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Kinetics and Stoichiometry of the Human Red Cell Na^+/H^+ Exchanger

Andrea Semplicini*, Anda Spalvins, and Mitzy Canessa
Endocrine-Hypertension Department, Brigham and Women's Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts

Summary. We have investigated the kinetic properties of the human red blood cell Na^+/H^+ exchanger to provide a tool to study the role of genetic, hormonal and environmental factors in its expression as well as its functional properties in several clinical conditions. The present study reports its stoichiometry and the kinetic effects of internal H^+ (H_i) and external Na^+ (Na_o) in red blood cells of normal subjects.

Red blood cells with different cell Na^+ (Na_i) and pH (pH_i) were prepared by nystatin and DIDS treatment of acid-loaded cells. Unidirectional and net Na^+ influx were measured by varying pH_i (from 5.7 to 7.4), external pH (pH_o), Na_i and Na_o and by incubating the cells in media containing ouabain, bumetanide and methazolamide. Net Na^+ influx ($Na_i < 2.0$ mmol/liter cell, Na_o = 150 mM) increased sigmoidally (Hill coefficient 2.5) when pH_i fell below 7.0 and the external pH_o was 8.0, but increased linearly at pH_o 6.0. The net Na^+ influx driven by an outward H^+ gradient was estimated from the difference of Na^+ influx at the two pH_o levels (pH_o 8 and pH_o 6). The H^+-driven Na^+ influx reached saturation between pH_i 5.9 and 6.1. The V_{max} had a wide interindividual variation (6 to 63 mmol/liter cell · hr, 31.0 ± 3, mean ± SEM, n = 20). The K_m for H_i to activate H^+-driven Na^+ influx was 347 ± 30 nM (n = 7). Amiloride (1 mM) or DMA (20 μM) partially (59 ± 10%) inhibited red cell Na^+/H^+ exchange. The stoichiometric ratio between H^+-driven Na^+ influx and Na^+-driven H^+ efflux was 1 : 1. The dependence of Na^+ influx from Na_o was studied at pH_i 6.0, and Na_i lower than 2 mmol/liter cell at pH_o 6.0 and 8.0. The mean K_m for Na_o of the H^+-gradient-driven Na^+ influx was 55 ± 7 mM.

An increase in Na_i from 2 to 20 mmol/liter cell did not change significantly H^+-driven net Na^+ influx as estimated from the difference between unidirectional ^{22}Na influx and efflux. Na^+/Na^+ exchange was negligible in acid-loaded, DIDS-treated cells. Na^+ and H^+ efflux from acid-loaded cells were inhibited by amiloride analogs in the absence of external Na^+ indicating that they may represent nonspecific effects of these compounds and/or uncoupled transport modes of the Na^+/H^+ exchanger.

It is concluded that human red cell Na^+/H^+ exchange performs 1 : 1 exchange of external Na^+ for internal protons, which is partially amiloride sensitive. Its kinetic dependence from internal H^+ and external Na^+ is similar to other cells, but it displays a larger variability in the V_{max} between individuals.

* *Present address:* Clinica Medica I, University of Padova, Padova, Italy.

Key Words human erythrocytes · transport modes · kinetics · Na^+/H^+ exchange

Introduction

Na^+/H^+ exchange is a transport system widely distributed in eukaryotic cells and implicated in many cellular functions such as control of internal pH and cell volume, response to mitogens, growth factors and hormones and Na^+ reabsorption by the kidney [*see* 2, 18, 21, 26 for reviews]. Recently, evidence has been provided for the presence of Na^+/H^+ exchange in the human red cell [10, 11]. It was shown that elevation of cytosolic calcium [10] and the imposition of an outward H^+ gradient in acid cells [11] stimulated an amiloride-sensitive Na^+ influx. The expression of this transporter in the mature anucleated red cell might be a remnant of its activity in the precursor stem cell, which at later stages of differentiation acquires hemoglobin and the anion exchanger [19] to transport H^+ from the tissues to the lungs.

The easy access to human red cells makes them a valuable tool for studying the functional properties of the Na^+/H^+ exchange in a variety of pathophysiological conditions. Therefore, the present study was designed to investigate the kinetics, stoichiometry and transport modes of the human red cell Na^+/H^+ exchanger. We report here the K_m[1] for internal and external H^+ and Na^+ and the maximal

[1] *Abbreviations:* DIDS: 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid; DMA: dimethyl-amiloride; DMSO: dimethyl-sulfoxide; Hb: hemoglobin; Hct: hematocrit; K_m: concentration for half-maximal velocity; MES: 2-[N-morpholino] ethanesulfonic acid; MOPS: 3-[N-morpholino] propanesulphonic acid; Tris: tris (hydroxymethyl) aminomethane; V_{max}: maximal rate of transportation. The subscript i is used for intracellular and o for extracellular conditions.

rate (V_{max}) of translocation. The stoichiometric ratio between external Na^+-driven H^+ efflux and H^+-gradient-driven Na^+ influx is 1:1, but not all the Na^+/H^+ activity is inhibited by amiloride and dimethyl-amiloride. Additional modes of operation of this transporter appear to be uncoupled Na^+ and H^+ efflux and a negligible Na^+/Na^+ exchange in the presence of the anion exchanger inhibitor DIDS. The study of the kinetic properties of this transporter will be useful in human clinical, genetical and epidemiological research.

Materials and Methods

All the experiments were performed in red cells obtained from 20 normotensive Caucasian donors (10 males, 10 females, 22–50 years old, mean age 34).

I. Red Cell Preparation

The blood was drawn into heparinized tubes and centrifuged at room temperature at 1850 × *g* for 10 min. Plasma and buffy coat were removed by aspiration and the red cells washed four times with cold washing solution containing (in mM): choline chloride 149, $MgCl_2$ 1, Tris MOPS 10, pH 7.4, 4°C (CWS). A 50% cell suspension was made in CWS and hematocrit (Hct) was measured after centrifuging capillary tubes in a microhematocrit (Autocrit Ultra 3) for 10 min. A 50-μl sample of the cell suspension in CWS was lysed (1/50) with Acationox detergent (0.02% in double distilled water) for hemoglobin (Hb) (1/500 dilution) and Na^+ (1/50 dilution) determination. The initial cell volume was estimated by measuring the Hct and the optical density of Hb at 540 nm in a Perkin Elmer Lambda 3 A spectrophotometer.

The Na^+ concentration of the cell lysate was measured with a Perkin Elmer 3030 B atomic absorption spectrophotometer using appropriate standards. The cell Na^+ content (mmol/liter cell) was determined by dividing the Na^+ concentration of the lysate by the Hct of the cell suspension.

A. Modification of Cell Na^+ Content

The nystatin method was used to bring cell Na_i^+ to the desired concentration as previously described [8]. One ml of washed packed red cells were slowly added, while mixing, to 5 ml cold loading solution (NLS) containing (in mM): (NaCl + KCl) 140, sucrose 50, and 45 μl of nystatin solution (5 mg in 1.3 ml of DMSO) to achieve a final concentration of 35–40 μg/ml. The Na^+ to K^+ ratio was varied from 0:140 to 80:60 to achieve a final cell Na^+ content between 1.5 to 70 mmol/liter cell. The nystatin powder had been weighed in 5-ml tubes, kept at 4°C, protected from light and DMSO added the day of the experiment. The cell suspension was kept at 4°C, in the dark, for 20 min, periodically vortexing. Subsequently, the cell suspension was centrifuged, the supernatant discarded, the loading solution (without nystatin) renewed and incubated for an additional 10 min in the cold.

For ^{22}Na loading, the cells were separated by centrifugation and incubated for an additional 20 min at 4°C in the dark in 0.6-ml loading solution containing 15 μCi/ml of ^{22}Na.

Nystatin was removed by adding 5 ml of warm (37°C) nystatin washing solution (NWS) and incubating the cell suspension for 10 min at 37°C [8]. NWS contained the same $Na^+ + K^+$ concentration of the loading solution: sucrose 50 mM, glucose 10, potassium phosphate buffer 1 (pH 7.40 at 37°C), and albumin 1 mg/ml. Nystatin removal was ensured by four more washes with NWS.

B. Modification of the Cell pH

In red cells, pH_i and pH_o rapidly equilibrate through the actions of carbonic anhydrase and the Cl^-/HCO_3^- exchange system [19]. The cell pH of fresh and nystatin-treated cells was modified by incubating the cells at 10% Hct in acid-loading solutions (ALS) for 10 min in a shaking water bath at 37°C (Table 1). The hypertonic medium (around 360 mOsm) avoided cell swelling induced by chloride redistribution in acid medium and the mean increase in red cell volume should not be greater than 5% of that of the original cells. All ALS contained (in mM): $MgCl_2$ 0.15, ouabain 0.1, bumetanide 0.01, glucose 10, and 20 Tris-MES for pH lower than 7.0 and Tris-MOPS for pH higher than 7.0. To prepare erythrocytes with cell pH 5.85, 6.2, 6.5, 6.7, 6.8 and 7.2, the ALS had pH 5.6, 5.8, 6.0, 6.2, 6.5, 7.0, respectively. Solutions with pH between 5.6 and 6.2 contained 170 mM KCl and those with pH over 6.5, 150 mM KCl. Solutions with pH below 6.5 also contained 40 mM sucrose and 360 mOsm; the osmolarity of the pH 6.5 and pH 7.0 solutions were 340 and 315 mOsm, respectively.

After 10 min incubation, the pH_i was clamped by addition of 1.0 ml of ALS containing 20 μl of 200 mM DIDS and 10 μl of 0.85 M methazolamide in DMSO to inhibit the anion exchanger and carbonic anhydrase, respectively. The final concentration of these drugs was 0.2 mM DIDS (1.75 mg/ml red blood cells) and 0.5 mM methazolamide. The cell suspensions were incubated in their respective ALS for an additional 30 min at 37°C.

Due to interindividual variability in cell loading and to ensure that the desired cell pH was achieved, the pH of the incubating media was measured prior to the addition of DIDS. If the pH of the cell suspension was too far from the desired pH, ALS could be replaced with fresh solution or the pH of media adjusted to the desired pH.

The acid-loaded cells (pH_i 5.9–6.9) were washed four times with five volumes of cold (4°C) unbuffered washing solution (AWS) containing (in mM) KCl 170, $MgCl_2$, 0.15, ouabain 0.1, glucose 10, sucrose 40, and kept cold until flux measurement. For cells with pH_i over 6.9, AWS was made isotonic by reducing KCl to 150 mM and eliminating sucrose.

The pH of the loaded cells was measured by lysing 0.2 ml of packed red cells in 2 ml of unbuffered Acationox (0.02%) and the pH of the lysate measured with a Radiometer pH meter at room temperature (within assay reproducibility was 2%). The cell pellet was kept on ice ready for flux measurements. An aliquot of the cell suspension (50% Hct) was used for the measurements of intracellular Na^+ and Hb as described in section IA.

II. Net Sodium Influx Into Acid-Loaded Cells

Na^+ influx was started by addition of packed acid-loaded cells (1% final Hct) to Na^+ media preincubated at 37°C in a shaking water bath. The Na^+ media contained (in mM): NaCl 0–150, choline-chloride 150–0, glucose 10, ouabain 0.1, bumetanide 0.01, methazolamide 0.5, sucrose 40, $MgCl_2$ 0.15 and either Tris

MOPS 10 (pH 8 at 37°C) or Tris MES 10 (pH 6 at 37°C). This procedure avoids significant dissipation of the H^+ gradient, which takes place even at 4°C using a previously described technique [11]. Similarly, to separate cells from the Na^+ incubation media, cell washing with cold solutions was avoided. At timed intervals, the transport reaction was stopped by pipetting triplicate aliquots (0.2 ml) of the warm cell suspension into previously chilled Eppendorff tubes (1.5 ml) containing 0.7 ml of cold Na^+-free solution layered over 0.4 ml dibutylphthalate (d = 1.04). The Na^+-free solution, used to dilute the Na^+ concentration of influx media and to reduce the trapping of Na^+ in the cell pellet, contained (in mM): choline Cl 80, KCl 80, $MgCl_2$ 0.25, sucrose 40, and Tris MOPS (pH 7.4 at 4°C). The tubes were immediately spun in a Fisher Scientific centrifuge at 12,000 × *g* for 15 sec; a longer time makes the subsequent lysing step more difficult. The supernatants were removed by aspiration, the tubes wiped to remove external Na^+ contamination and placed in plastic racks washed with distilled water. The bottom of the tubes were cut with a needle cutter Destruclip (Fisher, Fairlawn, NJ) into 3-ml plastic tubes containing 1 ml 0.02% Acationox to lyse the cells. The tubes were vortexed and kept overnight at 4°C. Hb concentration was measured by diluting (1/50) 50 μl of the cell lysate. The trapping of ^{22}Na was determined using cell-free solutions and 0 time samples and it was less than 1.0 μM Na^+ and 200 cpm/tube. The cell Na^+ content (mmol/liter cell) was computed according to the following equation:

$$\text{Cell } Na^+ \text{ (mmol/liter cell)} = \frac{Na_l \times OD_{cs}}{Hct_{cs} \times OD_l \times 1000} \quad (1)$$

where Na_l = Na^+ concentration (μM) of the lysate, OD_l = optical density of Hb from the lysate of the flux media sample, OD_{cs} = optical density of Hb from the lysate of the fresh cell suspension, and Hct_{cs} = Hct of the fresh cell suspension.

The slope of the regression line of cell Na^+ content *vs.* time was calculated with the least squares method [1] (*see* Fig. 1 as an example) and net Na^+ influx expressed in mmol/liter cell × hr. The coefficient of variation of the triplicate influx samples were 14% at pH_o 6.0 and 4% at pH_o 8.0, indicating that the Na^+ contamination of the tubes is negligible if appropriately manipulated.

III. Measurement of Unidirectional ^{22}Na Influx

The experimental design to measure the unidirectional Na^+ influx was the same as for the net Na^+ influx except that the acid-loaded cells were incubated in 3 ml of medium containing 3 μCi/ml of ^{22}Na at a final Hct of 10%. ^{22}Na in the lysate of the influx media was counted in a well-type LKB gammacounter. The cell Na^+ content was calculated according to

$$\text{Cell } Na^+ \text{ (mmol/liter cell)} = \frac{cpm_l \cdot OD_{cs}}{OD_l \cdot Hct_{cs} \cdot SA} \quad (2)$$

where cpm_l = cpm/liter of lysate of the influx sample, OD_l = optical density of the lysate of the influx media sample at 540 nm, OD_{cs} = optical density of the fresh cell lysate, Hct_{cs} = Hct of the fresh cell suspension, and SA = specific activity of the Na^+ influx media prior to flux measurement in cpm/mmol.

The unidirectional Na^+ influx (mmol/liter cell × hr) was computed from the slope of the regression line of cell Na^+ content *vs.* time with the least squares method.

IV. Measurement of ^{22}Na Efflux

^{22}Na efflux from cells loaded with different Na^+ and H^+ content was measured by incubating ^{22}Na-loaded cells at 2% Hct at 37°C in media containing (in mM) NaCl or KCl 150, glucose 10, sucrose 40, $MgCl_2$ 0.15, ouabain 0.1, bumetanide 0.01, and methazolamide 0.5. The pH of the efflux media was adjusted at 37°C with 10 mM Tris MOPS to pH 8.0 and with 10 mM Tris MES to pH 6.0. The efflux was stopped at timed intervals by layering 1-ml aliquots of the cell in triplicate samples over 0.7 ml cold dibutylphtalate oil in Eppendorff tubes, quickly spun in a Fisher Scientific centrifuge for 15 sec. The radioactivity of 0.8 ml of the supernatant was counted. The Na^+ content of the efflux medium was calculated according to

$$\text{Na}^+ \text{ content of the efflux media (mmol/liter cell)} = \frac{cpm_{sp}}{SA_{al} \times Hct_f} \quad (3)$$

where cpm_{sp} = radioactivity in the supernatant in cpm/liter, SA_{al} = specific activity of the acid-loaded cells prior to flux measurement in cpm/mmol of Na^+, and Hct_f = Hct of the efflux medium.

^{22}Na efflux was calculated from the slope of the regression line of the Na^+ content of the efflux media as a function of time with least squares method.

V. Measurement of H^+ Efflux

H^+ efflux was measured by titration of the flux media with KOH at constant pH_o 8 in a pH-stat apparatus (Brinkman 632). The unbuffered media (4 ml) was first equilibrated at 37°C in a water jacketed chamber. The media contained (in mM): either NaCl or KCl 150, glucose 10, sucrose 40, $MgCl_2$ 0.15, ouabain 0.1, bumetanide 0.01, methazolamide 0.5, and phloretin 0.1. Acid-loaded cells (0.3–0.4 ml of 50% cell suspension in AWS) were added to efflux media under constant stirring. The external pH was quickly (<30 sec) adjusted by the addition of 0.1 M KOH by the titrator to the set end-point (pH_o = 8.0) using the manual command. Thereafter, the pH stat was set up in the automatic mode and the amount of 0.1 M KOH (μl) added to keep the pH_o at the set end-point recorded for 10 min. Under these experimental conditions, the volume of KOH added is linear for 3–5 min and declines thereafter due to the dissipation of the outward H^+ gradient. A sample of efflux media cell suspension (50 μl) was diluted 1/50 in Acationox for Hb determination.

The initial rate of KOH solution added to the efflux media was calculated from the slope of the recording of volume of KOH as a function of time (μl/min) if it was linear for more than 5 min; when the recording was linear for a shorter time a nonlinear regression program was used to calculate IR from the whole recording period according to

$$IR = V (\ln t)^{-1} \quad (4)$$

where IR = initial rate of KOH addition (μl/min), V = volume of KOH (μl) added by time t, and t = elapsed time (min).

H^+ efflux was computed from the initial rate of KOH addition, i.e., of H^+ efflux, the concentration of the titrating solution and the Hct of the titrated cell suspension according to

$$H^+ \text{ efflux (mol/liter cell} \times \text{hr)} = \frac{IR \times 60 \times C \times OD_{cs}}{V_{cs} \times Hct_{cs} \times OD_f} \quad (5)$$

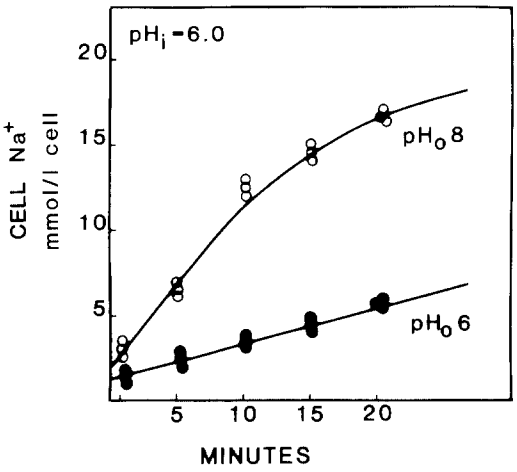

Fig. 1. Time course of Na^+ gain in Na^+-depleted (Na_i 0.4 mmol/liter cell), acid-loaded (pH_i 6.0) and DIDS-treated red cells. The influx media contained 150 mM NaCl with pH_o 6.0, (open dots) and pH_o 8.0, (filled dots). Net Na^+ influx, calculated from the initial (up to 10 min) linear rate of Na^+ gain, was 12.6 in acid medium and 62.3 mmol/liter cell × hr in alkaline medium. The difference between Na^+ influx at pH_o 8 and pH_o 6 was 49.7 mmol/liter cell × hr and provided an estimation of the net Na^+ influx driven by the imposition of an outward H^+ gradient

where IR = initial rate of KOH addition (μl/min), C = concentration of the titrating solution (M), OD_{cs} = optical density of Hb from the lysate of the acid-loaded cell suspension prior to flux measurement, V_{cs} = volume of the efflux media cell suspension (ml), OD_f = optical density of Hb from the lysate of the flux media sample, and Hct_{cs} = Hct of the acid-loaded cell suspension.

Statistical Analysis

The statistical analysis was performed with Student's t test for paired and unpaired data [1]. Linear and nonlinear regression analysis were computed with a Hewlett Packard calculator. P values smaller than 0.05 were regarded as statistically significant. The data are reported as mean ± SEM.

Chemicals

NaCl, $MgCl_2$, dibutylphthalate and glucose were obtained from Fisher Scientific Company (Fairlawn, NJ). Amiloride, ouabain, Tris, MES, MOPS, DIDS, nystatin and albumin (bovine fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). KCl from Mallinckrodt Inc. (St. Louis, MO). ^{22}Na was purchased from Amersham (Arlington Heights, IL). Dimethylamiloride (DMA) was kindly provided by Dr. E.J. Cragoe (Merck, Sharp and Dohme, Rahway, NJ), phloretin from Nutritional Biochemicals Corp. (Cleveland, OH) and methazolamide was from Lederle Laboratories Division of the American Cyanamide Co. (Pearl River, NY). Choline chloride was obtained from Calbiochem, Behring Diagnostic (San Diego, CA); this brand was tested against recrystallized reagent and showed to be ammonia free.

Table 1. Kinetic parameters of Na^+/H^+ exchange in human red cells: Dependence on internal protons

	K_m for cell H^+		Hill coefficient n_{app}	V_{max} net Na^+ influx (mmol/liter cell · hr)
	nM	pH_i		
Mean	347	6.46	2.71	30.1
± SEM	30	0.04	0.04	4.2
	(n = 7)		(n = 7)	(n = 20)

The V_{max} of Na^+/H^+ exchange was determined from the difference in net Na^+ influx from Na^+ media (150 mM) with pH_o 8.0 minus pH_o 6.0. Cell Na^+ was 0.86 ± 0.27 mmol/liter cell; pH_i was 5.93 ± 0.01 (n = 20).

Results

I. Net Na^+ Influx Driven by an Outward H^+ Gradient: Dependence on Cell pH

When acid-loaded Na^+-depleted cells (initial Na_i 0.4 mmol/liter cell and pH_i 6) were incubated in 150-mM Na^+ medium with pH_o 6.0, cell Na^+ content increased linearly with time up to 20 min while at pH_o 8.0 a rapid increase in cell Na^+ content occurred, which was linear only up to 10 min (Fig. 1). Therefore, the net Na^+ influx was computed with the linear regression analysis for the first 10 min. The difference between net Na^+ influx at pH_o 8.0 and at pH_o 6.0 represents the net Na^+ influx driven by an outward H^+ gradient (ΔpH_o).

The dependence of net Na^+ influx on cell H^+ concentration was investigated in Na^+-depleted cells; the influx incubation media contained 150 mM NaCl at pH_o 8.0 and 6.0 (Fig. 2*A*). The reduction of cell pH caused a sigmoidal increase in net Na^+ influx from media with pH_o 8, which reached a V_{max} of 58.0 mmol/liter cell × hr between pH_i 5.8 and 6.1 in this subject. In contrast, when the acid-loaded cells were incubated in pH_o 6.0 Na^+ media, Na^+ influx increased very slowly and linearly. The difference between Na^+ influx from alkaline and acid media (ΔpH_o) represents the net Na^+ transport driven by an outward H^+ gradient ($H_i > H_o$), i.e., Na^+/H^+ exchange. The H^+-gradient-driven Na^+ influx also increased sigmoidally with the increase in H_i (Fig. 2*A*) and reached V_{max} around pH_i 6.0. The Hill plot of log v/V_{max}^{-v} of the H^+-gradient-driven Na^+ influx (Na^+/H^+ exchange) *vs.* log H_i was used to calculate the K_m for cell H^+ to activate Na^+/H^+ exchange and the Hill coefficient (n_{app}) (Fig. 2*B*). In this experiment, the K_m for H_i was 320 nM and n_{app} 2.8, which indicates the presence of strong cooperativity between H_i sites. Table 1 shows the mean values of the kinetic parameters of the pH_i dependence of Na^+/H^+ exchange in six different subjects. The K_m

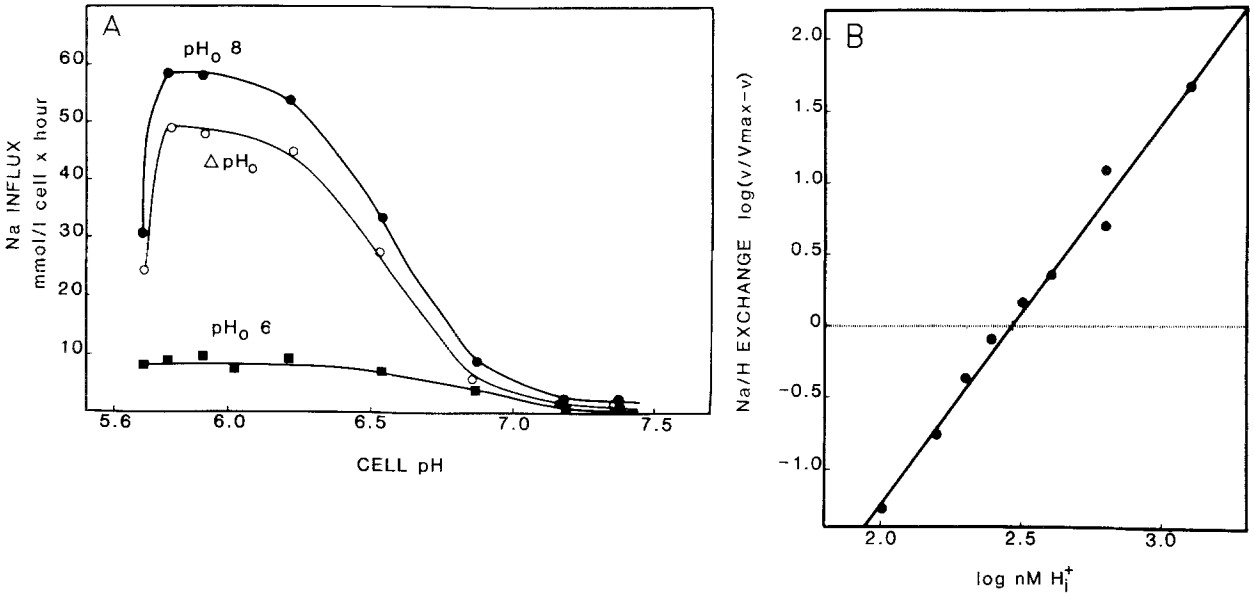

Fig. 2. The dependence of Na^+ influx on cell pH. (*A*) Na^+-depleted (Na_i < 2.0 mmol/liter cell) red cells were acid loaded and DIDS treated as described in Materials and Methods and incubated in 150 mM Na^+ media at pH_o 8.0 (filled dots) and 6.0 (filled squares). The reduction of intracellular pH sigmoidally stimulated net Na^+ influx in alkaline Na^+ medium. ΔpH_o (open dots) is the difference between fluxes at pH_o 8 and pH_o 6 and represents the outward H^+ gradient stimulated Na^+ influx (Na^+/H^+ exchange). The V_{max} (50 mmol/liter cell · hr) was reached at pH_i 6. (*B*) Hill plot for the dependence of H^+-gradient-driven Na^+ influx (Na^+/H^+ exchange) on cell H^+. The K_m for H_i is 320 nM and the Hill coefficient (n_{app}) 2.8

for H_i activation of Na^+ influx was 347 ± 30 nM, which corresponds to pH_i 6.47 ± 0.04 was similar to that observed in other cells.

From these experiments, we selected the conditions for measurements of the V_{max} of Na^+ influx driven by an outward H^+ gradient (i.e., Na_i 0.5–2.0 mmol/liter cell, Na_o 150 mM, pH_i 6, pH_o 8.0 and 6.0, at 37°C). As shown in Table 1, the V_{max} was 31 ± 3.3 mmol/liter cell × hr (mean ± SE) with a large interindividual variation (6–63 mmol/liter cell × hr). No significant differences were observed between males and females in this study group.

The within assay reproducibility of the V_{max} of H^+-driven Na^+ influx was determined by measuring duplicate samples from 10 subjects assayed blindly. The technical error was calculated according to the formula $(d^2/2N)^{1/2}$ where d is the difference in value between a split pair and N is the number of pairs. The absolute error was 3.3 mmol/liter cell × hr, which was 12% of the mean value of Na^+/H^+ exchange in this group (28.2 mmol/liter cell × hr). The measurement of the V_{max} of Na^+/H^+ exchange was repeated one month apart in four subjects and appeared to be stable (*data not shown*).

II. The Dependence of Na^+ Influx Driven by Na^+/H^+ Exchange on External Na^+ and H^+

The K_m for external Na^+ of the Na^+/H^+ exchange was measured in red cells suspended in media with increasing NaCl concentration. The rate of ^{22}Na influx into acid-loaded, Na_i-depleted cells increased with Na_o to a greater extent in alkaline than in acid medium, where the increase was linear (Fig. 3*A*). Since Na^+ influx at pH_o 6.0 did not reach saturation and it could be accounted for by a diffusional component, it was substracted from the influx at pH_o 8.0. The Na^+ influx promoted by the outward H^+ gradient, i.e., the difference between Na^+ influx in pH_o 8 and pH_o 6 media (ΔpH_o), increased sigmoidally with Na_o, reaching saturation around 150 mM. According to Hill's plot of the ΔpH_o data (Fig. 3*B*), the K_m for Na_o was 57 mM and the n of Hill 2.0. In three similar experiments, the K_m for Na_o ranged from 42 to 65 mM (55 ± 7) with a n_{app} 2.12 ± 0.09.

III. The Dependence of H^+ Efflux on External Na^+: Stoichiometry of the Red Cell Na^+/H^+ Exchanger

To determine the stoichiometry of the red cell Na^+/H^+ exchanger, the Na_o-dependent H^+ efflux and the outward H^+-gradient-driven Na^+ influx were measured in acid-loaded cells. Acid-loaded red cells (pH_i 6.0) were incubated in unbuffered Na^+ and K^+ media and the H^+ efflux was measured using the pH-stat apparatus to clamp pH_o at 8.0. H^+ efflux was calculated from the timed recording of the volume of KOH added to maintain the pH of the flux media at 8.0 as reported in Materials and Methods. H^+ efflux into K^+ media was very high, but it was stimulated by external Na^+ (Table 2). The stoichi-

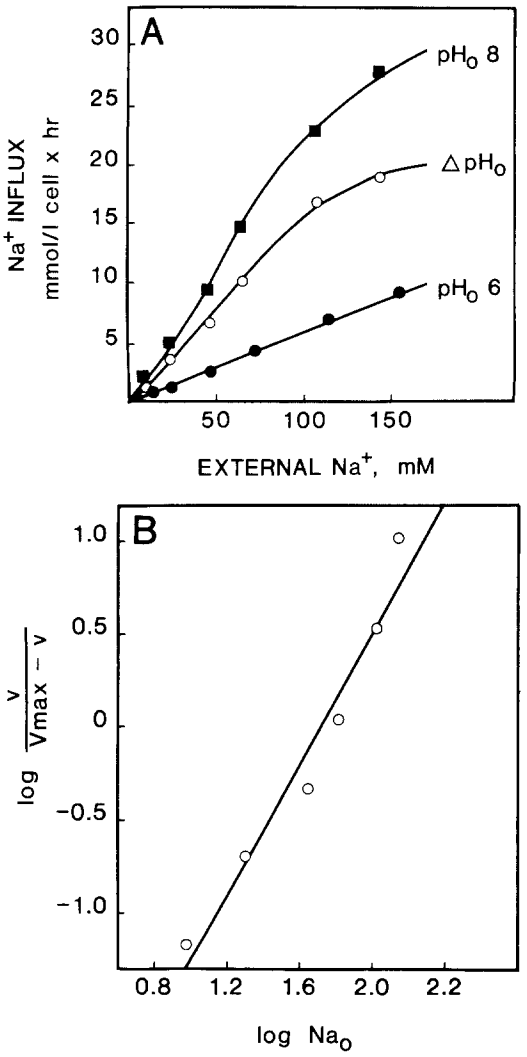

Fig. 3. The dependence of unidirectional Na^+ influx on external Na^+. (*A*) Na^+ influx into acid-loaded (pH_i 6.0), Na^+-depleted (Na_i 0.4 mmol/liter cell) and DIDS-treated red cells incubated in flux media with increasing Na^+ concentrations balanced with K^+ at pH_o 6.0 and pH_o 8.0. ΔpH_o represents the difference between pH_o 8 and pH_o 6 influx and it is the Na^+ influx promoted by the outward H^+ gradient. (*B*) Hill plot for the dependence of ΔpH_o Na^+ influx on external Na^+. The K_m for external Na^+ was 57 mM and the Hill's n_{app} 2.0

ometry of Na^+/H^+ exchanger, computed from the ratio between the Na_o-dependent H^+ efflux and H^+-gradient-driven Na^+ influx at comparable Na_i, at pH_i 6 and pH_o 8, was 1.13 ± 0.22, not statistically different from 1 : 1 stoichiometry (Table 2).

IV. Amiloride Sensitivity of Red Cell Na^+/H^+ Exchanger

Extensive literature has shown that amiloride inhibits Na^+/H^+ exchange in diverse cell types [13, 20 and 18, 21, 26 for reviews]. In acid-loaded red cells,

Table 2. The stoichiometric ratio between H^+ and Na^+ movements driven by Na^+/H^+ exchanger in human red cells

	Flux media composition		
	K^+	Na^+	Na_o-stimulated
H^+ efflux (mmol/liter cell · hr)	279 ± 117	313 ± 130[a]	34 ± 13
	pH_o 6	pH_o 8	H_o-stimulated
Na^+ influx (mmol/liter cell · hr)	17.1 ± 5.8	50.3 ± 4.6[b]	33.2 ± 5.5
Stoichiometric ratio:			ΔpH_o:ΔNa 1.13 ± 0.22

pH_i 6, Na_i 14 ± 14 mmol/liter cell, Na_o 150 mM, K_o 170 mM, n = 3, mean ± SEM. pH_o was 8.0 in H^+ efflux measurements. [a] $0.05 < P < 0.10$ *vs.* K^+ medium. [b] $P < 0.025$ *vs.* pH_o 6 medium.

amiloride (1 mM) only partially inhibited unidirectional and net Na^+ influx as well as unidirectional Na^+ efflux in alkaline media (Table 3). The mean inhibition of unidirectional Na^+ influx was 59 ± 10% (90% confidence limits: 40–77%). A similar fraction of H^+-gradient-driven Na^+ influx was inhibited by 20 μM DMA (*data not shown*) as previously reported in a comparative study of the effect of different amiloride-analogs [11].

We also studied the effects of DMA (an inhibitor of Na^+/H^+ exchange with higher affinity than amiloride) on H^+ efflux from acid-loaded cells. H^+ efflux was not stimulated by Na_o in the presence of maximal doses of DMA (20 μM), which does not change the buffering capacity of the flux media. However, there was also DMA-sensitive H^+ efflux into K^+ media of 25 mmol/liter cell × hr. These results indicate that DMA inhibits also H^+ efflux uncoupled from Na^+ influx or that the drug exerts nonspecific effects on this ion movement.

V. The Effect of Internal Na^+ on the V_{max} of Na^+/H^+ Exchange

Unidirectional and net Na^+ influx were measured in acid (pH_i 6.0) and Na^+-loaded (11 and 29 mmol/liter cell) erythrocytes (Table 4). At both intracellular Na^+ concentrations, the unidirectional and net Na^+ influx were greater at pH_o 8.0 than at 6.0, the difference being the Na^+ influx driven by an outward H^+ gradient (ΔpH_o). No significant differences were seen between net and unidirectional Na^+ influx at 11 mmol/liter cell Na^+. The net Na^+ influx at Na_i 29 mmol/liter cell was not significantly different from the one measured at Na_i 11 mmol/liter cell, but in alkaline medium (pH_o 8), it was significantly smaller than the unidirectional influx due to the stimulation of Na^+ efflux by increased cell Na^+. The elevation

Table 3. The effect of amiloride (1 mM) on unidirectional and net Na^+ fluxes in acid-loaded cells

Na^+ fluxes (mmol/liter cell · hr)	Composition of the flux media		
	pH_o 6.0	pH_o 8.0	pH_o 8 + amiloride
Influx	11.5 ± 3.8	42.6 ± 10.3[a]	24.7 ± 8.9[d]
Efflux	4.1 ± 0.2	8.9 ± 1.5[b]	5.8 ± 0.9[d]
Net	8.3 ± 1.4	30.8 ± 0.7[c]	15.4 ± 3.1[e]

pH_i 6.0, Na_i 18 ± 3 mmol/liter cell; Na_o 150 mM. Mean ± SEM, $n = 3$. [a]$P < 0.025$; [b]$P < 0.05$; [c]$P < 0.005$ *vs.* pH_o 6; [d]$P < 0.025$ *vs.* pH_o 8; [e]$P < 0.05$.

of cell Na^+ stimulated Na^+ efflux to a greater extent when the external pH was alkaline than when acid, indicating that Na^+ can be transported uphill if an outward H^+ gradient is imposed.

In separate experiments under the same conditions, net and unidirectional Na^+ influx into acid-loaded (pH_i 6.0) cells, containing 0.5–1.5 and 10 mmol/liter cell Na^+, were comparable. At pH_o 8, the net Na^+ influx was 41 ± 10 mmol/liter cell · hr and the unidirectional Na^+ influx 43 ± 10 ($n = 3$). At pH_o 6, the corresponding fluxes were 10 ± 4 and 12 ± 4 mmol/liter cell · hr.

The influence of low to physiological cell Na^+ concentration on the measurement of net Na^+ influx V_{max} was further investigated in Na^+-depleted (Na_i 0.8 ± 0.2 mmol/liter cell) and Na^+-repleted (Na_i 9.0 ± 0.4 mmol/liter cell) erythrocytes. The V_{max} of net Na^+ influx were 33 ± 6 mmol/liter cell × hr in the former and 34 ± 6 in the latter ($n = 5$). These data confirm that at low cell Na^+, Na^+ efflux is negligible and suggest that the transport pathway for Na^+ efflux has a very low affinity for cell Na^+ at pH_i 6.0. At Na_i lower than 20 mmol/liter cell both measurements of Na^+ influx may therefore provide an estimate of Na^+/H^+ exchange V_{max} within the 9% coefficient of variation of the assay.

To investigate whether internal Na^+ exchanges with external Na^+ in a Na^+/Na^+ exchange mode, we measured Na^+ efflux from acid-loaded cells into K^+ and Na^+ media (*data not shown*). Na^+ efflux was smaller in Na^+ than in K^+ media (significantly at pH_o 8), thus ruling out the presence of a Na^+/Na^+ exchange pathway in acid-loaded cells and providing further evidence that the Na^+ influx is coupled to an outward movement of H^+ and not of Na^+. As shown in Table 3, Na^+ efflux from acid-loaded cells was significantly greater at pH_o 8.0 than at pH_o 6.0, confirming that an outward H^+ gradient also stimulates Na^+ efflux (*see* section V in Materials and Methods). This behavior of Na^+ efflux is consistent with an uncoupled mode of Na^+ transport stimulated by H_i or with cotransport with H^+, partially inhibited by external Na^+ and H^+. Uncoupled Na^+ efflux was partially inhibited by 1 mM amiloride (67 ± 4%; 90% confidence limits: 59–76%) (*data not shown*). The values of the "uncoupled" Na^+ efflux stimulated by outward H^+ gradient, in comparison to the V_{max} of the Na^+ influx, confirms that the cell Na^+ content of fresh cells is an unlikely source of error in the study of the Na^+/H^+ exchanger in human red cells.

Discussion

I. Kinetic Properties of Red Cell Na^+/H^+ Exchange

Several studies have shown the presence of Na^+/H^+ exchange in nucleated *Amphiuma* [7], dog [24, 25] and rabbit red cells [12, 22]. The present study indicates that the human red cell Na^+/H^+ exchanger has an activity 30 times lower than rat thymocytes [17] (V_{max} 0.5 *vs.* 15 mmol/liter cell · min), but kinetic dependence on internal H^+ similar to nonerythroid cells [4, 14, 17]. The V_{max} of red cell Na^+/H^+ exchange displayed large variability between individuals (6–63 mmol/liter cell · hr), but it remains to be determined which factors (genetic or environmental) account for these interindividual differences. Notably, the V_{max} of the Na^+/H^+ exchanger is 8 to 10 times higher than the V_{max} of the Na^+ pump (4.5 ± 1.0 mmol/liter cell · hr), which was believed to be the most active Na^+ transport system in human red cells. However, the Na^+ pump is extruding Na^+ under physiological conditions while the Na^+/H^+ exchanger probably is not playing a functional role in mature cells because the pH gradient is effectively and rapidly clamped by the even more active anion exchange system. Since it has been shown that the activity of the Na^+ pump decays rapidly upon maturation of the reticulocyte [6], our data suggest that Na^+/H^+ exchange may decay at slower rate than the Na^+ pump.

The red cell Na^+/H^+ exchanger showed asymmetric response to H^+ and Na^+ ions because H^+ ions were stimulatory from inside and inhibitory from outside while Na^+ ions stimulated only from outside.

The V_{max} of Na^+/H^+ exchange was reached at pH_i 6, pH_o 8 and 150 mM external Na^+. The K_m for internal H^+ to stimulate Na^+ influx varied very little around pH_i 6.5, a value very similar to that reported in studies of rat thymic lymphocytes [16], kidney vesicles [3] and vascular smooth muscle cells [5]. Na^+ influx was sigmoidally stimulated by internal H^+ in acid-loaded cells when an outward H^+ gradient ($H_i > H_o$) was imposed (Fig. 2). This behavior was reflected by a high Hill coefficient (>2.0) for H_i-

activation of Na^+ influx indicating the presence of strong positive cooperativity for H_i binding sites. As previously proposed by Aronson [3], the occupancy of a H^+ regulatory site can activate, but not transport, H^+ in exchange for external Na^+. This kinetic property of Na^+/H^+ exchange has important physiological implications for the regulation of cell pH because it determines how rapidly the transporter will respond to a small fall in cell pH.

The dependence of Na^+ influx on external Na^+ was studied at $Na_i < 2.0$ mmol/liter cell, pH_i 6.0 and pH_o 6.0 and 8.0. Na^+ influx at pH_o 6.0 was linearly dependent on Na_o, a finding compatible with a diffusional process while at pH_o 8.0 it was markedly stimulated and showed saturation kinetics. Na^+ influx at pH_o 6.0 was, therefore, subtracted from Na^+ influx at pH_o 8.0 to estimate the H^+-driven Na^+ influx (i.e., Na^+/H^+ exchange), which had a K_m of 55 mM for Na_o. In other cell types, acid pH_o markedly increased the K_m for Na_o without changes in V_{max}, thus indicating that H_o acted as competitive inhibitors of external Na^+ sites [2, 14, 17].

The kinetic dependence on Na_o of ΔpH_o Na^+ influx was better fitted with an Hill plot (n of Hill 2.12) than with a Lineweaver- Burke plot, indicating that there might be interaction between Na_o sites in the absence of internal Na^+. Similar results have been obtained for rabbit red cell Na^+/H^+ exchange by Morgan and Canessa [22]. As far as we know, the K_m for external Na^+ has not been studied in other cell types in the absence of, or at very low, Na_i to exclude other components of Na^+ transport such Na^+/Na^+ exchange or *trans*-effects of Na_i. In Na-containing lymphocytes [16] and microvillus membrane vesicles from rabbit kidney [2], the kinetics for Na_o-dependence of Na^+/H^+ exchange have been shown to obey Michaelis-Menten kinetics. However, in agreement with our results, recent studies by Otsu et al. [23] indicate that the Na^+/H^+ exchanger might be an oligomer with two distinct classes of Na^+ transport sites, each having at least two sites interacting cooperatively.

II. The Stoichiometry and Amiloride Sensitivity of Red Cell Na^+/H^+ Exchange

An outward H^+ gradient stimulated Na^+ influx and an inward Na^+ gradient stimulated H^+ efflux from acid-loaded human red cells. The stoichiometric ratio between these components of Na^+ influx and H^+ efflux (Table 2) is consistent with a 1 : 1 stoichiometry, already reported [2, 7, 13]. The high Hill coefficient observed for H_i to stimulate Na^+ influx together with a ratio of Na^+ influx to H^+ efflux of 1 : 1 is more likely compatible with a coupling ratio of counter-transport of 2 : 2, 3 : 3, or 4 : 4 ions.

It appears, therefore, from this study that both H^+ efflux and Na^+ influx in human red cells are only partially (59–77%) inhibited by amiloride. Escobales and Canessa [10, 11] previously reported that Na^+ influx into acid-loaded (pH_i 6.8), ATP-rich human red cell was only partially inhibited by 50 μM 5-N-methyl-butylamiloride, 20 μM 5-N,N-dimethyl-amiloride and 1 mM amiloride. Thus, amiloride and the high affinity amiloride analogs inhibit a similar component of Na^+ influx driven by Na^+/H^+ exchange. The IC_{50} for amiloride inhibition of Na^+ influx into acid-loaded, ATP-rich cells was, however, significantly higher (10 μM) than the inhibition of Na^+ influx in acid-loaded, DIDS-treated cells [10, 11]. This finding suggests that the amiloride-sensitivity of Na^+/H^+ exchanger may depend also on the metabolic state of the cells.

As previously shown also by Grinstein et al. [16], rat thymic lymphocytes have also an amiloride-insensitive Na^+/H^+ exchanger.

In a recent study designed to estimate the number and turnover rate of Na^+/H^+ exchange, Dixon et al. [9] reported no significant amiloride displacement binding of 3H-labeled 5-(N-methyl-N-isobutyl)-amiloride (MIA) in human red cells. It seems, therefore, that an estimation of the number of Na^+/H^+ exchange units by that binding assay fails to detect an amiloride-sensitive pathway with a V_{max} of 0.5 mmol/liter cell · min such as that of the human red cells.

III. Effect of Internal Na^+ on Red Cell Na^+/H^+ Exchange

The amiloride sensitivity of Na^+ influx has often been used to measure Na^+/H^+ exchange. In view of the partial amiloride sensitivity of this transporter in human red cells, our strategy has been to determine the coupling between Na^+ and H^+ efflux and the kinetic effects of H_i and Na_o and Na^+ influx and H^+ efflux in order to determine the kinetic parameters (V_{max} and K_m) of human red cells Na^+/H^+ exchange. We have shown that measurements of Na^+ influx into acid-loaded cells at two different pH_o levels provide a good estimation of the Na^+ movement promoted by an outward H^+ gradient (Table 2). Similarly, under physiological conditions, a fall in red cell pH may provide the stimulus for the exchange of H_i for Na_o.

The measurement of cell Na^+ content and cell pH can be easily performed in most laboratories. Therefore, the assay of kinetic properties of red cell Na^+/H^+ exchange can be used to study this transporter in a variety of physiological and pathophysiological conditions. Red cells are easily available in large quantities to perform kinetic measurements,

Table 4. The effect of internal Na on the unidirectional and net Na^+ fluxes driven by an outward H^+ gradient

	Cell Na content			
	(11 mmol/liter cell)		(29 mmol/liter cell)	
	pH_o 6	pH_o 8	pH_o 6	pH_o 8
Net influx	10 ± 4	41 ± 10[a]	12 ± 1	39 ± 9[b,c]
ΔpH_o	31 ± 7		27 ± 9	
Unidirectional Influx	12 ± 4	43 ± 10[a]	14 ± 4[d]	50 ± 11[a]
ΔpH_o	34 ± 7		35 ± 7	
Unidirectional Efflux	2 ± 0.2	3 ± 0.4	4 ± 0.2[e]	9 ± 1[b,d]
ΔpH_o	1 ± 0.1		4 ± 0.4	

Na fluxes in mmol/liter cell · hr; cell pH 6.0, Na_o 150 mM; $n = 3$, mean ± SEM. [a] $P < 0.025$ *vs.* pH_o 6; [b] $P < 0.05$; [c] $P < 0.05$ *vs.* unidirectional influx at pH_o 8; [d] $P < 0.05$; [e] $P < 0.005$ *vs.* Na_i 11, same pH_o.

they can be more easily prepared than other blood cells such as platelets and leukocytes [15], they can be preserved for several days and Na_i, H_i and cell volume can be manipulated precisely. The net Na^+ influx measurement provides a more simple and accurate determination of Na^+/H^+ exchange than Na_o-dependent H^+ efflux because the Na_o-independent H^+ efflux is very large and several assays can be carried out simultaneously while H^+ efflux measurements are performed only sequentially. Finally, the determination of the V_{max} of Na^+/H^+ exchange measuring the H^+-driven Na^+ influx also provides a good estimation of the Na^+-driven H^+ efflux given its 1 : 1 stoichiometric ratio and the good reproducibility within assay and within subject.

Under V_{max} conditions, a raise in Na_i from 0.5 to 25 mmol/liter cell had no significant effects on Na^+ influx driven by Na^+/H^+ exchange. This finding implies that removal of cell Na^+ by nystatin treatment of red cells might not be required, but the assay of net Na^+ entry has greater precision in Na_i-depleted cells. Moreover, it appears that internal Na^+ has different effects on Na^+/H^+ exchange depending on the cell type. Reduction of the Na^+/H^+ exchange activity has been observed in rat thymocytes when Na_i was increased [17] while the opposite was reported by Morgan and Canessa in rabbit erythrocytes [22].

Experiments designed to study the effect of internal Na^+ on the V_{max} of the Na^+/H^+ exchange indicated that an outward H^+ gradient also stimulated Na^+ efflux, which was partially inhibited by amiloride (Table 3). The Na^+ efflux from acid-loaded cells driven by an outward H^+ gradient (pH_o 8.0) may represent an uncoupled Na^+ transport mode of the Na^+/H^+ exchange or, alternatively, a Na^+-H^+ cotransport. Na^+ efflux was not stimulated by external Na^+ and, under our experimental conditions, the transport of Na^+ by Na^+/Na^+ exchange could be ruled out. According to previous observations, Na^+/Na^+ exchange in human red cell may be performed through DIDS-sensitive and DIDS-insensitive pathways [27]. At $pH_o = pH_i = 7.4$, DIDS-sensitive Na^+/Na^+ exchange can be inhibited also by furosemide, phloretin and dypiridamole [27], which suggests that it is performed through the anion exchanger. It seems, therefore, that DIDS-insensitive Na^+/Na^+ exchange is performed through the Na^+/H^+ exchange.

In summary, our study of the kinetics and stoichiometry of human red cell Na^+/H^+ exchange provides an assessment of its functional properties, which can be further investigated in a variety of physiological, pathophysiological and pharmacological conditions to determine the role of hormonal, genetic and environmental factors modulating its expression.

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