Amiloride-Insensitive Na\textsuperscript{+}–H\textsuperscript{+} Exchange: A Candidate Mediator of Erythrocyte Na\textsuperscript{+}–Li\textsuperscript{+} Countertransport

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Abstract. Erythrocyte Na\textsuperscript{+}–Li\textsuperscript{+} countertransport shows an increased activity in essential hypertension and diabetic nephropathy, but its nature remains unknown. This amiloride-insensitive membrane transport may not be a mode of operation of the amiloride-sensitive NHE1, the only Na\textsuperscript{+}–H\textsuperscript{+} exchange isoform found in human erythrocytes. Whether an independent, although unknown, amiloride-insensitive isoform mediates Na\textsuperscript{+}–Li\textsuperscript{+} countertransport is unclear. Na\textsuperscript{+}–H\textsuperscript{+} exchange activity was measured in acid-loaded erythrocytes. Dimethylamiloride, a specific inhibitor of Na\textsuperscript{+}–H\textsuperscript{+} exchange and phloretin, a known inhibitor of Na\textsuperscript{+}–Li\textsuperscript{+} countertransport, gave a reduction in H\textsuperscript{+}-driven Na\textsuperscript{+} influx (by 31 and 37%, respectively). This effect was additive, and a 66% reduction in H\textsuperscript{+}-driven Na\textsuperscript{+} influx was found in the presence of both inhibitors. Internal acidification, a stimulus for Na\textsuperscript{+}–H\textsuperscript{+} exchange, enhanced Na\textsuperscript{+}–Li\textsuperscript{+} countertransport activity (from 287 ± 55 to 1213 ± 165 μmol·L\textsubscript{cell}\textsuperscript{-1}·h\textsuperscript{-1}, mean ± SEM, P = 0.003). This transport remained sensitive to phloretin under both conditions. Conversely, external acidification decreased Na\textsuperscript{+}–Li\textsuperscript{+} countertransport activity (as expected for a Na\textsuperscript{+}–H\textsuperscript{+} exchanger). Competition between internal H\textsuperscript{+} and Li\textsuperscript{+} or Na\textsuperscript{+} for a common binding site was present. Finally, similar kinetic parameters for external Na\textsuperscript{+} characterized Na\textsuperscript{+}–Li\textsuperscript{+} countertransport and the phloretin-sensitive component of H\textsuperscript{+}-driven Na\textsuperscript{+} influx. These findings suggest that both Na\textsuperscript{+}–Li\textsuperscript{+} countertransport and the amiloride-insensitive, phloretin-sensitive component of H\textsuperscript{+}-driven Na\textsuperscript{+} influx can be mediated by a previously unrecognized novel amiloride-insensitive Na\textsuperscript{+}–H\textsuperscript{+} exchange isoform in human erythrocytes.

Erythrocyte Na\textsuperscript{+}–Li\textsuperscript{+} countertransport (SLC) is a membrane transport that mediates a bidirectional 1:1 exchange of Li\textsuperscript{+} for Na\textsuperscript{+} (1–3). This exchanger is an established property of the erythrocyte of humans and of several animal species (1,4). The prospective relevance of this membrane function follows the observation that elevated activity rates of SLC are a consistent feature of essential hypertension (5–8) and may also characterize diabetic nephropathy (9,10), under a prominent although probably the best characterized intermediate phenotype of essential hypertension (11,13), its nature and role in human physiology are still unclear.

Recent molecular cloning studies have identified an NHE gene family from which five isoforms have been cloned and sequenced in humans (NHE1, 2, 3, 4, and 5). Functionally, NHE isoforms show a range of sensitivity to amiloride and its analogues (24–27), and it is theoretically possible that an amiloride-resistant isoform of NHE might account for the activity of SLC.

Although only the amiloride-sensitive NHE1 isoform has been presently identified in human erythrocytes (28–31), the idea that an otherwise unrecognized, amiloride-insensitive NHE isoform might characterize the membrane of the erythrocyte, as suggested for other kinds of cells (32–34), cannot be ruled out. This possibility would be in line with functional evidence that H\textsuperscript{+}-driven Na\textsuperscript{+} influx in human erythrocytes is only partially (59 ± 10%) inhibited by amiloride (35–37).

To clarify whether SLC activity is mediated by an amiloride-insensitive NHE in human erythrocytes, we tested the effect of phloretin, the strongest inhibitor of SLC (4,20,38), on the amiloride-insensitive component of H\textsuperscript{+}-driven Na\textsuperscript{+} influx in these cells. Furthermore, we investigated whether the activity of SLC, as expected for a mode of operation of NHE, could be activated by an established stimulus of this transport, such as internal acidification, and consequently reduced by an inhibitor, such as external acidification (14,39). These studies led to the identification of an amiloride-insensitive, phloretin-sensitive component of NHE and proved that SLC is regulated by pH\textsubscript{i}, in line with the possibility that both transports may be mediated by a single carrier. Thus, we focused on the functional characteristics of these exchanges, evaluated the interaction of different H\textsubscript{i}, Li\textsubscript{i}, and Na\textsubscript{i} concentrations on SLC
activity, and measured \( N_a \) kinetics of SLC and of the phlorotretin-sensitive component of NHE. Finally, the effect of valinomycin (known to alter the membrane potential) (16,40) and 4,4'-di-isothiocyanostilbene-2,2'-disulfonic acid (DIDS) (known to activate NHE4) (41,42) on the activity of these two exchanges was also tested.

**Materials and Methods**

**Reagents**

Ouabain, bumetanide, DIDS, dimethylamiloride (DMA), nystatin, valinomycin, \( \text{Me}_2\text{SO} \), Tris, \( 3-(N\text{-morpholino})\text{propanesulfonic acid (MOPS)} \), 2-\( (N\text{-morpholino})\text{ethanesulfonic acid (MES)} \), and bovine serum albumin (BSA) were purchased from Sigma Aldrich (Milan, Italy). LiCl monohydrate was purchased from Merck (Darmstadt, Germany). All other reagents and chemicals were purchased from BDH Laboratory Supplies (Milan, Italy).

**\( N^+ \) Influx Studies**

**\( \text{H}^+ \)-Driven \( N^+ \) Influx: Activation Curve for \( H^+ \).** \( N^+ \)--\( H^+ \) exchange was estimated as net \( N^+ \) influx into \( N^+ \)-depleted, acid-loaded cells driven by an outward proton gradient according to the technique described by Escobales and Canessa (36). Briefly, after the removal of plasma anduffy coat, erythrocytes were washed four times at 4°C in a neutral (pH 7.4) 10 mM Tris-MOPS buffer containing 150 mM choline chloride and 1.0 mM MgCl\(_2\). Aliquots of this suspension were then used for the determination of hematocrit, hemoglobin, and \( N_a \) concentration. Erythrocytes were then \( N^+ \)-depleted by incubating a 15% hematocrit suspension for 20 min at 4°C in an unbuffered solution containing 40 \( \mu \text{g} \)/ml nystatin, 50 mM sucrose, and 150 mM KCl. After centrifugation, the cell pellet was resuspended for 10 min in the same solution, although without nystatin. The ionophore was removed by washing cells four times at 37°C in a 1 mM K-phosphate buffer (pH 7.4) containing 50 mM sucrose, 150 mM KCl, 10 mM glucose, and 1 mg/L BSA.

\( N^+ \)-depleted cells (10% hematocrit) were incubated at 37°C in six different acid-loading solutions containing 0.15 mM MgCl\(_2\), 0.1 mM ouabain, 0.1 mM bumetanide, and 10 mM glucose in a 20 mM Tris-MES buffer for pH lower than 7.0 or Tris-MOPS buffer for pH higher than 7.0 adjusted to pH 5.6, 6.0, 6.4, 6.7, and 7.0 at 37°C. Hypertonic media were used to change the membrane potential (14). For the purpose of this study, we determined the activity of this exchange in erythrocytes from three individuals in the presence of valinomycin (50 \( \mu \text{g} \)/ml) and of an outward K\(^+\) gradient, a technique known to induce cell hyperpolarization (16).

**Effect of DIDS.** Amiloride-resistant NHE4 can be activated by DIDS (41,42). To clarify whether the phlorotretin-sensitive \( \text{H}^+ \)-driven \( N^+ \) influx could be explained by this NHE isoform, we studied the effect of DIDS (0.1 mM added to the influx medium) on the activity of this exchange in erythrocytes from three individuals (pH 5.9, \( N_a = 150 \text{ mM} \)). Osmolarity was adjusted to 360 ± 5 mosmol by choline chloride. Kinetic parameters were evaluated by using the Eznfitter software computer program (Elsevier Science Publishers). The \( N_a \) activation of \( N^+ \) influx was fitted by nonlinear regression to determine the \( N_a \) concentration for \( K_a \) and \( V_{\text{max}} \) (45).

**Membrane Potential.** To evaluate the possible electronegativity of the phlorotretin-sensitive component of \( \text{H}^+ \)-driven \( N^+ \) influx, we tested whether changing the membrane potential alters the rate of this ion transport (14). For the purpose of this study, we determined the activity of this exchange in erythrocytes from three individuals in the presence of valinomycin (50 \( \mu \text{g} \)/ml) and of an outward K\(^+\) gradient.

**Li\(^+\) Efflux Studies**

**\( \text{Na}^+ \)-Driven Li\(^+\) Efflux.** Properties of erythrocyte Li\(^+\) efflux were addressed following the original technique described by Canessa et al. (5,46), as subsequently modified by the use of nystatin to load cells with Li\(^+\) (47). Acidification of Li\(^+\)-loaded cells was obtained modifying the erythrocyte acid loading procedure described by Escobales and Canessa (36). In brief, after the removal of plasma anduffy
coat, erythrocytes were washed as described previously for Na⁺ influx studies; aliquots of this suspension were used for the determination of hematocrit, hemoglobin, and Na⁺ concentration. Erythrocytes were then incubated for 20 min at 4°C in an unbuffered solution containing 40 µg/ml nystatin, 50 mM sucrose, 130 mM KCl, and 20 mM LiCl, followed by centrifugation and resuspension of the cell pellet for 10 min in the same solution, although without nystatin. The ionophore was removed by washing cells four times at 37°C in a 1 mM K-phosphate buffer (pH 7.4) containing 50 mM sucrose, 130 mM KCl, 20 mM LiCl, 10 mM glucose, and 1 mg/L BSA.

Li⁺-loaded cells (10% hematocrit) were incubated at 37°C in six different acid-loading solutions containing 0.15 mM MgCl₂, 0.1 mM ouabain, 0.1 mM bumetanide, 10 mM glucose, and 20 mM LiCl in a 20 mM Tris-MES buffer for pH lower than 7.0 or Tris-MOPS buffer for pH higher than 7.0 adjusted to pH 5.6, 5.8, 6.0, 6.4, 6.7, and 7.0 at 37°C (to prepare erythrocytes with cell pH 5.9, 6.2, 6.5, 6.7, 6.9, and 7.2). Solutions with pH lower than 6.4 contained 150 mM KCl and those with pH over 6.4 contained 130 mM KCl. Solutions with pH below 6.7 also contained 40 mM sucrose. The final osmolality of the acid-loading solutions was 360 ± 5 (pH between 5.6 and 6.4), 340 ± 5 (pH 6.7), and 315 ± 5 (pH 7.0). Hypertonic media were used to avoid cell swelling induced by low pH.

After a 10-min incubation, the pH was clamped by addition of 0.2 mM DIDS and 0.5 mM methazolamide to inhibit anion exchanger (43) and carbonic anhydrase (44). The cells were then incubated for an additional 30 min at 37°C and then washed four times with ice-cold unbuffered washing solution containing 170 mM KCl, 40 mM sucrose, and 0.15 mM MgCl₂. Aliquots of the cell suspensions were used for the determination of hematocrit, hemoglobin, and Na⁺ and Li⁺ concentrations. The pH was measured by lysing 0.2 ml of packed erythrocytes in 2 ml of distilled water. The pH of the lysate was then determined with a pH meter at room temperature.

Li⁺-loaded erythrocytes were finally resuspended in ice-cold washing buffer. Aliquots of erythrocytes with different pH were transferred into full set of efflux media, suitably matching a hematocrit of 3 to 4% in each efflux incubate. Na⁺ and choline (Na⁺-free) efflux media contained 150 mM NaCl or equimolar choline chloride, 40 mM sucrose, 10 mM glucose, 0.1 mM ouabain, 0.1 mM bumetanide, and 0.15 mM MgCl₂ in a 10 mM Tris-MOPS buffer (pH 7.4 at 37°C).

Duplicate aliquots of each incubate were taken at the beginning of the incubation and after 15 and 30 min at 37°C, were chilled in melting ice, and then centrifuged at 4°C. Supernatants were taken for the measurement of Li⁺ concentrations by atomic absorption spectrophotometry (Perkin Elmer 4000).

To evaluate the effect of DMA and phloretin on SLC activity, these inhibitors were added to the efflux solutions without preincubation. Na⁺ and choline efflux media at different pH values (8.0, 7.4, 7.0, 6.7, 6.3, and 6.0) were prepared to evaluate the effect of external acidification on the activity of SLC.

Li⁺ efflux rates were calculated in each medium as the change in Li⁺ concentration over time by simple linear regression analysis, and expressed as µmol · L⁻¹ · cell⁻¹ · h⁻¹. The correlation coefficient was higher than 0.98 in all studies. As usual, SLC was taken as the difference between Li⁺ efflux rates in Na⁺ and choline media.

Interaction Between Li⁺ and H⁺ on the Na⁺-Driven Li⁺ Efflux. To achieve different Li⁺ concentrations, erythrocytes from three individuals were loaded with Li⁺ by incubation in increasing amounts of Li⁺ (Liᵦ = 0.2, 0.5, 1.0, 3.0, and 5.0 mM). Osmolarity was kept constant by choline chloride as appropriate. Li⁺-loaded erythrocytes were divided into two aliquots and underwent internal acidification (pHᵦ = 5.9 or 7.2) as described above. Liᵦ concentrations (0.2, 0.5, 1.0, 3.0, and 5.0 mM) were maintained constant in every solution until the end of the acidification procedure. Li⁺ efflux rates were measured as described above.

Interaction Between Na⁺ and H⁺ on the Na⁺-Driven Li⁺ Efflux. To achieve different Na⁺ concentrations, erythrocytes from three individuals were loaded with Na⁺ by incubation in increasing amounts of Na⁺ (Naᵦ = 0, 5, 10, 20, and 50 mM) in the presence of ouabain (0.1 mM) to inhibit the Na⁺ pump (48). Osmolarity was kept constant by adjusting with choline chloride as appropriate. Once Na⁺-loaded, erythrocytes were divided in two aliquots and underwent internal acidification (pHᵦ = 5.9 or 7.2) as described above. Ouabain (0.1 mM) and Naᵦ concentrations (0, 5, 10, 20, and 50 mM) were maintained constant in every solution until the end of the acidification procedure. Li⁺ efflux rates were measured as described previously.

Kinetic Parameters for the Na⁺ Activation of Na⁺-Driven Li⁺ Efflux. Activation curves for Naᵦ were evaluated in erythrocytes from three individuals. As described above, Li⁺-loaded cells were added to efflux media containing increasing concentrations of Na⁺ (Naᵦ = 0, 10, 30, 50, 80, 120, and 150 mM). Kinetic parameters were evaluated by using the Enzfitter software computer program (Elsevier Science Publishers). The Naᵦ activation of Li⁺ efflux was fitted by a nonlinear regression program to determine the Na⁺ concentrations for Kᵦ and V_max (45).

Membrane Potential. To evaluate the possible electrogenicity of Na⁺- and H⁺-driven Li⁺ efflux, we tested whether changing the membrane potential alters the rate of this ion transport (14). We determined the activity of this exchange in erythrocytes from three individuals (Naᵦ = 150 mM) in the presence of valinomycin (50 µg/ml) and of an outwardly directed K⁺ gradient, a technique known to induce hyperpolarization of the cell (16).

Effect of DIDS. To clarify whether Na⁺- and H⁺-driven Li⁺ efflux could be explained by NHE4 isofrom, we studied the effect of DIDS (0.1 mM added to the influx medium) on the activity of this exchange in erythrocytes from three individuals (Naᵦ = 150 mM).

Statistical Analyses

Data are shown as arithmetical mean with SEM. Comparisons between paired or unpaired observations were made by t test. Correlations were sought by simple linear regression. The null hypothesis was rejected for two-tailed P values <5%.

Results

Effect of Inhibitors on Na⁺—H⁺ Exchange

The effects of DMA and phloretin were tested on the activity of the erythrocyte NHE. Figure 1 shows the activation curve for Hᵦ of NHE and the inhibitory effect of 0.1 mM DMA and 0.2 mM phloretin. The use of higher concentrations of DMA up to 1 mM did not further inhibit the H⁺-driven Na⁺ influx (data not shown). In acid-loaded (pHᵦ = 5.92 ± 0.03) erythrocytes from seven different individuals (Figure 1), 0.1 mM DMA significantly inhibited the H⁺-driven Na⁺ influx from 27.4 ± 7 to 18.9 ± 5 mmol Na⁺ · L⁻¹ · cell⁻¹ · h⁻¹, P = 0.02 (mean inhibition = 31 ± 9%). Phloretin (0.2 mM) reduced the H⁺-driven Na⁺ influx to 17.2 ± 3 mmol Na⁺ · L⁻¹ · cell⁻¹ · h⁻¹, P = 0.04 (mean inhibition = 37 ± 6%). The simultaneous presence of DMA and phloretin, however, induced a further inhibition of NHE activity to 9.4 ± 3 mmol Na⁺ · L⁻¹ · cell⁻¹ · h⁻¹, P = 0.007 (mean inhibition = 66 ± 7%), suggesting the independence of inhibition sites for DMA and phloretin.
Figure 1. (A) Representative experiment showing the activation curve of H⁺-driven Na⁺ influx by H⁺. Control cells (●) are compared to cells treated with dimethylamiloride (DMA) (○) and with cells treated with DMA and phloretin (▲). (B) H⁺-driven Na⁺ influx at pH₇ = 5.92 ± 0.03 in human erythrocytes from seven normotensive individuals, without further inhibitors (Control) and in the presence of DMA, phloretin (Phl), or both (DMA + Phl). When compared to control, H⁺-driven Na⁺ influx was inhibited by DMA (***P = 0.02), Phl (**P = 0.04), and DMA + Phl (**P = 0.007). The association of DMA + Phl also reduced H⁺-driven Na⁺ influx with respect to DMA (P = 0.005) and Phl (P = 0.01). Vertical bars are the SEM of duplicate within-assay observations.

Kinetic Parameters for Na₉ Activation of the DMA-Insensitive, Phloretin-Sensitive Component of H⁺-Driven Na⁺ Influx

The activation curve for Na₉ of the DMA-insensitive, phloretin-sensitive component of H⁺-driven Na⁺ influx followed Michaelis-Menten saturation kinetics in the erythrocytes of the three subjects considered with a mean Kₘ for Na₉ of 69.8 ± 11 mM and a calculated Vₘₐₓ of 10.4 ± 1 mmol Na⁺ · L₉⁻¹ h⁻¹, n = 3 (Figure 2A).

Membrane Potential

The addition of valinomycin to influx solutions did not affect the activity of phloretin-sensitive H⁺-driven Na⁺ influx (measured at pH₇ = 5.95 ± 0.07, Na₉ = 150 mM, Kᵢ = 154.3 ± 26 mmol · L₉⁻¹, Kₒ = 0 mM) from 7.1 ± 0.9 to 7.0 ± 0.8 mmol Na⁺ · L₉⁻¹ h⁻¹, P = NS, n = 3.

Effect of DIDS

The addition of DIDS to influx solutions did not affect the activity of phloretin-sensitive H⁺-driven Na⁺ influx (measured at pH₇ = 5.95 ± 0.07 and Na₉ = 150 mM) from 7.1 ± 0.9 to 6.8 ± 1.0 mmol Na⁺ · L₉⁻¹ h⁻¹, P = NS, n = 3.

Effect of Internal Acidification on Na⁺–Li⁺ Countertransport

Na⁺-driven Li⁺ efflux was progressively enhanced by reductions in pH, from 7.4 to 6.0, as shown in the representative experiment of Figure 3. However, stimulation of Li⁺ efflux was largely inhibited by the addition of 0.2 mM phloretin, the strongest inhibitor of SLC (4,20,38).

In erythrocytes from five different individuals, internal acidification (from pH₇ 7.32 ± 0.08 to pH₇ 5.91 ± 0.04) significantly increased the Na⁺-driven Li⁺ efflux from 287 ± 55 to
Effect of External Acidification on Na\(^+\)-Li\(^+\) Countertransport

Extracellular acidification progressively inhibited Na\(^+\)-driven Li\(^+\) efflux. A representative experiment is shown in Figure 4. This phenomenon could be demonstrated both at physiologic pH\(_i\) and after internal acidification. In erythrocytes from three different individuals (Figure 4), external acidification (from pH\(_0\) 8.00 to pH\(_0\) 6.00) significantly decreased the Na\(^+\)-driven Li\(^+\) efflux from 276 ± 31 to 84 ± 26 pmol L\(^{-1}\) h\(^{-1}\), \(P = 0.002\) at pH\(_i\) = 7.51 ± 0.03 and from 592 ± 1213 ± 165 μmol L\(^{-1}\) h\(^{-1}\), \(P = 0.003\) (Figure 3). In both conditions, phloretin significantly reduced the Na\(^+\)-driven Li\(^+\) efflux (respectively to 34 ± 20 μmol L\(^{-1}\) h\(^{-1}\), \(P = 0.005\), and 211 ± 44 μmol L\(^{-1}\) h\(^{-1}\), \(P = 0.006\)). The activity of SLC was similarly inhibited at physiological and acidified pH\(_i\) (88 ± 6% versus 83 ± 5%, \(P = NS\)). 0.1 mM DMA, the specific inhibitor of NHE (14,15,23), did not affect Na\(^+\)-driven Li\(^+\) efflux at physiologic pH\(_i\) (to 307 ± 60 μmol L\(^{-1}\) h\(^{-1}\), \(P = NS\), thus confirming the amiloride insensitivity of this transport (20–22). However, after acidification, a DMA-sensitive component of Na\(^+\)-driven Li\(^+\) efflux could be appreciated (to 762 ± 126 μmol L\(^{-1}\) h\(^{-1}\), \(P = 0.01\), corresponding to a reduction of 37 ± 2%), suggesting that acidification might induce the exchange of Na\(^+\) for Li\(^+\) also through an amiloride-inhibitable transporter. The acid-stimulated, amiloride-insensitive component of the Na\(^+\)-driven Li\(^+\) efflux, however, was still significantly higher than the Na\(^+\)-driven Li\(^+\) efflux at physiologic pH\(_i\) (762 ± 126 versus 287 ± 55 μmol L\(^{-1}\) h\(^{-1}\), \(P = 0.01\)), indicating that SLC was effectively activated by this maneuver, and its increased activity was not simply due to the activation of the DMA-sensitive component.

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Interaction Between Li\textsuperscript{+} and H\textsubscript{i} on Na\textsuperscript{+}-Driven Li\textsuperscript{+} Efflux

The effect of H\textsubscript{i} on Na\textsuperscript{+}-driven Li\textsuperscript{+} efflux depends on the concentration of Li\textsubscript{i}. As shown in Figure 5, cellular acidification caused inhibition of the exchange at low Li\textsubscript{i}, and activation at high Li\textsubscript{i}. The activation curve for Li\textsubscript{i} was therefore modified by internal acidification with a consequent increase of both calculated V\textsubscript{max} (from 226.3 ± 35 at pH\textsubscript{i} = 7.28 ± 0.04 to 541.6 ± 46 μmol L\textsubscript{cell}\textsuperscript{-1} h\textsuperscript{-1} at pH\textsubscript{i} = 6.01 ± 0.06, P = 0.02, n = 3) and K\textsubscript{m} for Li\textsubscript{i} (from 0.11 ± 0.09 at pH\textsubscript{i} = 7.28 ± 0.04 to 0.74 ± 0.15 mmol Li\textsubscript{i} L\textsubscript{cell}\textsuperscript{-1} at pH\textsubscript{i} = 6.01 ± 0.06, P = 0.02, n = 3).

Kinetic Parameters for Na\textsubscript{0} Activation of Na\textsuperscript{+}-Driven Li\textsuperscript{+} Efflux

The activation curve for Na\textsubscript{0} of Na\textsuperscript{+}-driven Li\textsuperscript{+} efflux followed Michaelis-Menten saturation kinetics in the erythrocytes of the three subjects considered with a mean K\textsubscript{m} for Na\textsubscript{0} of 78.8 ± 6 mM and a mean calculated V\textsubscript{max} of 559.6 ± 62 μmol L\textsubscript{cell}\textsuperscript{-1} h\textsuperscript{-1}, n = 3 (Figure 2B).

Membrane Potential

The addition of valinomycin to influx solutions did not affect the activity of Na\textsuperscript{+}-driven Li\textsuperscript{+} efflux from 411.3 ± 28.5 to 405.1 ± 27.7 μmol L\textsubscript{cell}\textsuperscript{-1} h\textsuperscript{-1}, P = NS, n = 3, K\textsubscript{i} = 118.3 ± 25 mmol L\textsubscript{cell}\textsuperscript{-1}, K\textsubscript{o} = 0 mM.

Effect of DIDS

The addition of DIDS to influx solutions did not change the activity of Na\textsuperscript{+}-driven Li\textsuperscript{+} efflux from 411.3 ± 28.5 to 399.9 ± 39.3 μmol L\textsubscript{cell}\textsuperscript{-1} h\textsuperscript{-1}, P = NS, n = 3.

Discussion

The results of our study demonstrate for the first time the coexistence of three different components in the H\textsuperscript{+}-driven Na\textsuperscript{+} influx of human erythrocytes, as defined by pharmacologic challenge. The first component is sensitive to DMA, which gave a mean inhibition by 31 ± 9%. This may be explained by NHE1, the amiloride-sensitive NHE isoform that has been recently identified in the erythrocyte membrane (28–31). A second component appears to be sensitive to phloretin, which gave a mean inhibition of 37 ± 6%. This indicates the presence of a previously unrecognized phloretin-sensitive H\textsuperscript{+}-driven Na\textsuperscript{+} influx in these cells, which is independent of the first component because the use of DMA and phloretin...
together gave an additive effect on the inhibition of H⁺-driven Na⁺ influx (mean reduction by 66 ± 7%). Finally, these data are consistent with the existence of a third component in erythrocyte H⁺-driven Na⁺ influx, characterized by DMA- and phloretin insensitivity.

The second component of the H⁺-driven Na⁺ influx described above (i.e., the amiloride-insensitive component, which can be inhibited by phloretin) might be a good candidate to explain the activity of SLC. This membrane transport mediates Na⁺-driven Li⁺ efflux and is consistently insensitive to DMA (20–22), but inhibited by phloretin (4,20,38). This hypothesis is supported by the following observations: (1) SLC is activated by internal and inhibited by external acidification, as would be expected for a mode of operation of NHE (14,39). (2) There is competition between H⁺ and Li⁺ and between Li⁺ and Na⁺ at different H⁺ for a common internal SLC binding site, as already suggested for another mode of operation of NHE, i.e., erythrocyte Na⁺→Na⁺ exchange (49). (3) SLC and the phloretin-sensitive component of NHE share similar kinetic parameters for the Na⁺ activation. (4) Both exchanges are electroneutral, confirming previous studies (4,14).

To our knowledge, this is the first report suggesting that SLC may be mediated by a carrier with a pH-sensitive regulatory subunit, just like any known NHE isoform (27). Evidence that external acidification inhibits SLC is in line with previous reports showing that, at variance with other cells (15,50), the erythrocyte does not express a functionally reversible NHE (51,52). Although H⁺-driven Na⁺(Li⁺) influx has been demonstrated (36,37), no H⁺-driven Na⁺(Li⁺) efflux is detectable in these cells. This phenomenon was explained by a high affinity of the transport site for H⁺ (almost saturated at pH₀ = 7.4) coupled to an inhibitory H⁺ binding site (52). For this same reason, external acidification is therefore expected to inhibit an NHE-mediated SLC.

Human erythrocytes are known to possess an amiloride-insensitive Na⁺-driven Li⁺ efflux, i.e., SLC (1,2,20), along with the amiloride-sensitive NHE isoform (NHE1) (28–31). This apparently is not consistent with the notion that amiloride-sensitive NHE is known to mediate amiloride-sensitive Na⁺-driven Na⁺(Li⁺) efflux, at least in nonerythroid cells (14,15,39,50). In other words, part of Na⁺-driven Li⁺ efflux should be sensitive to amiloride also in erythrocytes. Interestingly, in our experiments Na⁺-driven Li⁺ efflux remained unaffected by DMA at physiologic pH₀, in line with all previous observations (20–22), but developed a partial sensitivity to this inhibitor upon internal acidification. This suggests that in human erythrocytes, Na⁺-driven Li⁺ efflux through DMA-resistant pathways (SLC) is active at a higher pH than through DMA-sensitive pathways (NHE1).

The amiloride-sensitive component of H⁺-driven Na⁺ influx in our study is only apparently lower than that detected previously by other authors (31 ± 9% versus 59 ± 10%) (36). However, in that study by Escobales and Canessa (36), phloretin was present in all efflux media. This results in the suppression of the phloretin-sensitive component in all assays and may thus lead to an overestimation of the proportion of H⁺-driven Na⁺ influx that can be inhibited by amiloride. Actually, when we tested the simultaneous effect of DMA and phloretin on H⁺-driven Na⁺ influx, we observed a similar inhibition (66 ± 7%).

SLC is not the only Na transport possibly mediated by an amiloride-insensitive NHE. Functional studies have recently suggested the presence of amiloride-insensitive NHE in several cells (32–34,42). Among the gene(s) encoding amiloride-insensitive components, only NHE4 has been identified in rat hippocampus (53) and in rat renal cortical tubules (41). We have therefore addressed the possibility that NHE4 could mediate SLC in human erythrocytes by testing the effect of DIDS (known to stimulate NHE4 activity) (41,42) on SLC activity. However, no effect could be seen.

Another important functional difference between NHE4 and SLC seems to be their different responses to hyperosmotic challenge. NHE4 is activated by hyperosmolarity-induced cell shrinkage (53) that vice versa leaves SLC unaffected (54). Finally, the presence of NHE4 on the erythrocyte membrane could not be confirmed in a recent report using specific antibodies (31).

A new NHE5 isoform has been recently cloned (55), however, it is unclear whether this isoform is expressed by the erythrocyte membrane. Also, its pharmacologic properties are unknown. Taken together, these findings suggest that the NHE family has not yet been thoroughly described and that one or more amiloride-insensitive exchangers may still be missing.

Almost 20 years have passed since SLC was first identified in human erythrocytes (1) and since its activity was found to be elevated in essential hypertension (5), yet very little is known about its nature. The hypothesis that SLC might be a mode of operation of NHE (18) remains most intriguing, although it is still unconfirmed (31). The recent identification of amiloride-resistant NHE isoforms is in line with this possibility. Our findings, although restricted to a functional level, suggest that SLC are mediated by an amiloride-insensitive isoform of NHE that cannot be listed among those that have already been cloned. Its identification and molecular cloning could help to clarify the genetic basis of essential hypertension and, possibly, diabetic nephropathy.

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