03	1.32 ± 0.02	0.86 ± 0.04
	Amiloride	1.06 ± 0.01	0.89 ± 0.02	0.17 ± 0.02
$pH_o = 6.0$	None	0.43 ± 0.01	0.50 ± 0.01	−0.07 ± 0.01
	Amiloride	0.34 ± 0.01	0.42 ± 0.01	−0.08 ± 0.01

[a] The values shown are the means ± SE of three determinations. Na^+ influx and efflux was measured as described in Materials and Methods. pH_i was within 6.10 and 6.42, Em = +6.7 mV, $Na_o^+ = Na_i^+ = 15$ mM. The negative sign in the net flux column indicates a net Na^+ efflux.

creased when pH_i was further decreased to 6.42 (from 1.36 ± 0.25 to 5.50 ± 0.44 mmol/liter cell · hr). Moreover, Na^+ influx is higher at $pH_i = 6.4$ and $pH_o = 7$ than at $pH_i = 7$ and $pH_o = 8$, even though the proton gradient is steeper in the latter case.

The possibility that a Na^+/H^+ exchange was responsible for the pH-induced Na^+ uptake was further strengthened by the effect of amiloride on the H^+-induced Na^+ uptake. As shown in Table 2, amiloride (1 mM) inhibited about 50% of the H^+-induced-Na^+ entry (from 5.50 ± 0.44 to 2.86 ± 0.23 mmol/liter cell · hr). Similar effects were found with 5-N-methyl-N-butyl-amiloride (20 μM), a potent amiloride analog. The drugs, however, only slightly affected Na^+ transport in the absence of a H^+ gradient. In addition, the pH-dependent and amiloride-sensitive Na^+ uptake (about 2.64 ± 0.49 mmol/liter cell · hr) was abolished, at constant pH_o (8.0), when pH_i was increased from 6.42 to 7.84. As can be observed in Table 2, an amiloride-insensitive Na^+ entry was also stimulated by outward pH gradients (from 0.98 ± 0.11 to 2.86 ± 0.23 mmol/liter cell · hr). However, that the effect of H^+ was mainly on the amiloride-sensitive Na uptake was suggested by the 18-fold stimulation of this fraction of Na entry versus the threefold enhancement of amiloride-insensitive Na transport. Since amiloride has been shown to inhibit Na^+/H^+ exchange in other membranes (Rindler & Saier, 1981; Benos, 1982; Grinstein et al., 1983) the results strongly support the presence of a Na^+/H^+ exchanger in human red cells.

Net Na^+ Movement Driven by an Outward H^+ Gradient

If Na^+ and H^+ movements are coupled in a Na^+/H^+ exchange mechanism, the outward movement of H^+ should drive Na^+ against its electrochemical gradient. The experiment in Table 3 illustrates this property of H^+-induced Na^+ entry. Cells preloaded by the nystatin method to contain 15 mM Na^+ were acid-loaded ($pH_i = 6.42$). Na influx and efflux were measured at a pH_o of 6.0 and 8.0. In this case, however, there was no concentration gradient of Na^+ since $Na_o^+ = 15$ mM. Since acid-loaded cells, have an Em of +6.7 mV (inside positive, Table 1) a net outward Na^+ movement should occur in the virtual absence of a H^+ gradient ($pH_i = 6.4$, $pH_o = 6.0$). On the contrary, in the presence of an outward H^+ gradient ($pH_i = 6.4$, $pH_o = 8.0$) a net amiloride-sensitive Na^+ influx should take place. As depicted in Table 3, all these predictions were satisfied. Thus in the absence of a H^+ gradient a small amiloride-insensitive net Na^+ efflux took place (about 0.07 ± 0.01 mmol/liter cell · hr). However, a net Na^+ entry (about 0.86 ± 0.04 mmol/liter cell · hr) was observed in the presence of a pH gradient. Moreover, the H^+-induced net Na^+ influx was amiloride sensitive. Therefore, the data is consistent with a coupled Na^+/H^+ exchange process in red cell membranes.

Table 3 also shows that Na^+ efflux ($pH_i = 6.42$) was stimulated when pH_o was raised from 6.0 to 8.0 (from 0.50 ± 0.01 to 1.32 ± 0.02 mmol/liter cell · hr). In addition, such effect of H^+ on Na^+ efflux was also sensitive to amiloride (from 1.32 ± 0.01 to 0.89 ± 0.02 mmol/liter cell · hr).

Net H^+ Movements Driven by an Inward Na^+ Gradient

The above studies have described the effect of an outward pH gradient on Na transport. If the stimulation of Na influx takes places through a Na^+/H^+ exchange then the imposition of an inward Na^+ gradient should stimulate H^+ efflux. To determine this, we measured H^+ efflux from acid-loaded cells ($pH_i = 6.42$) into a medium lightly buffered with 0.25 mM Tris base. Thus, cells were added to the medium and pH_o measured. Under these conditions pH_o

Table 4. Effect of an inward Na^+ gradient on H^+ efflux into a lightly buffered medium

Conditions	H^+ efflux	
	None	N-methyl-N-butyl amiloride (60 μM)
	(mmol/liter cell · hr)	
K^+ media	7.58 ± 0.62	8.05 ± 0.90
Na^+ media	10.87 ± 1.6	9.04 ± 0.44
Na^+-stimulated	3.29 ± 0.91	0.99 ± 0.44

[a] The values shown are the means ± SE of three experiments in different subjects. DIDS (125 μM) and methazolamide (0.4 mM)-treated cells were incubated (20% hematocrit at 37°C in media only buffered with 0.25 mM Tris-base. Na_i^+ = 10 mM; Na_o^+ or K_o^+ = 75 mM.

slowly equilibrated to about 6.3 (initial $pH_o \simeq 6.2$) where the relation $[Cl^-]_i/[Cl^-]_o = [H^+]_o/[H^+]_i$ is satisfied. In order to measure H^+ efflux under similar conditions in which Na^+ transport was measured (pH_i = 6.4, pH_o = 8.0), the pH_o was brought to 8.0 by the addition of 0.1 N KOH. Following, there was a slight but constant acidification (assumed to represent a net H^+ efflux) of the medium which was allowed to proceed for about 3 min. To determine the effect of Na^+ on H^+ efflux, the rate of acidification of the medium was determined with K^+ or Na^+ (75 mM) as the main cation. Table 4 shows that the substitution of K^+ or Na^+ stimulated a net H^+ efflux from 7.58 ± 0.62 to 10.87 ± 1.60 mmol/liter cell · hr. Thus, the imposition of a Na^+ gradient (Na_o^+ = 75 mM, Na_i^+ = 15 mM) stimulated a net H^+ efflux of about 3.29 ± 0.91 mmol/liter cell · hr. Similar results were found under conditions in which H^+ efflux was measured at constant pH_o (8.0) by using a pH titrator.

When the effect of amiloride (1 mM) on the Na^+-induced H^+ efflux was studied it was found to stimulate the basal H^+ efflux while only marginally inhibiting the Na^+-induced H^+ transport. Such effect of amiloride (1 mM) is probably due to cell lysis that blurs its effect on H^+ transport by releasing H_i^+. This side effect was overcome by the use of 5-N-methyl-N-butyl-amiloride (60 μM). The amiloride analog was found to be about 50 times more potent than the parent compound in inhibiting the H^+-induced Na^+ influx (*data not shown*). 5-N-methyl-N-butyl-amiloride inhibited the Na^+-stimulated H^+ efflux from 3.29 ± 0.91 to 0.98 ± 0.44 mmol/liter cell · hr (Table 4), thus yielding an amiloride-sensitive fraction of about 2.30 ± 0.88 mmol/liter cell · hr. In two of four subjects studied a full inhibition of the Na^+ efflux was observed.

Discussion

In this study we have presented evidence compatible with the presence of a Na/H exchange in human red cells: 1) pH gradients (inside acid) stimulated Na^+ uptake; 2) inward Na^+ gradients stimulated H^+ efflux; 3) in the absence of chemical gradients for Na^+ an outward H^+ gradient stimulated a net Na^+ uptake against an electrical potential, and 4) both the H^+-induced Na^+ influx and the Na^+-induced H^+ efflux were sensitive to amiloride or its high-affinity analog: 5-N-methyl-N-butyl amiloride. Our data is thus consistent with preliminary findings by Dissing and Hoffman (1982), and Milanick, Dissing & Hoffman (1985) indicating the existence of H^+-induced Na^+ efflux in DIDS-treated and SO_4^{2-}-loaded human red cells. These findings, together with the results presented here would appear to indicate that as in other preparations the Na^+/H^+ exchanger present in human red cells is reversible. Although the effect of membrane potential on the H^+-induced Na^+ entry was not evaluated, some observations suggest that the process is not significantly affected by an electrical potential. Thus, Na^+ uptake was stimulated in acid-loaded cells which had an inside-positive membrane potential (+6.7 mV) but it was not significantly affected in alkaline cells with a negative membrane potential (−12 mV). Therefore, the data is consistent with the idea that the H^+-stimulated Na^+ transport represents an electroneutral transport system.

If the H^+-induced Na^+ entry is indeed an electroneutral process the stoichiometry of the H^+-induced Na^+ entry to the Na^+-stimulated H^+ efflux should be 1 : 1, 2 : 2 etc. As indicated in Table 2, in acid-loaded cells (pH_i = 6.4) the increase in pH_o stimulated the entry of Na^+ by about 4.4 mmol/liter cell · hr, of which 2.64 mmol/liter cell · hr were amiloride sensitive. Under similar conditions Na^+ stimulated a H^+ efflux of 3.29 mmol/liter cell · hr (Table 4) of which 2.3 mmol/liter cell · hr were sensitive to 5-N-methyl-N-butyl-amiloride. The close similarity of both total and amiloride-sensitive Na^+ and H^+ transport rates support the notion that the pH-induced Na^+ entry occurs as a Na/H exchange with a stoichiometry not different from 1 : 1. Similar results were found when the stoichiometry was estimated from H^+-induced net Na entry ($[Na^+]_o$ = 75 mM) and Na^+-induced net H^+ efflux (*data not shown*).

The finding that amiloride or its high-affinity analog inhibited both the H^+-dependent Na^+ influx and the Na^+-activated H^+ efflux is compatible with the presence of a Na^+/H^+ exchange system in red cell membranes. The mechanism of amiloride inhibition of Na^+ uptake has been postulated to be due to a collapse of the pH gradient since amiloride is a

weak base (Dubinsky & Frizzell, 1982). The possibility is unlikely since under our experimental conditions the pH clamp was not affected by amiloride.

Since the effect of H^+ on Na^+ influx and that of Na^+ on H^+ efflux were observed in the presence of 0.1 mM phloretin, a well-known inhibitor of Na^+/Na^+ or Na^+/Li^+ exchange in red cells (Pandey et al., 1978), our results suggest that the Na^+/H^+ exchange activity takes place through a transport molecule different from that of the Na^+/Li^+ exchanger. The fact that the Na^+/Na^+ exchange is insensitive to amiloride (Pandey et al., 1978) is consistent with such interpretation. However, a definite conclusion in this regard should await experiments on the effects of pH gradients on the activity of the Na^+/Na^+ exchanger under our experimental conditions.

As previously stated in the introduction, evidence obtained in our laboratory indicates that an increase in Ca_i^{2+} by using A23187 activates an amiloride-sensitive Na^+ entry pathway. Since the role of H^+ on the activation of amiloride-sensitive Na^+ transport can be accounted for by the presence of a Na^+/H^+ antiport, it is conceivable that in human erythrocytes the effect of Ca^{2+} on Na^+ transport represents the activation of an otherwise silent Na^+/H^+ exchange system. One possible mechanism by which the increase in Ca^{2+} could result in an activation of Na^+/H^+ exchange is through an increase in the $[H^+]_i/[H^+]_o$ ratio. The data presented here are consistent with this mechanism. However, under our experimental conditions, the effects of Ca^{2+} loading on pH_i were negligible and in the opposite direction required to activate the Na^+/H^+ exchange (*unpublished observations*). Therefore, the data would appear to indicate that at least two different routes for activation of Na^+/H^+ exchange exist in red cells: namely a Ca^{2+}-dependent one and another which is sensitive to changes in the $[H^+]_i/[H^+]_o$ ratio. It is possible that Ca^{2+} modulates the affinities of the transport molecule for H^+ and/or Na^+, resulting in activation of the exchanger in the virtual absence of pH gradients. Therefore, although both signals (Ca^{2+} and H^+) would result in enhanced rates of Na^+/H^+ exchange, that mediated by Ca^{2+} would promote cytosolic alkalinization whereas that mediated by H^+ would only be compensatory to acid shifts.

Although the present work did not attempt to evaluate the kinetic properties of the Na^+/H^+ antiport in human red cells, our data also suggest that in addition to the thermodynamic effects of H^+ gradients on Na^+ transport, kinetic effects (competitive or allosteric) are also present. As shown in Table 2, Na^+ influx was higher at pH_i/pH_o = 6.4/7.0 than at pH_i/pH_o = 7.0/8.0 even though the gradient is steeper in the latter case. In addition, at pH_o = 8.0, Na^+ entry was not affected when pH_i was decreased from 7.84 to 7.0 but was markedly stimulated when pH_i was further reduced from 7.0 to 6.4. These findings would appear to indicate that H^+ interacts at both transport and activator sites as found by Aronson et al. (1982) and Grinstein et al. (1984) in renal microvillus membrane vesicles and rat thymic lymphocytes, respectively. However, additional experiments are needed to settle this important point.

The stimulation of amiloride-sensitive Na^+ efflux by an outward H^+ gradient is also consistent with kinetic effects of H^+ on Na^+ transport (i.e. competitive inhibition). However, it may also represent the activation of amiloride-sensitive Na^+/H^+ or Na^+/Na^+ exchange by H_i^+ as reported by Aronson et al. (1982) in renal microvillus membrane vesicles. Further studies are required to establish the mechanism(s) by which this effect may be produced.

Consistent with Na^+/H^+ antiporters in other preparations (Villereal, 1981; Grinstein et al., 1984; Moolenaar, Tertoolen & de Laat, 1984) the exchanger present in red cells is minimally active under basal conditions (Escobales & Canessa, 1984*a,b*) or in the absence of pH gradients (present paper). Since in human red cells the operation of the Jacobs-Stewart cycle and the anion exchanger essentially clamp pH_i to $pH_o + \log[Cl^-]_i/[Cl^-]_o$, a physiological role of this transport system is questionable. Similarly, a role of the exchanger in volume regulation is unlikely since red cell volume decrease does not activate amiloride-sensitive Na^+ transport (*data not shown*). The finding that even at normal pH_i serum and growth factors rapidly activate Na^+/H^+ exchange (Moolenaar et al., 1983; Paris & Pouyssegur, 1984), raises the possibility that the exchanger present in human red cells may be regulated by substances present in human serum. Since in human red cells pH_i is essentially constant, such an activation would likely produce changes in the activity of Na^+-dependent transport systems (i.e. Na^+/K^+ pump) through changes in the internal Na^+ concentration. Indeed, the stimulation of Na^+/H^+ exchange by serum and growth factors results in an increased rate of ouabain-sensitive K^+ or Rb influx (Rozengurt, 1981). However, since changes in pH_i have been correlated with the turning on and off of the cell activities (Nuccitelli & Heiple, 1982) it seems most likely that the Na^+/H^+ exchange in red cells may represent a "vestigial" transport system that was left over from the processes of cellular differentiation and growth in the bone marrow.

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