Na^+-H^+ and Na^+-Li^+ Exchange Are Mediated by the Same Membrane Transport Protein in Human Red Blood Cells: An NMR Investigation†

Yuling Chi, Suilan Mo, and Duarte Mota de Freitas*

Department of Chemistry, Loyola University of Chicago, Chicago, Illinois 60626

Received April 4, 1996; Revised Manuscript Received June 25, 1996⊗

ABSTRACT: Na^+-H^+ exchange is a transport system present in erythrocytes which plays an important role in the regulation of intracellular pH, cellular volume, and transmembrane ion transport. Na^+-Li^+ exchange has received much attention and has been investigated in more detail than have any of the other ion transport systems, because of its high reproducibility. Both red blood cell (RBC) Na^+-H^+ and Na^+-Li^+ exchange are elevated in essential hypertensive patients relative to normotensive individuals. RBC Na^+-Li^+ exchange may be a mode of operation of Na^+-H^+ exchange. Amiloride and its analogue, 5-(*N*,*N*-hexamethylene)amiloride (HMA), are well-known inhibitors of Na^+-H^+ exchange, whereas phloretin strongly inhibits Na^+-Li^+ exchange. In this study, we tested the effects of amiloride, HMA, and phloretin on Na^+-Li^+ exchange activity in intact RBCs by using atomic absorption. We investigated by using ^7Li nuclear magnetic resonance (NMR) spectroscopy the effects of HMA and phloretin inhibition on Li^+ efflux across resealed H^+- and Li^+-loaded RBC ghosts in the absence and presence of pH gradients. Amiloride inhibitory activities on both Na^+ and Li^+ binding to exposed RBC membranes under different pH conditions were also studied by ^{23}Na and ^7Li NMR relaxation time measurements. We found that Na^+-Li^+ exchange activity was inhibited by amiloride, HMA, and phloretin in suspensions of intact RBCs and of resealed RBC ghosts. Li^+ efflux rates across resealed H^+- and Li^+-loaded RBC ghosts were significantly lower when a pH gradient was present, presumably because of the competition between Li^+ and H^+ for transport by the same transport protein. Amiloride had similar inhibitory constants on both Na^+ and Li^+ binding to RBC membranes (1021 ± 48 M^{-1} vs 964 ± 40 M^{-1} at pH 8.0; 731 ± 147 M^{-1} vs 716 ± 27 M^{-1} at pH 7.0). These results suggest that Na^+-H^+ exchange and Na^+-Li^+ exchange are mediated by the same RBC membrane transport protein.

Na^+-H^+ exchange occurs in the human body. It is very important in the regulation of intracellular pH, cellular volume, and transmembrane ion transport (Nakhoul & Boron, 1988). Na^+-Li^+ exchange is observable in human red blood cells (RBCs)[1] and has been considered as the most reproducible Na^+ transport pathway in primary hypertension (Hilton, 1986). Na^+-Li^+ exchange has been used as a marker of genetic predisposition to primary hypertension in childhood (Houtman et al., 1993). Both RBC Na^+-H^+ and RBC Na^+-Li^+ exchange rates are higher for hypertensive than for normotensive individuals (Semplicini et al., 1989b). Na^+-H^+ and Na^+-Li^+ exchanger genes have been used as intermediate phenotypes in linkage studies for the investigation of genetic factors in essential hypertension (Lifton, 1993). Na^+-H^+ exchange and Na^+-Li^+ countertransport were significantly and positively correlated in RBCs from hypertensive patients with insulin-dependent diabetes mellitus (Semplicini et al., 1989a). Na^+-H^+ exchange may also relate the genetics of Na^+-Li^+ countertransport to hyperinsulinemia and dyslipidemic hypertension (Williams et al., 1994). H, Li, and Na are in the same group in the periodic table, and their ions are the only substrates for most of the monovalent cation transport systems (Jennings et al., 1985). Therefore, we hypothesized that these two ionic exchange processes are mediated by the same RBC membrane transport protein.

Amiloride and its analogues, HMA, EIPA, and MIA, are specific Na^+-H^+ exchange inhibitors (Kleyman & Cragoe, 1988), whereas phloretin is a very effective Na^+-Li^+ exchange inhibitor (Pandey et al., 1978). In sarcolemmal vesicles from the bovine superior mesenteric artery, Na^+-H^+ and Na^+-Li^+ exchange are mediated by the same transport system, as demonstrated by inhibition studies with EIPA (Kahn et al., 1989). In human RBCs, however, Na^+-H^+ exchange, but not Na^+-Li^+ countertransport, was inhibited by amiloride (Kahn, 1987). Inhibitors were therefore used previously for determination of whether or not Na^+-H^+ and Na^+-Li^+ exchange processes were mediated by the same RBC transport protein. The inhibitory activities were tested by measurement of the rates (V_{std}) of RBC Na^+-H^+ or Na^+-Li^+ exchange under standard assay conditions (Canessa et al., 1980, 1992; Kahn, 1987, 1989). Under the standard assay conditions, the extracellular side of the RBC membranes was, however, far from being saturated with Na^+ (Canessa et al., 1992; Rutherford et al., 1990, 1992). The kinetic parameters of ion exchange (V_{max} and K_m) are now measured by varying the Na^+ concentration in the suspension medium (Rutherford et al., 1990, 1992).

† Financial support from USPHS Grant MH45926 from the National Institute of Mental Health is gratefully acknowledged by D.M.deF.

* Author to whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1996.

[1] Abbreviations: RBC, red blood cell; AA, atomic absorption; NMR, nuclear magnetic resonance; T_1, spin−lattice relaxation time; HMA, 5-(*N*,*N*-hexamethylene)amiloride; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; MIA, 5-(*N*-methyl-*N*-isobutyl)amiloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TRIS, tris(hydroxymethyl)-aminomethane; MES, 2-[*N*-morpholino]ethanesulfonic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid; CWS, choline wash solution; V_{std}, rate of Na^+-Li^+ exchange measured by the standard transport assay; V_{max} and K_m, kinetic parameters of Na^+-Li^+ exchange.

S0006-2960(96)00814-8 CCC: $12.00

In our investigation, we studied whether the transport inhibitors changed the V_{max} and K_m values, but not V_{std}, in suspensions of intact human RBCs.

Na^+-H^+ and Na^+-Li^+ exchange processes are operational across the membranes in intact RBCs and in RBC ghosts. Hemoglobin is the major nonmembrane protein in these cells. Resealed RBC ghosts are devoid of hemoglobin and of other factors, but they still maintain most transport characteristics of native erythrocyte membranes (Klonk & Deuticke, 1992). They can be loaded with the required concentrations of ions and are ideal models for investigation of ion transport without the interference of hemoglobin and of other factors. In a set of experiments with resealed RBC ghosts, we tested whether or not there was competition between Na^+-Li^+ and Na^+-H^+ exchange processes. By comparing Li^+ efflux rates across H^+- and Li^+-loaded, resealed RBC ghosts induced by an outside Na^+ gradient in the absence and in the presence of a pH gradient, we were able to determine whether Li^+ and H^+ interacted with the same RBC membrane transport protein. Both Na^+-H^+ and Na^+-Li^+ exchange inhibitors, HMA and phloretin, were also applied to the Li^+ efflux rate measurements. The overall rate of ion transport depends on the rate of translocation of ions from the cis to the trans sides of the RBC membrane and on the rates of uptake and release of ions from opposite sides of the RBC membrane. To address the ion-membrane interactions fully, we investigated the inhibitory activities of amiloride on both Na^+ and Li^+ binding to exposed RBC membranes at two different pH values.

EXPERIMENTAL PROCEDURES

Materials. Choline chloride, glucose, sucrose, $MgCl_2$ hexahydrate, LiCl, NaCl, dimethyl sulfoxide (DMSO), sodium hydroxide, and dysprosium chloride ($DyCl_3$) were supplied by Aldrich (Milwaukee, WI). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), 2-[*N*-morpholino]ethanesulfonic acid (MES), 3-[*N*-morpholino]propanesulfonic acid (MOPS), bovine serum albumin (BSA), ouabain, phloretin, bumetanide, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), methazolamide, and the detergent octyl β-D-glucopyranoside were purchased from Sigma Chemical Company (St. Louis, MO). Amiloride, HMA, EIPA, and MIA were supplied by Research Biochemicals International (Natick, MA). Sodium hydrogen thulium 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetramethylenephosphonate sodium chloride (Na_3H_2TmDOTP·3NaCl) was purchased from Magnetic Resonance Solutions (Dallas, TX). The protein assay dye reagent was obtained from Bio-Rad Chemical Co. Fresh-packed RBCs were supplied from a blood bank (Chicago Chapter of Life Source).

Preparation of Li^+-Loaded RBCs. Washed and packed RBCs were added to a lithium-loading solution (150 mM LiCl, 10 mM glucose, and 10 mM HEPES, pH 7.4) at 10% hematocrit and incubated at 37 °C for 3 h. After incubation, extracellular Li^+ was removed from the Li^+-loaded RBCs by washing four times with choline wash solution (CWS) containing 112 mM choline chloride, 80 mM sucrose, 10 mM glucose, and 10 mM HEPES, pH 7.4 (Canessa et al., 1980, 1989).

Preparation of Acid-Loaded and of Sodium-Modified RBCs. RBCs were packed by centrifugation at 3928*g* and washed at 4 °C with CWS; the osmolarity was 300 ± 10 mosM. Washed packed RBCs were added to fresh acid-loading solution (150 mM KCl, 10 mM glucose, 0.15 mM $MgCl_2$, 0.1 mM ouabain, 0.01 mM bumetanide, 20 mM Tris-MES, pH 6.0, 350 mOsm) at 10% hematocrit and incubated at 37 °C for 10 min. We then added 1 mL of acid-loading solution containing DIDS and methazolamide to the cell suspension to lock the intracellular pH. The final concentration of DIDS was 0.2 mM, and that of methazolamide was 0.5 mM. The cell suspension was incubated for an additional 30 min. After incubation, the suspension was centrifuged at 3928*g* and washed four times at 4 °C with acid-washing solution (170 mM KCl, 40 mM sucrose, and 0.15 mM $MgCl_2$) (Canessa, 1989). With this procedure, the intracellular pH was 5.8, as determined by measurement of the pH of the lysate obtained by addition of 0.2 mL of packed RBCs to 2.0 mL of double-distilled water and allowing 5 min to reach equilibrium. We loaded packed RBCs with different intracellular pH values by conducting acid loading in suspension media of various pH values, as described by Canessa (1989). Because the RBC volume is dependent on the intracellular and medium pH, we used hypertonic solutions in the preparation and washing of acid-loaded RBCs to prevent cell volume changes from occurring (Canessa, 1989).

We used nystatin, a channel-forming Na^+-selective ionophore, to bring the Na^+ concentration in RBCs to the desired levels. The procedures used for the preparation of sodium-modified RBCs have been reported (Canessa, 1989) and were followed exactly in this study. The final intracellular Na^+ concentrations were measured by atomic absorption.

Preparation of H^+- and Li^+-Loaded Resealed RBC Ghosts. RBCs were washed three times with CWS. Ice-cold packed RBCs were added to ice-cold hemolyzing medium (5 mM Tris-MES, pH 6.0, 4 mM $MgCl_2$, 21 mM LiCl, 70 ± 10 mosM) at 10% hematocrit. After mixing for 10 min at 0−2 °C, the isotonicity was restored to 300 ± 10 mOsm by addition of choline chloride. Subsequent to stirring for an additional 10 min at 0−2 °C, the suspensions were incubated for 1 h at 37 °C for resealing the ghosts. The pH was maintained at 6.0 during the incubation. After incubation, the light pink ghosts were washed twice with CWS (pH 6.0) at 2 °C (Bodemann & Passow, 1972; Duhm, et al., 1976).

Preparation of Unsealed RBC Membranes. Unsealed RBC membranes were prepared by lysing of the washed packed RBCs with 5 mM HEPES buffer, pH 8.0. The membranes were washed three more times with buffer and centrifuged at 51 948*g* at 4 °C until the membranes were pale white (Steck & Kant, 1974). The desired pH values of the membrane suspensions were obtained by washing of the membranes with 10 mM Tris-MES or Tris-MOPS; the pH was adjusted with Tris-MES- or Tris-MOPS-buffered solutions.

Determination of Protein Concentration. The membrane protein concentration was measured by the Bradford assay (Bollag & Edelstein, 1991) with the detergent hexyl β-D-glucopyranoside or octyl β-D-glucopyranoside (Fanger, 1987) at 595 nm on an IBM UV/vis 9420 or on a JASCO model V-500 UV/vis spectrophotometer. 5 μL of membranes was added to 45 μL of 5 mM HEPES solution, pH 8.0, containing 10 μL 50% detergent, and the resulting solution was incubated for 5 min at room temperature. Then 2.5 mL of dye reagent, which was purchased from Bio-Rad Chemical

Co., was diluted with 4-fold deionized water and added to the membrane suspension. The dye reagent was filtered through Whatman No. 1 paper before being added to the membrane suspension.

NMR, AA, and Hematocrit Measurements. ^{23}Na and ^{7}Li measurements were obtained at 79.4 and 116.5 MHz, respectively, on a Varian VXR-300 NMR spectrometer. The instrument was equipped with 10 mm multinuclear probes and a variable temperature unit. All NMR experiments were conducted under identical gain settings. ^{23}Na NMR spectra were recorded at 37 °C at a flip angle of 60° (20 μs) and with an acquisition time of 0.94 s, a delay of 0.3 s, a spectral width of 15974.4 Hz, and 95 transients, without spinning. ^{7}Li NMR spectra for Li^+ efflux across resealed RBC ghosts were obtained at 37 °C at a flip angle of 60° (18 μs), an acquisition time of 0.981 s, a delay of 30 s, a spectral width of 4500.5 Hz, and 29 transients, with 10 Hz spinning. No significant sedimentation was observed in our samples without spinning or with the low spinning rate used in some of our measurements. We obtained T_1 and T_2 values by using the inversion recovery and the Carr−Purcell−Meiboom−Gill pulse sequences, respectively (Gadian, 1982); the errors involved in the T_1 and T_2 measurements reported in this study are within 10%, which is standard for this type of measurements.

Li^+ and Na^+ absorptions were measured at 670.8 and 589.0 nm, respectively, on a Perkin Elmer 5000 spectrophotometer equipped with a flame source and a graphite furnace. The fuel and oxidant were acetylene and compressed air, respectively. Hematocrit measurements were obtained with a microcentrifuge (IEC, model MB IM116).

Calculation of RBC Na^+-Li^+ Exchange Rates. The Li^+-loaded RBCs were suspended at 10% hematocrit in six media containing 150, 100, 70, 40, 20, and 0 mM NaCl that were made isotonic with choline chloride and also contained 10 mM glucose, 0.1 mM ouabain, and 10 mM HEPES, pH 7.4. Aliquots were taken every 20 min from each of the Li-loaded RBC suspensions and centrifuged at 10 519*g* for 1.5 min at 4 °C. The supernatants were collected and analyzed by AA. The kinetic parameters of Na^+-Li^+ exchange V_{std}, V_{max}, and K_m were obtained from the following equations (Canessa et al., 1980; Rutherford et al., 1990):

$$V = \text{slope}_{NaCl}(1 - \text{Ht}_{NaCl})/\text{Ht}_{NaCl} - \text{slope}_{Choline}(1 - \text{Ht}_{Choline})/\text{Ht}_{Choline} \quad (1)$$

$$V_{std} = V_{([Na^+]=150\ mM)} - V_{([Choline]=150\ mM)} \quad (2)$$

$$1/V = K_m/(V_{max}[Na^+]) + 1/V_{max} \quad (3)$$

where slope_{NaCl} and $\text{slope}_{Choline}$ represent the slopes of the time dependencies of the extracellular Li^+ concentrations measured in NaCl and choline chloride, respectively.

Calculation of Li^+ Efflux Rates across H^+- and Li^+-Loaded Resealed RBC Ghosts. Li^+-loaded resealed RBC ghosts at 20% hematocrit were added to a NaCl medium containing 120 mM NaCl, 10 mM glucose, 0.1 mM ouabain, 3 mM $Na_3H_2TmDOTP{\cdot}3NaCl$, 10 mM Tris-MES for pH 6.0, or 10 mM Tris-MOPS for pH 8.0, with or without 0.1 mM phloretin or 0.1 mM HMA. ^{7}Li NMR measurements were made every 15 min. Extracellular Li^+ concentrations were calculated from the following equation:

$$[Li^+]_{out} = A_{out}[Li^+]_s/(A_s(1 - Ht)) \quad (4)$$

where A_{out} is the peak area under the extracellular Li^+ NMR resonance, $[Li^+]_s$ and A_s are the known concentration and peak area, respectively, of a standard Li^+ solution, which were measured separately, and Ht is the hematocrit. Li^+ efflux rates were obtained from the equation

$$\text{rate} = \text{slope}(1 - Ht)/Ht \quad (5)$$

where "slope" denotes the slope of the time dependence of the extracellular Li^+ concentration.

Calculation of Observed Na^+ and Li^+ Binding Constants. The observed ^{23}Na or ^{7}Li T_1 values measured with RBC membrane suspensions represent the weighted average of free and bound Na^+ or Li^+ ions; these values are thus sensitive to ion binding to the RBC membrane and total intracellular ion concentrations. Free and bound Na^+ or Li^+ ions undergo exchange which is represented by a time-averaged expression:

$$R_{1obs} = (1/T_1) = R_{1f}X_f + R_{1b}X_b \quad (6)$$

James and Noggle (1969) introduced graphic methods for calculating metal ion binding constants which express the interactions between metal ions and cell membranes and assume one-to-one stoichiometry for binding at a microscopic level when $[B] \ll [M^+]$:

$$(\Delta R)^{-1} = (R_{1obs} - R_{1f})^{-1} = K_b^{-1}\{[B](R_{1b} - R_{1f})\}^{-1} + [M^+]\{[B](R_{1b} - R_{1f})\}^{-1} \quad (7)$$

where R_{1obs}, R_{1f}, and R_{1b} are the observed, free, and bound relaxation rates of M^+ ions in RBC membrane suspension, X_f and X_b are the mole fractions of free and bound M^+, $[M^+]$ and [B] are the total concentrations of M^+ and of membrane binding sites, and K_b is the binding constant of the metal ion to the RBC membrane, respectively.

Calculation of Actual Na^+ Binding Constants. A series of ^{7}Li T_1 values were measured for unsealed RBC membrane suspensions containing varying concentrations of Na^+ and titrated with increasing Li^+ concentrations. Apparent Li^+ binding constants, K_{Li}^{app}, to unsealed RBC membranes were obtained from James−Noggle plots (James & Noggle, 1969).

Because Na^+ and Li^+ bind competitively to RBC membranes, the Li^+ binding constant, K_{Li}, and the actual Na^+ binding constant, $K_{Na(a)}$, were determined from the following equation:

$$1/K_{Li}^{app} = (1 + K_{Na(a)}[Na^+])/K_{Li} \quad (8)$$

The observed Na^+ binding constants to the same membrane preparations were calculated from the observed ^{23}Na T_1 values for the membrane suspensions titrated with Na^+ at increasing concentrations by James−Noggle plots (James & Noggle, 1969).

The correction factor, which is the ratio of the actual Na^+ binding constant to the observed Na^+ binding constant, was calculated from the equation

$$r = K_{Na(a)}/K_{Na(o)} \quad (9)$$

Table 1: Na^+-Li^+ Exchange Rates [in mmol of Li^+/(L of RBCs h)] and Na^+ Dissociation Constants (in mM) in the Presence and in the Absence of Inhibitors[a]

	without inhibitor	with 0.1 mM amiloride	with 0.1 mM HMA	with 0.1 mM phloretin
V_{std}	0.19 ± 0.03	0.18 ± 0.02 (0.60)	0.19 ± 0.05 (0.89)	0.079 ± 0.014 (0.01)
V_{max}	0.34 ± 0.05	0.23 ± 0.01 (0.04)	0.20 ± 0.03 (0.03)	0.10 ± 0.01 (0.03)
K_m	117 ± 13	69 ± 32 (0.12)	48 ± 17 (0.01)	45 ± 13 (0.01)

[a] The kinetic parameters listed in the table were generated from measurements conducted on three separately prepared samples from the same blood batch. The values in parentheses are the *p* values that were calculated from a Student's paired *t*-test. $p \leq 0.05$ was considered to indicate significance.

Calculation of Inhibitor Binding Constants. The inhibitory activity of amiloride for Na^+ or Li^+ binding to RBC membranes was determined by calculation of the amiloride binding constants to RBC membrane suspensions containing either Na^+ or Li^+. The kinetic behavior of amiloride as a competitive inhibitor (Potts, 1994) can be expressed by the equation

$$1/K_M^{app} = (1 + K_{Ami}[Ami])/K_M \qquad (10)$$

where K_M^{app} is the apparent binding constant of the metal ion M^+, K_M is the metal ion binding constant, [Ami] is the amiloride concentration, and K_{Ami} is the amiloride binding constant. The apparent Na^+ or Li^+ binding constants to unsealed RBC membranes were calculated from James–Noggle plots (James & Noggle, 1969) from the observed ^{23}Na or ^7Li T_1 values in RBC membrane suspensions containing varying concentration of amiloride, and titrated with increasing concentrations of either Na^+ or Li^+.

Because Li^+ is 100% visible by ^7Li NMR, we used the apparent Li^+ binding constants directly in eq 10 to generate the amiloride binding constants. In contrast, Na^+ is not 100% visible by ^{23}Na NMR (vide infra). The apparent Na^+ binding constants were corrected by multiplication with the corresponding correction factors obtained from the curve shown in Figure 4. We used the corrected Na^+ binding constants to calculate the amiloride binding constants to the RBC membranes in the presence of Na^+ by using eq 10.

Statistical analyses were obtained by Student's paired *t*-test because the experiments were conducted under the same conditions except for the presence and absence of inhibitors.

RESULTS

Na^+-Li^+ RBC Exchange in Intact RBCs. Table 1 shows the Na^+-Li^+ exchange rates and the Na^+ dissociation constants in intact RBCs in the presence and absence of inhibitors as measured by AA spectroscopy, and the corresponding statistical analysis. Amiloride had a significant effect on V_{max} [0.23 ± 0.01 vs 0.34 ± 0.05 mmol of Li^+/(L cell h); $p = 0.04$, $n = 3$], but had no significant effects on V_{std} or K_m. HMA had significant effects on V_{max} and K_m [V_{max} and K_m were 0.20 ± 0.03 mmol of Li^+/(L cell h) and 48 ± 17 mM with HMA vs 0.34 ± 0.05 mmol of Li^+/(L cell h), and 117 ± 13 mM without inhibitors; $p \leq 0.03$, $n = 3$], but had no significant effect on V_{std}. Phloretin had significant effects on V_{std}, V_{max}, and K_m [V_{std}, V_{max}, and K_m were 0.079 ± 0.014, 0.10 ± 0.01 mmol of Li^+/(L cell h), and 45 ± 13 mM with phloretin vs 0.19 ± 0.04, 0.34 ± 0.05 mmol of Li^+/(L cell h), and 117 ± 13 mM without inhibitors; $p \leq 0.03$, $n = 3$].

From Table 1, one can see that HMA, an amiloride analogue, is more effective than amiloride. Both HMA, which is an Na^-H^+ exchange inhibitor, and phloretin, which is an Na^+-Li^+ exchange inhibitor, inhibited Na^+-Li^+ exchange activity in intact RBCs.

Li^+ Efflux across H^+- and Li^+-Loaded Resealed RBC Ghosts. The pH inside the ghosts was 6.0. When the H^+- and Li^+-loaded, resealed RBC ghosts were suspended in the NaCl medium at pH 8.0, a pH gradient was present. Both Li^+ and H^+ came out of the ghosts and exchanged with extracellular Na^+, indicating that Na^+-Li^+ and Na^+-H^+ exchange occurred simultaneously. As seen in Figure 1, ^7Li NMR spectra showed that Li^+ came out more slowly when Na^+-H^+ exchange occurred at the same time, with or without inhibitors. Both HMA and phloretin decreased Li^+ efflux. The Li^+ efflux rates were calculated and are listed in Table 2. Li^+ efflux rates across the resealed H^+- and Li^+-loaded RBC ghosts were significantly lower in the presence of a pH gradient than in its absence [0.48 ± 0.09 vs 0.81 ± 0.09 without inhibitors; 0.28 ± 0.05 vs 0.49 ± 0.08 with 0.1 mM HMA; 0.25 ± 0.04 vs 0.41 ± 0.06 mmol of Li^+/(L cell h) with phloretin; $p \leq 0.03$, $n = 3$], presumably because of the competition between Li^+ and H^+ ions for transport by the same transport protein. HMA significantly decreased the Li^+ efflux rates [0.49 ± 0.08 vs 0.81 ± 0.09 in the absence of a pH gradient; 0.28 ± 0.05 vs 0.48 ± 0.09 mmol of Li^+/(L cell h) in the presence of a pH gradient; $p \leq 0.05$, $n = 3$]. Phloretin also significantly decreased Li^+ efflux rates [0.41 ± 0.06 vs 0.81 ± 0.09 in the absence of a pH gradient; 0.25 ± 0.04 vs 0.41 ± 0.06 mmol of Li^+/(L ghosts h) in the presence of a pH gradient; $p \leq 0.05$, $n = 3$].

^{23}Na NMR spectra were also obtained for the six RBC ghost samples corresponding to the ^7Li NMR spectra shown in Figure 1 (data not shown); in all cases, the areas of the intraghost ^{23}Na NMR resonances increased, indicating that the area changes shown in Figure 1 are primarily due to the Na^+-Li^+ exchange processes and not to ion leakage from RBC ghosts during the course of transport measurements.

^{23}Na NMR Relaxation Measurements in Acid-Loaded and Na^+-Modified Cells. We conducted ^{23}Na NMR T_1 and T_2 relaxation measurements with packed acid-loaded RBCs with different intracellular pH values (Figure 2). When the intracellular pH decreased, the ^{23}Na T_1 and T_2 values increased because H^+ competed with Na^+ for the same binding sites in acid-loaded RBCs. The T_2 values were significantly smaller than the T_1 values, indicating that ^{23}Na NMR relaxation values are sensitive to Na^+ binding in RBCs. The changes in ^{23}Na NMR relaxation values observed for acid-loaded RBCs are not due to fluctuations in RBC volume because the hypertonic solutions used prevent cell volume changes from occurring (see Experimental Procedures). By varying the total intracellular Na^+ concentration and the oxygenation state of hemoglobin in acid-loaded RBCs (data not shown), we found that the pH-induced

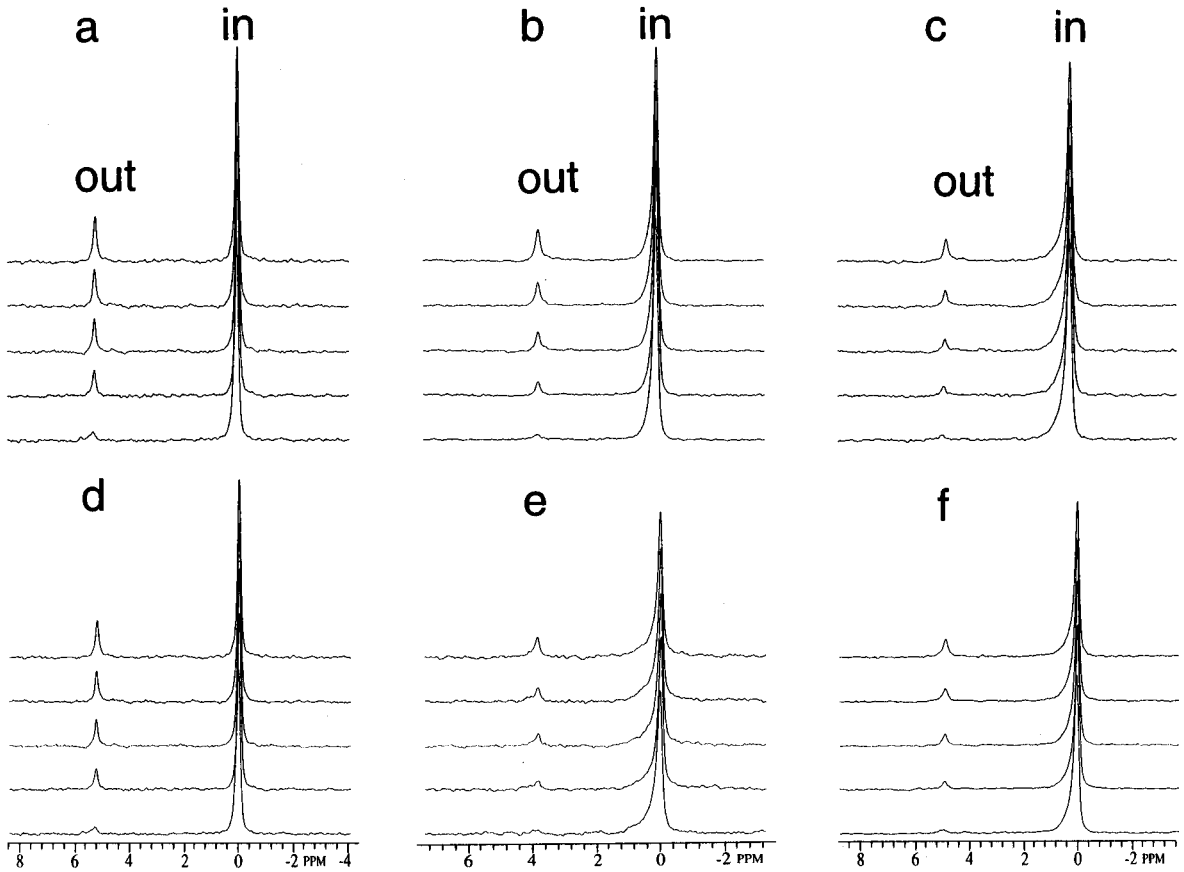

FIGURE 1: ^{7}Li NMR spectra of Li^+ efflux across Li^+-loaded ghosts suspended in 120 mM NaCl medium. The acquisitions were accumulated every 15 min; the spectra shown represent the midpoints of the acquisitions at 7.5, 37.5, 67.5, 97.5, and 187.5 min. The pH inside the resealed ghosts was 6.0 in all samples. The pH outside the resealed ghosts was 6.0 for spectrum sets a−c, whereas the pH value outside of the resealed ghosts was 8.0 for spectrum sets d−f. No inhibitor was present in the NaCl media for spectrum sets a and d. For spectrum sets b and e, the suspension medium contained 0.1 mM HMA, whereas for spectrum sets c and f, 0.1 mM phloretin was present.

Table 2: Li^+ Efflux Rates [in mmol of Li^+/(L of Ghosts h)] across H^+- and Li^+-Loaded Resealed RBC Ghosts in NaCl Medium with and without Inhibitors[a]

pH_{out}	without inhibitor	with 0.1 mM HMA	with 0.1 mM phloretin
6.0	0.81 ± 0.09	0.49 ± 0.08	0.41 ± 0.06
8.0	0.48 ± 0.09	0.28 ± 0.05	0.25 ± 0.04

[a] The composition of the NaCl media was 120 mM NaCl, 10 mM glucose, 0.1 mM ouabain, 3 mM Na_3H_2TmDOTP·3NaCl, and either 10 mM Tris-MES for pH 6.0 or 10 mM Tris-MOPS for pH 8.0. The hematocrit and the intracellular pH_{in} values were 20% and 6.0, respectively, for all samples.

changes in ^{23}Na NMR relaxation time values shown in Figure 2 are not associated either with variations in intracellular Na^+ concentration or with the magnetic properties of hemoglobin.

Na^+, Li^+, and H^+ Interactions with Exposed RBC Membranes. As shown in Figure 3, ^{23}Na T_1 relaxation times increased as the pH decreased in RBC membrane suspensions with or without LiCl. For the same pH values, the ^{23}Na T_1 relaxation times were larger in the presence of 3 mM LiCl than in its absence; the observed increases in ^{23}Na T_1 values are not due to dilution because a small volume (15 μL) from a 0.5 M LiCl stock solution was added to 2.5 mL of a suspension of RBC membranes. The ^{23}Na T_1 data shown in Figure 3 are representative of two RBC membrane suspensions obtained from the same blood batch; for both samples, the ^{23}Na T_1 values were significantly larger in the presence of 3 mM LiCl than in its absence for every pH value studied (Student's paired *t*-test, $p < 0.05$). A similar pH dependence was observed in ^{7}Li T_1 values for RBC membrane suspensions containing 3 mM LiCl (data not shown). Whereas the percentage change in the ^{23}Na T_1 values measured in RBC membrane suspensions containing NaCl (but no LiCl) was of the order of 20% in the pH range studied, the corresponding percentage change for the ^{7}Li T_1 values measured in RBC membrane suspensions containing LiCl was in the range of 40% to 50%.

Na^+ Visibility in the RBC Membranes. The visibility of the ^{23}Na NMR resonance in RBC membrane suspensions was determined by comparison of the signal intensities of Na^+ in RBC membranes with those in water solutions at the same Na^+ concentrations. Table 3 shows the results of two trials for the Na^+ visibility and the ^{23}Na T_1 values in RBC membranes without and with 150 mM LiCl present. When no LiCl was present, the ^{23}Na NMR resonance was not 100% visible. The lower the Na^+ concentration, the lower the visibility. The ^{23}Na T_1 values at lower Na^+ concentrations were lower than the free ^{23}Na T_1 value when no LiCl was present. Upon addition of 150 mM LiCl, the ^{23}Na T_1 values were close to the free ^{23}Na T_1 value, and Na^+ was almost fully visible at any Na^+ concentration. The small decrease in the visibility of the ^{23}Na NMR resonance observed in RBC membrane suspensions containing 150 mM LiCl and high concentrations of NaCl (Table 3) is presumably associated with competition between Na^+ and Li^+ binding to RBC membranes under these conditions.

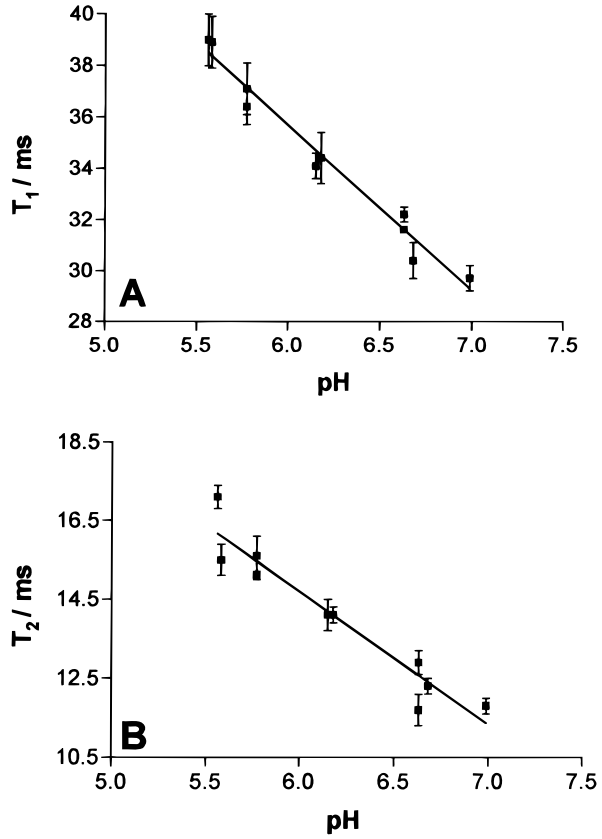

FIGURE 2: pH dependence of ^{23}Na T_1 (A) and of ^{23}Na T_2 (B) values for packed acid-loaded RBCs.

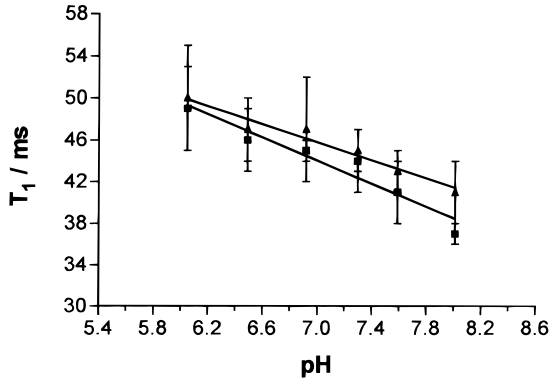

FIGURE 3: pH dependence of ^{23}Na T_1 relaxation times. NaCl at 3 mM was added to each membrane sample prior to measurement of the ^{23}Na T_1 values (squares). After these measurements, 3 mM LiCl was added from a 0.5 M LiCl stock solution to the same membrane samples and the ^{23}Na T_1 values were remeasured (triangles). The protein concentration in the membrane samples before and after addition of LiCl was 6.58 ± 0.80 mg/mL.

Because Na^+ was not 100% visible by ^{23}Na NMR, the Na^+ binding constants generated from the observed ^{23}Na T_1 values by use of James−Noggle plots are not the actual Na^+ binding constants. We measured the ^{7}Li T_1 relaxation times with increasing Li^+ concentrations in the presence of various concentrations of Na^+ (data not shown). Because Na^+ and Li^+ bind competitively to RBC membranes, the actual Na^+ binding constant, $K_{Na(a)}$, was calculated from eq 8. The $K_{Na(a)}$ value was 618 M^{-1} ($r^2 = 0.94$). By using ^{23}Na NMR with the same membrane preparation, we calculated the observed Na^+ binding constant, $K_{Na(o)}$, from eq 7 and found it to be 254 M^{-1} ($r^2 = 0.98$). We used five batches of blood to obtain the correction curve shown in Figure 4. We used

Table 3: Visibility of the ^{23}Na Resonance and ^{23}Na T_1 Values in RBC Membrane Suspensions[a]

	membranes without LiCl		membranes with 150 mM LiCl	
$[Na^+]$/mM	visibility (%)	T_1 (ms)	visibility (%)	T_1 (ms)
4	52.8 ± 0.4	39.9 ± 3.0	100 ± 3.0	61.4 ± 1.0
12	78.6 ± 0.5	46.9 ± 2.0	99.8 ± 5.0	62.5 ± 2.0
20	95.2 ± 3.2	50.5 ± 0.6	96.0 ± 5.6	63.8 ± 2.0
500	91.6 ± 2.8	65.1 ± 0.4	92.4 ± 2.1	65.3 ± 0.2

[a] The values shown are the average of the values obtained from two separate experiments conducted with RBC membranes obtained from the same blood batch. The errors shown indicate the range of values obtained.

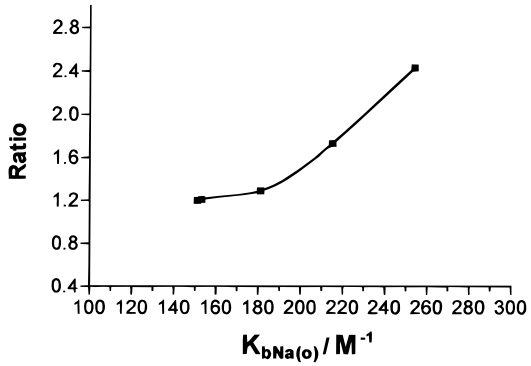

FIGURE 4: Correction factors, as defined by eq 9, for Na^+ binding constants to RBC membranes. The pH value was 7.6 ± 0.2, and the protein concentration was 6.11 ± 1.70 mg/mL for all membrane samples.

the correction factors r to correct the observed Na^+ binding constants ($K_{Na(o)}$) and then to calculate the amiloride binding constants shown below to RBC membrane suspensions containing Na^+.

Inhibitory Effects of Amiloride and Its Analogues and of Phloretin on Na^+ and Li^+ Binding to RBC Membranes. The inhibitory effects of amiloride and its analogues, EIPA, HMA, and MIA, on both Na^+ and Li^+ interacting with RBC membranes were tested by measurements of ^{23}Na and ^{7}Li T_1 values. All of these inhibitors increased both the ^{23}Na and the ^{7}Li T_1 values by inhibiting both Na^+ and Li^+ binding to the RBC membranes (data not shown). HMA had the highest inhibition activity; the effects of all inhibitors were, however, of the same order of magnitude.

Figure 5 shows ^{23}Na and ^{7}Li T_1 measurements in RBC membrane suspensions containing increasing concentrations of Na^+ or Li^+ in the presence of various concentrations of amiloride (0, 0.5, 1.0, 1.5, 2.0, and 2.5 mM). For a given amiloride concentration, the ^{23}Na or the ^{7}Li T_1 values increased as the Na^+ or the Li^+ concentrations increased. For the same Na^+ or Li^+ concentrations, as the amiloride concentration increased, both the ^{23}Na and the ^{7}Li T_1 values increased. The percentage changes in ^{23}Na T_1 values upon addition of amiloride were smaller than those observed for ^{7}Li T_1 values, presumably because Na^+ binds more weakly to the RBC membrane than does Li^+ (Canessa et al., 1988). Although phloretin at 6.0 mM increased the ^{7}Li T_1 values of Li^+-treated RBC membrane suspensions by 32%, it showed no significant effect on the ^{23}Na T_1 values in Na^+-treated RBC membranes (data not shown). Phloretin is a transport inhibitor which is not as specific as amiloride for Na^+-H^+ exchange, and it presumably binds more weakly to the RBC membrane than does amiloride, resulting in no appreciable effect on the weakly bound Na^+.

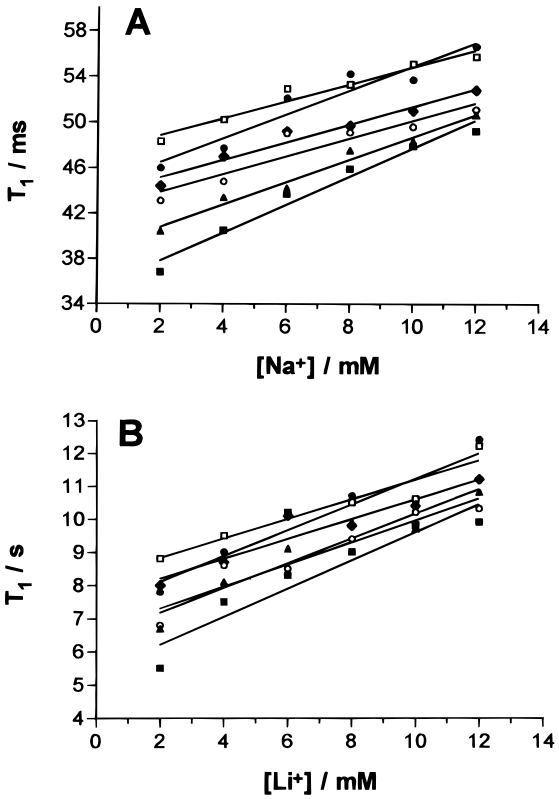

FIGURE 5: ^{23}Na (A) and ^7Li (B) T_1 measurements with 0 (solid squares), 0.5 (triangles), 1.0 (circles), 1.5 (diamonds), 2.0 (dots), and 2.5 mM (open squares) amiloride. The protein concentration in the RBC membrane suspensions was 7.03 ± 1.44 mg/mL, and the pH value was 8.0. The errors in the T_1 measurements are within 10%.

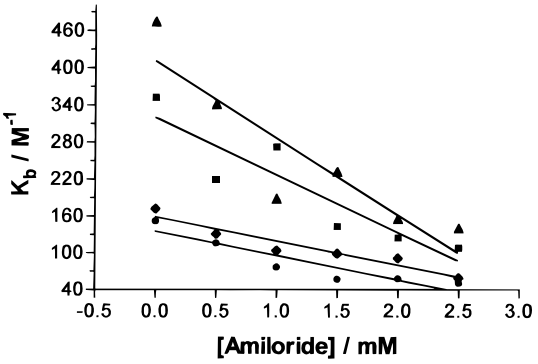

FIGURE 6: Amiloride inhibitory activities on Na^+ and Li^+ binding to RBC membranes. Squares, $K_{Na(a)}$, pH 7.0; triangles, $K_{Na(a)}$, pH 8.0; dots, K_{Li}, pH 7.0; diamonds, K_{Li}, pH 8.0.

In the absence of RBC membrane, the ^{23}Na T_1 (70 ± 2 ms) and the ^7Li T_1 (21 ± 1 s) values were the same in the absence or presence of 6.0 mM amiloride or of 6.0 mM phloretin, indicating that there was no specific interaction between Na^+ or Li^+ and amiloride or phloretin.

The ^{23}Na $T_{1(free)}$ values for calculation of the apparent Na^+ binding constants at pH 7.0 and pH 8.0 were 67.1 ± 0.8 and 65.8 ± 0.4 ms, respectively. The ^7Li $T_{1(free)}$ values for calculating the Li^+ binding constants at pH 7.0 and pH 8.0 were 20.6 ± 0.9 and 21.0 ± 0.9 s, respectively. From each James−Noggle plot, we calculated the Na^+ and the Li^+ binding constants at each amiloride concentration by using eq 7. The Na^+ binding constants were actual Na^+ binding constants which we obtained by multiplying the observed Na^+ binding constants by the corresponding correction factors from the curve in Figure 4. At both pH values of 7.0 and 8.0, both the Na^+ and the Li^+ binding constants decreased when the amiloride concentration increased, as shown in Figure 6.

The amiloride binding constants to RBC membranes with Na^+ present were 731 ± 147 M^{-1} (mean ± range); $n = 2$ at pH 7.0, and 1021 ± 48 M^{-1}; $n = 2$ at pH 8.0. With Li^+ present, the amiloride binding constants were 716 ± 27 M^{-1}; $n = 2$ at pH 7.0, and 964 ± 40 M^{-1}; $n = 2$ at pH 8.0. The amiloride binding constants calculated from either Na^+ or Li^+ binding studies in RBC membrane suspensions were larger at pH 8.0 than at pH 7.0. For the two pH values studied, the average values of the amiloride inhibition constants to RBC membranes (obtained from duplicate membrane preparations) with either Na^+ or Li^+ present in the suspension media were similar. Amiloride therefore has similar inhibitory effects on binding of both Na^+ and Li^+ to RBC membranes.

DISCUSSION

The osmolarity inside normal red blood cells is 290 mosM. To maintain the cells intact in the process of transport experiments, one has to maintain the osmolarities in the media at 300 ± 10 mosM. Therefore, the highest Na^+ concentration that could be used in the suspension media was 150 mM. Prior to 1990, the maximal rates of Na^+-Li^+ exchange in human RBCs were generally assayed by subtraction of the Li^+ efflux rates of Li^+-loaded RBCs in an Na^+-free medium from the rates in 150 mM NaCl medium (Canessa et al., 1980). Mechanistic studies of RBC Na^+-Li^+ exchange have indicated that the RBC membrane transport protein is asymmetric, with higher Na^+ and Li^+ affinities on the intracellular than on the extracellular side of the membrane (Hannert & Garay, 1986; Sarkadi et al., 1978). The dissociation constants (K_m) for extracellular Na^+ are, at least for some individuals, of the same order of magnitude as the extracellular Na^+ concentration (140 mmol/L) used in the standard transport assay, suggesting that the RBC Na^+-Li^+ exchange protein is far from saturated with Na^+ on the extracellular side of the RBC membrane. The rates under standard transport assay conditions may not, therefore, be maximal rates of RBC Na^+-Li^+ exchange; variations in Na^+ affinity (K_m) and maximal velocity (V_{max}) could change the observed rates. Only by varying the Na^+ concentration in an isotonic suspension medium can one measure the true kinetic parameters of RBC Na^+-Li^+ exchange (Aronson, 1990; Rutherford et al., 1990, 1992).

It was reported that amiloride had no effect on Na^+-Li^+ exchange in human intact RBCs (Carr et al., 1988; Kahn, 1987). However, the inhibitory effects of amiloride and its analogues were reported only for the standard rates of Na^+-Li^+ exchange. There have been no reports about the effects of amiloride on V_{max} and K_m. As was noted in previous studies, we found that amiloride and HMA at 0.1 mM concentration had no significant effects on the V_{std} values; we noted, however, that amiloride significantly decreased the V_{max} value and that HMA had significant effects on both V_{max} and K_m values (Table 1). HMA had greater inhibitory effects than did amiloride, in agreement with previous reports (Kleyman & Cragoe, 1988; Simchowitz & Cragoe, 1986). Gende and Cingolani (1993) found that EIPA blocked the exchanges of Li^+ or Na^+ with H^+. They also found that

thrombin, an activator of Na^+-H^+ exchange, could stimulate Li^+ as well as Na^+ exchange (Gende & Cingolani, 1993). All of these results suggest that the Na^+-Li^+ exchange protein is very likely to be the same as the Na^+-H^+ exchange protein.

Measurements of Li^+ movements across resealed RBC ghosts, and not through intact RBCs, simplify the study of RBC transport proteins. If Na^+-H^+ and Na^+-Li^+ exchange are mediated by the same transport protein, one would expect that they would compete against each other for interaction with the same membrane transport protein. The observed Li^+ efflux rates were significantly lower when H^+ efflux occurred simultaneously with and without inhibitors (Figure 1 and Table 2). This means that H^+ and Li^+ competed for binding to the same membrane protein to be transported out of the ghosts. Previous studies on interactions of lithium and protons with the sodium−proton exchanger in intact RBCs indicated that lithium behaved like protons (Parker, 1986). Both Li^+ and H^+ can stimulate Na^+-H^+ exchange when they are loaded inside cells, and they can inhibit Na^+-H^+ exchange when they are added to the exterior of the cells (Parker, 1986).

Phloretin significantly decreased the Li^+ efflux rates no matter whether or not there was a pH gradient (Figure 1 and Table 2); this observation is consistent with previous work (Pandey et al., 1978). HMA, which is a specific Na^+-H^+ exchange inhibitor, also significantly inhibited Li^+ efflux across resealed RBC ghosts. This result is in agreement with the inhibitory effect of HMA on Na^+-Li^+ exchange in intact RBCs and, furthermore, suggests that Na^+-Li^+ exchange and Na^+-H^+ exchange are mediated by the same membrane protein. In our ghost experiments (Figure 1 and Table 2), intraghost Li^+ exchanges with extraghost Na^+ in the medium; the inhibitors HMA and phloretin are present primarily in the medium. When a pH gradient was present, the competition between intraghost H^+ and Li^+ was presumably larger than the differences in the binding activities of the extraghost inhibitors at different pH values.

By using resealed RBC ghosts, one can avoid binding of hemoglobin to H^+ and alkali metal ions, such as Li^+ and Na^+. However, the membrane constituents might change during the preparation, especially in the process of hemolysis. Although no lipids are lost during the preparation of resealed ghosts, a considerable structural change occurs (Schwoch & Passow, 1973). The fluidity of the phospholipid bilayer also changes. Some enzymes and enzyme activities might be lost during the preparation. By keeping the osmolarity in the range of 60−80 mosM, one can retain most of the enzymes and the enzyme activities (Schwoch & Passow, 1973). Ionic strength and pH also affect the loss of membrane proteins and their structures (Bodemann & Passow, 1972; Schwoch & Passow, 1973). To remove most of the hemoglobin and keep the membranes resealable, one must maintain the temperature at 0−2 °C during hemolysis. However, resealed RBC ghosts still contain primary cell membranes and maintain most of the characteristics of the native erythrocyte membranes (Klonk & Deuticke, 1992). Despite these sample limitations, the data that we report here for Li^+ efflux across resealed RBC ghosts still provide strong evidence that Na^+-H^+ exchange and Na^+-Li^+ exchange are mediated by the same membrane protein.

The rate of translocation of ions from the cis to the trans sides of membranes, and the rates of uptake and release from opposite sides of the RBC membrane depend mainly on the interactions between ions and the RBC membranes. Exposure of white ghosts (exposed RBC membranes) to ions makes it possible to test these interactions directly. There are two states in which ions can exist in biological systems: in free motion or in slow motion. NMR T_1 relaxation measurements, in particular, provide information about molecular motions. The more ions are bound to the membranes, the lower the T_1 value. As shown in Figure 3, the ^{23}Na T_1 values increased as the pH decreased in either the presence or the absence of 3 mM LiCl. Similar trends in pH dependence were observed by ^7Li T_1 measurements with RBC membrane suspensions containing 3 mM LiCl. H^+ replaced Na^+ (or Li^+) in binding to the membranes and left more free Na^+ (or Li^+). The lower the pH, the larger the fraction of free Na^+ (or Li^+). For the same pH values, the ^{23}Na T_1 values were larger in the presence of Li^+ than its absence, because Li^+ occupied most membrane-binding sites, leaving more free Na^+ in the suspension medium. Because Na^+ binds more weakly than does Li^+ to RBC membranes (Canessa et al., 1988), the pH-induced changes in ^{23}Na NMR relaxation times were, not surprisingly, smaller than those observed for ^7Li. From the data in Figure 3, we can conclude that H^+, Li^+, and Na^+ competed among each other for binding to RBC membranes. Because the pH-induced changes in the ^{23}Na and ^7Li NMR relaxation values were observed both in acid-loaded packed RBCs (Figure 2) and in RBC membrane suspensions (Figure 3), we conclude that one can use NMR relaxation measurements to understand the competition between Na^+ or Li^+ and H^+ for binding sites in the human RBC membrane.

It has been shown that lithium is 100% visible in ^7Li NMR experiments on RBC suspensions by comparing the measurements of intracellular lithium concentrations with both atomic absorption and NMR methods (Mota de Freitas et al., 1990). In intact human RBCs, however, the visibility of the $^{23}Na^+$ NMR resonance is only 75% (Nissen et al., 1989). In RBC membrane supensions, the visibility of the NMR resonance increased as the Na^+ concentration increased (Table 3). The lower the Na^+ concentration, the lower the visibility. Sodium is a quadrupolar nucleus with a nuclear spin of $^3/_2$, which is typically under conditions of extreme narrowing (Bull, 1972). When sodium ions are exposed to RBC membrane suspensions, the ions experience two states of motion and undergo exchange between two sites, free and bound. One site is the hydrated ion in aqueous solution which is under the extreme narrowing condition and is called the narrow component, and the other site is the membranous peptide binding site which is not under the extreme narrowing condition and is called the broad component (Bull, 1972; Urry et al., 1989).

The visibility of the ^{23}Na signal is also dependent on instrumental parameters (Pekar and Leigh, 1986). Under our experimental conditions, the sum of the pulse width (27 μs) with the preacquisition delay and offset parameters α (20 μs) and R of 2 (10 μs) is small compared with the relaxation time T_{2f} (of the order of 200 μs); we may, however, have lost signal from the early part of the free induction decay. The observed T_1 values are therefore dominated by the T_{1s} values, which, in turn, are weighted averages of the values for free and bound Na^+ (Bull, 1972; Urry et al., 1989). The observed increases in T_1 values after addition of 150 mM LiCl confirmed the release of bound sodium (Table 3). Once

the bound sodium was released by lithium, sodium was almost 100% visible by ^{23}Na NMR spectroscopy.

We generated a correction curve in the $K_{Na(0)}$ range 150−260 M^{-1} (Figure 4). For lower binding constants, the correction factors were close to 1.0, and the actual Na^+ binding constants were close to the observed values of the Na^+ binding constants. The interactions between Na^+ and the membranes were weak at the lower binding constants, and there was a large fraction of free sodium. For larger binding constants, however, the correction factors were significantly greater than 1.0 because of the larger fraction of invisible bound Na^+.

Amiloride is a weak base with a pK_a value of 8.7 (Kleyman & Cragoe, 1988). Much work has been done on the inhibitory effects of amiloride analogues on Na^+-H^+ exchange in intact RBCs (Kleyman & Cragoe, 1988; Simchowitz & Cragoe, 1986; Vigne et al., 1983). Studies on structure−activity relationships of amiloride analogues indicated that the guanidinium moiety of amiloride is the active group that recognizes and binds to the Na^+ transport site of the exchanger (Simchowitz & Cragoe, 1986). Increasing the number of carbon atoms in the alkyl chain at the 5-N position increased the activity (Simchowitz & Cragoe, 1986). The inhibitory activity can be ascertained from the apparent K_I value. Thus, the lower the apparent K_I, the higher the activity. For Na^+-H^+ exchange in intact RBCs, the apparent K_I values of amiloride, HMA, EIPA, and MIA are 83.8, 0.16, 0.38, and 0.44 μM, respectively (Kleyman & Cragoe, 1988; Simchowitz & Cragoe, 1986).

In this study, we measured both ^{23}Na and ^7Li T_1 values with varied concentrations of these inhibitors (data not shown). All of these inhibitors decreased the interaction of Na^+ with the membranes, in agreement with a recent report (Spruth et al., 1995) which showed that amiloride derivatives and Na^+ compete for a common binding site. We also found that all of these inhibitors inhibited the interaction of Li^+ with the membranes. The effects of these inhibitors were of the same order of magnitude, although HMA had the highest effect. Because of its availability, we chose amiloride to determine its inhibitory constants in Na^+ and Li^+ binding studies of RBC membranes. Senyk et al. (1995) reported that the inhibitory constants for amiloride and EIPA are 8 and 1 μM, respectively, in rabbit alveolar type-II Na^+ channels reconstituted in planar lipid bilayers.

^{23}Na or ^7Li T_1 values increased as the Na^+ or the Li^+ concentration increased in RBC membrane suspensions containing increasing amiloride concentrations (Figure 5), because there was more free Na^+ or Li^+ as the Na^+ or Li^+ concentration increased. At the same Na^+ or Li^+ concentrations, the ^{23}Na or the ^7Li T_1 values increased as the amiloride concentration increased; a less clear dependence of ^{23}Na or ^7Li T_1 measurements on amiloride concentration was observed at high Na^+ or Li^+ concentrations because the fractions of free ions are significantly larger. This indicated that amiloride inhibited both Na^+ and Li^+ binding to RBC membranes, leaving more free Na^+ or Li^+ in the suspension media. Not surprisingly, both the Na^+ and the Li^+ binding constants decreased as the amiloride concentration increased at pH values of both 7.0 and 8.0 (Figure 6). Both Na^+ and Li^+ binding constants were higher at pH 8.0 than at pH 7.0 (Figure 6) because H^+ competed with both Na^+ and Li^+ for binding to RBC membranes (see Results). Another reason for this observation is that the inhibitory effect of amiloride is higher at pH 7.0 than at pH 8.0. Amiloride is a weak base, and only its protonated form can interact with the membrane protein (Kleyman & Cragoe, 1988). Therefore, the lower the pH, the higher the inhibitory activity.

K_{Ami} is a measure of the interaction between amiloride and the RBC membrane exchange protein. We (see Results) and others (Canessa et al., 1988) found that Na^+ binds more weakly than does Li^+ to RBC membranes. No matter how strongly or weakly Na^+ or Li^+ ions bind to the RBC membrane protein, if they bind to the same protein, $K_{Ami(Na)}$ and $K_{Ami(Li)}$ are expected to be the same, as found in this study. Amiloride had the same inhibitory effects on both Na^+ and Li^+ binding to the RBC membranes, once again suggesting that Na^+-Li^+ exchange and Na^+-H^+ exchange are mediated by the same RBC membrane protein.

The amiloride binding constants to the exposed RBC membranes at pH 7.0 and 8.0 are much lower than the reciprocal of the amiloride apparent K_I values for Na^+-H^+ exchange in intact RBCs. Several reasons can explain this difference. Amiloride is known to have a high blockage effect when it is applied to the outer face of membranes but has no effect when it is applied to the inner face (Small & Morris, 1995). When amiloride was applied to both sides of the membranes, the inhibitory effect must have been lower than the effect when it was applied to only the outside of the membranes. Although RBC membranes maintain the characteristics of the native erythrocyte membranes, the Na^+-H^+ and Na^+-Li^+ exchange proteins might change in amount and structure after lysis from intact RBCs. The distribution, the amount, and the structure of the protein in the exposed membranes might be different from that in intact RBCs, resulting in different inhibitory effects for amiloride. The amiloride inhibitory activity also varies from one to another isoform of the Na^+-H^+ exchanger. For example, the 50% inhibition concentration values for amiloride and EIPA of the NHE mutant are significantly larger than that of the wild type NHE1 (Wang et al., 1995).

ACKNOWLEDGMENT

We are grateful to the Loyola University Center for Instructional Design for the preparation of the figures included in this article. The authors are also grateful to Professor Carlos Geraldes (University of Coimbra, Portugal) and Dr. Ravichandran Ramasamy (University of California, Davis) for useful suggestions during the course of this project.

REFERENCES

Aronson, J. K. (1990) *Clin. Sci. 78*, 247−254.
Bodemann, H., & Passow, H. (1972) *J. Membr. Biol. 8*, 1−26.
Bollag, D. M., & Edelstein, S. J. (1991) in *Protein Methods*, pp 50−55, Wiley-Liss, New York.
Bull, T. E. (1972) *J. Magn. Reson. 8*, 344−353.
Canessa, M. (1989) *Methods Enzymol. 173*, 176−191.
Canessa, M., Adragna, N., Solomon, H. S., Connolly, T. M., & Tosteson, D. C. (1980) *N. Engl. J. Med. 302*, 772−776.
Canessa, M., Morgan, K., Semplicini, A. (1988) *J. Cardiovasc. Pharmacol. 12*, S92−S98.
Canessa, M., Zerbini, G., & Laffel, L. M. B. (1992) *J. Am. Soc. Nephrol. 3*, S41−S49.
Carr, S., Thomas, T. H., & Wilkinson, R. (1988) *Clin. Chim. Acta 178*, 51−58.
Duhm, J., Eisenried, F., Becker, B. F., & Greil, W. (1976) *Pflügers Archiv. 364*, 147−155.
Fanger, B. O. (1987) *Anal. Biochem. 162*, 11−17.

Gadian, D. G. (1982) in *NMR and Its Applications to Living Systems*, pp 109−122, Claredon Press, Oxford, U.K.
Gende, O. A., & Cingolani, H. E. (1993) *Biochim. Biophys. Acta 1152*, 219−224.
Hannaert, P. A., & Garay, R. P. (1986) *J. Gen. Physiol. 87*, 353−368.
Hilton, P. (1986) *N. Engl. J. Med. 314*, 222−229.
Houtman, P. N., Shah, V., & Dillon, M. J. (1993) *Acta Paediatrica 82*, 1057−60.
James, T. L., & Noggle, J. H. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 644−649.
Jennings, M. L., Adams-Lackey, M., & Cook, K. W. (1985) *Am. J. Physiol. 249*, C63−C68.
Kahn, A. M. (1987) *Hypertension 9*, 7−12.
Kahn, A. M., Allen, J. C., Cragoe, Jr., E. J., & Shelat, H. (1989) *Circ. Res. 65*, 818−828.
Kleyman, T. R., & Cragoe, E. J., Jr. (1988) *J. Membr. Biol. 105*, 1−21.
Klonk, S., & Deuticke, B. (1992) *Biochim. Biophys. Acta 1106*, 126−136.
Lifton, R. P. (1993) *Curr. Opin. Nephr. Hypertens. 2*, 258−264.
Mota de Freitas, D., Espanol, M. T., Ramasamy, R., & Labotka, R. J. (1990) *Inorg. Chem. 29*, 3972−3979.
Nakhoul, N. L., & Boron, W. F. (1988) in *Na^+−H^+ Exchange* (Grinstein, S., Ed.) pp 167−178, CRC Press, Inc., Boca Raton, FL.
Nissen, H., Jacobsen, J. P., & Horder, M. (1989) *Magn. Reson. Med. 10*, 388−398.
Pandey, G. N., Sarkadi, B., Haas, M., Gunn, R. B., Davis, J. M., & Tosteson, D. C. (1978) *J. Gen. Physiol. 72*, 233−247.
Parker, J. G. (1986) *J. Gen. Physiol. 87*, 189−200.
Pekar, J., & Leigh, J. S., Jr. (1986) *J. Magn. Reson. 69*, 582−584.
Potts, W. T,. W. (1994) *Am. J. Physiol. 266*, R315−R320.
Rutherford, P. A., Thomas, T. H., & Wilkinson, R. (1990) *Clin. Sci. 79*, 365−369.
Rutherford, P. A., Thomas, T. H., & Wilkinson, R. (1992) *Clin. Sci. 82*, 291−299.
Sarkadi, B., Alifimoff, J. K., Gunn, R. B., & Tosteson, D. C. (1978) *J. Gen. Physiol. 72*, 249−265.
Schwoch, G., & Passow, H. (1973) *Mol. Cell. Biochem. 2*, 197−218.
Semplicini, A., Mozzato, M., Sama, B., Nosadini, R., Fioretto, P., Trevisan, R., Pessina, A. C., Crepaldi, G., & Palu, C. D. (1989a) *Am. J. Hypertens. 2*, 174−177.
Semplicini, A., Canessa, M., Mozzato, M. C., Ceolotto, G., Marzola, M., Buzzaccarini, F., Casolino, P., & Pessina, A. C. (1989b) *Am. J. Hypertens. 2*, 903−908.
Senyk, O., Ismailov, I., Bradford, A. L., Baker, R. R., Matalon, S., & Benos, D. J. (1995) *Am. J. Physiol. 37*, C1148−C1156.
Simchowitz, L., & Cragoe, E. J., Jr. (1986) *Mol. Pharmacology 30*, 112−120.
Small, D. L., & Morris, C. E. (1995) *Br. J. Pharmacol. 114*, 180−186.
Spruth, M., Reidlinger, J., & Muller, V. (1995) *Biochim. Biophys. Acta 1229*, 96−102.
Steck, T. L., & Kant, J. A. (1974) *Methods Enzymol. 31*, 172−180.
Urry, D. W., Trapane, T. L., Venkatachalam, C. M., & McMichens, R. B. (1989) *Methods Enzymol. 171*, 286−342.
Vigne, P., Frelin, C., Cragoe, E. J., Jr., & Lazdunski, M. (1983) *Mol. Pharmacol. 25*, 131−136.
Wang, D. H., Balkovetz, D. F., & Warnock, D. G. (1995) *Am. J. Physiol. 38*, C392−C402.
Williams, R. R., Hunt, S. C., Hopkins, P. N., Wu, L. L., & Lalouel, J. M. (1994) *Clin. Genet. 46*, 80−87.

BI960814L