A Thermodynamic Study of Electroneutral K-Cl Cotransport in pH- and Volume-clamped low K Sheep Erythrocytes with Normal and Low Internal Magnesium

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Abstract

Swelling-induced human erythrocyte K-Cl cotransport is membrane potential independent and capable of uphill transport. However, a complete thermodynamic analysis of basal and stimulated K-Cl cotransport, at constant cell volume, is missing. This study was performed in low K sheep red blood cells before and after reducing cellular free Mg into the nanomolar range with the divalent cation ionophore A23187 and a chelator, an intervention known to stimulate K-Cl cotransport. The anion exchange inhibitor 4,4'diisothiocyanato-2,2'disulfonic stilbene was used to clamp intracellular pH and Cl or NO₃ concentrations. Cell volume was maintained constant as external and internal pH differed by more than two units. K-Cl cotransport was calculated from the K effluxes and Rb (as K congener) influxes measured in Cl and NO₃, at constant internal K and external anions, and variable concentrations of extracellular Rb and internal anions, respectively. The external Rb concentration at which net K-Cl cotransport is zero was defined as flux reversal point which changed with internal pH and hence Cl. Plots of the ratio of external Rb concentrations corresponding to the flux reversal points and the internal K concentration versus the ratio of the internal and external Cl concentrations (i.e., the Donnan ratio of the transported ions) yielded slopes near unity for both control and low internal Mg cells. Thus, basal as well as low internal Mg-stimulated net K-Cl cotransport depends on the electrochemical potential gradient of Cl⁻.

Keywords: K-Cl cotransport • sheep erythrocytes • thermodynamics • magnesium • DIDS-pH-clamp

Introduction

K-Cl cotransport, a secondary active transport pathway, occurs in cells of the erythron such as nucleated red blood cells, reticulocytes, and young red blood cells of a variety of species, in endothelial cells, in certain epithelial cells, and in fish liver cells (reviewed by Lauf et al., 1992). In mature red blood cells, K-Cl cotransport declines with cell aging (Hall and Ellory, 1986; O’Neill, 1989) but less so in low K (LK) red blood cells of ruminants (Lauf et al., 1992) and carnivores (Parker and Dunham, 1989). Because of the apparent persistence of K-Cl cotransport in human red blood cells with certain hemoglobinopathies, such as hemoglobin SS disease, and its potential contribution to abnormal cellular dehydration (Bookchin et al., 1991), interest has been focused on regulatory aspects using rabbit and sheep red cells as models (Jennings and Al-Rohil, 1990; Dunham et al., 1993).

In human red blood cells, K-Cl cotransport is considered to operate in an electroneutral fashion: Brugnara et al. (1989) showed Cl⁻-driven uphill K transport at constant membrane potential, \( \Phi_m \), and Kaji (1993) varied \( \Delta \psi_m \) with the Na ionophore hemisodium without affecting K-Cl cotransport. In these two studies, the effect of \( \Delta \psi_m \) was assessed without deliberate efforts to maintain constant cell volume. For swelling-induced K flux of LK sheep red blood cells in Cl⁻, the experimentally obtained and theoretically predicted net K fluxes disagreed at equilibrium (Delpire and Lauf, 1991a). A similar conclusion was reached in volume-clamped LK sheep red cells with normal and low Mg (Lauf et al., 1994). Hence, a thorough thermodynamic evaluation of basal and activated K-Cl cotransport, still lacking for any cell system, was undertaken.

The present study uses the stilbene-derivative DIDS which irreversibly inhibits band 3 protein-mediated anion, i.e., chloride/bicarbonate, exchange (Ship et al., 1977; Gasbjerg et al., 1993), and therefore pH equilibration (Cala, 1980) but only reversibly blocks K-Cl cotransport in LK sheep red cells (Delpire and Lauf, 1992). After pH₂ titration with CO₂ or Na₂CO₃ DIDS should permit clamping of the internal Cl concentration.
tions, [Cl], at different levels. In such cells K effluxes and influxes may be studied at variable [Cl], and external K concentrations, [K]o, and with constant internal K and external Cl concentrations, [K], and [Cl], respectively. Intracellular NO3 concentrations were varied accordingly. Since there is no kinetic evidence for Cl-dependent K/Rb exchange (Delpire and Lauf, 1991a, b), the effect on K/Cl cotransport of variable ion products on both sides of the plasma membrane can thus be assessed. By combining this technique with osmotic volume clamping (Zade-Oppen and Lauf, 1990; Lauf et al., 1994), we unequivocally demonstrate that the electrochemical potentials of both K and Cl are indeed the driving forces for K-Cl cotransport.

**Materials and Methods**

**Red Blood Cells**

For each experiment, blood was freshly drawn by professional and licensed personnel from the Laboratory of Animal Resources through venipuncture from mixed breed sheep (consistent with laboratory animal guidelines and license #A91). Red cells were homozygous for the LK (LL) genotype as determined by cation and immunologic analysis (Lauf, 1984). Red cells were separated from plasma by centrifugation at 10,000 rpm and washed at that speed twice with 295 mosM NaCl before suspension in further experimental media (see below). Cell suspensions for ion flux determinations were 2.5% (vol/vol).

**Chemicals and Solutions**

The following solutes of analytic grade were purchased from either Sigma Chemical Co. (St. Louis, MO) or from Fisher Scientific (Fair Lawn, NJ): NaCl, NaNO3, glucose, ouabain, EDTA, PIPES, BICINE, (N,N-bis[2-hydroxyethyl]glycine), Tris, sucrose, and MgCl2. A23187 was obtained from Calbiochem Corp., San Diego, CA. Stock solutions were made for A23187 (1.91 mM) in ethanol. DIDS (4,4'-dicycloxyanate-2,2'-disulfonil stibene), dissolved in buffer solutions, and diamox (Acetazolamide), dissolved in DMSO, were obtained from Sigma Chemical Co., and ultrapure (Purartronic) RbCl and RbNO3 were obtained from Johnson Mathematical Chemicals, Rosyton, UK. Osmolarities were measured with an Advanced Digimatic Osmometer (model 3DH; Advanced Instruments, Needham Heights, MA), and pH was measured using an Orion Research Ionalyzer 901 (Orion Research Incorporated, Cambridge, MA) and Fisher Microprobe or Orion combination electrodes. Dry weights were determined using a Mettler M5 microbalance on ~150 µl triplicate samples of packed red cells, dried for 48 h in a drying oven at 80°C (Lauf, 1982). The pH values are given for 37°C if not otherwise stated. Treatment and flux solutions are described below.

**pH4-titration with Osmotic Clamping, Mg Removal, and DIDS-pH4 Clamping**

The bulk of the experiments required a three-step procedure for osmotic clamping, pH-titration and Mg removal. The osmolarities needed to maintain constant cell volume at various pH, after pH-clamping with DIDS were calculated based on the approaches chosen by Zade-Oppen and Lauf (1990) and Lauf et al. (1994) and experimentally confirmed. From a plot of K cell water/Kg dry cell solids (dcs) versus variable osmolality (range: 350-220 mosM) for each pH, (6.5, 7.4/7.5, and 9), the osmolarities were extrapolated yielding close to 1.85 Kg water/kg dcs. This water content was close to that obtained in 250 mosM solutions (see composition below) of pH 9 and only ~3% below the original cell volume in plasma. The external anion concentration was fixed at ~100 meq/liter. The salt and water shifts across the cell membrane induced by pH titration were balanced by addition of sucrose at pH 7.4/7.5 and 6.5. Samples were taken within the first 10 min of the flux experiment for determination of cell water, [Cl], and pHi, [Cl], was measured with a Buchler chloridometer (Buchler Instruments, Fort Lee, NJ) as published earlier (Lauf, 1982), and pHi on freeze-thawed red blood cells. [K], was measured with a Perkin-Elmer 5000 Atomic Absorption Spectrophotometer (Perkin Elmer Instruments, Norwalk, CT). Ionic concentrations are expressed in meq/liter of original cells (loc) or meq/Kg cell water, and pH in units. Final solution osmolarities are given after appropriate adjustments with sucrose.

To obtain pHi ~6.5, LK red cells washed in 295 mosM NaCl were resuspended in 330 mosM NaCl and titrated with CO2 to pH 6.5 in a jacketed chamber equilibrated at 0°C. Cells were washed by centrifugation at 7,500 rpm until pHi, was stable (Gunn et al., 1973). Cells were then subjected to Mg removal as described below and resuspended in a 10 mM PIPES-buffered solution (4°C) of the same pH as above, containing either 100 mM NaCl (a) or NaNO3 (b) with final osmolalities of 330 mosM in a and 320 mosM in b, respectively. The packed cells were then resuspended in solutions a and b containing 0.1 mM DIDS and 10 µM diamox, a carbonic anhydrase inhibitor, at 37°C, and incubated for 45 min in a shaker bath. Cells were washed twice at 4°C in the same solutions to remove the inhibitors.

For pHi ~7.4 the titration step was omitted, and washed cells were suspended in 10 mM PIPES-buffered solutions of pH 7.4 or 7.5 at 4°C containing 100 mM NaCl with a final osmolality of 295 mosM. After Mg removal (see below), cells were resuspended and incubated for 45 min in fresh PIPES-buffered pH 7.4 or 7.5 media at 37°C with either 100 mM NaCl or NaNO3, with final osmolarities at 295 and 277 mosM, respectively, and containing 0.1 mM DIDS and 10 µM diamox. DIDS and diamox were removed by washing, as described above.

For pHi ~9, washed LK red cells were loaded into a jacketed chamber, equilibrated at 0°C, and titrated to pH 9 with 100 mM Na2CO3 with intermittent washes in 250 mosM NaCl until pHi, was stable. Cells were then suspended in cold 100 mM NaCl buffered to pH 9 with 10 mM BICINE, final osmolality 250 mosM, for Mg removal by A23187 and EDTA as described below. Cells were then divided into two aliquots and resuspended in 10 mM BI-

**Measurements of Ion Fluxes, Statistics, and Calculations**

K efflux and Rb influx were assessed simultaneously, as described previously (Lauf, 1983; Lauf et al., 1994). Flux solutions contained 0.1 mM ouabain and were buffered with 10 mM PIPES for pH 6.5, with 10 mM PIPES/BICINE for pH 7.4/7.5 and with 10 mM BICINE for pH 9. For cells titrated to pH4, 6.5, ~7.4, and ~9, the osmolarities of the CI and NO3 flux media were maintained at 390 and 320 mosM, 295 and 277, and 250 and 240 mosM, respectively, at all pHi values during the flux experiment. External Rb concentrations, [Rb]o, were 0, 5, 10, 15, 20, 30, 40,
and 60 mM replacing equimolar quantities of [Na], keeping the total cation concentration, [Rb + Na], constant in both CI and NO₃ media. For measurement of K loss, samples were taken at about 10, 20, 30, 40, and 50 min, and the supernatants were removed after centrifugation at 10,000 rpm. K loss was measured by atomic absorption spectrophotometry and the pseudo-first-order rate constants (1/h) of K loss were calculated from the supernatant [K]ₙ at five time points, corrected for spontaneous hemolysis (<2%), and [K], at equilibrium, using a linear regression program essentially as published earlier (Lauf, 1983). Data are presented as K efflux in mmol/(locxh) by multiplying the optical density of the hemolyzed cell aliquot, and (iii) of 1 ml of the original packed cells measured at 527 nm (Lauf, 1989), respectively. Rb influx in mmol/loc/h was calculated from the initial velocity of Rb uptake at 5 time points using linear regression programs available through STATISTIX 4 or the Slide Write Programs. The CI-dependent Rb influx (also referred to as K-CI influx or cotransport) is the calculated difference between K efflux measured in CI and NO₃.

For assessment of ouabain-resistant Rb uptake, 1-ml aliquots of cell suspension were removed at about the same time intervals and placed into test tubes with 8 ml ice-cold 295 mosM Tris-buffered MgCI₂, pH 7.4. Cells were separated by centrifugation, washed twice in the same media to remove any Rb₀, and hemolyzed in “hemolyzing solution” (Lauf, 1983; Lauf et al., 1994). Rb uptake was calculated in mmol/(locxh) based on (i) the hemolysate Rb measured by flame emission spectrophotometry in a Perkin-Elmer 5000 Atomic Absorption Spectrophotometer, (ii) the optical density of the hemolyzed cell aliquot, and (iii) of 1 ml of the original packed cells measured at 527 nm (Lauf, 1989), respectively. Rb influx in mmol/loc/h was calculated from the initial velocity of Rb uptake at 5 time points using linear regression programs available through STATISTIX 4 or the Slide Write Programs. The CI-dependent Rb influx (also referred to as K-CI influx or cotransport) is the calculated difference between Rb influx in CI and NO₃. The kinetic parameters V_max and K_m were obtained from Wolf-Hanes or Linewaier-Burke plots.

Thermodynamics

We derived the conditions for flux equilibrium, i.e., when the net flux is zero, from the standard equation defining the electrochemical potential (µₐ) of the transported ions (see Heinz, 1978). Accordingly:

\[ \mu_j = \mu_j^0(T) + RT \ln a_j + z_j F \Phi \]  

where \( j \) signifies K, Cl, and CI, for internal and external K and Cl ions, \( \mu_j^0 \) = standard chemical potential of ion \( j \), \( T \) = absolute temperature, \( R \) = gas constant, \( a_j \) = activity of ion \( j \), \( z_j \) = concentration of ion \( j \), \( [K]_j, [Cl]_j \) = charge of ion \( j \), \( F \) = Faraday constant, \( \Phi \) = electrical potential, i.e., difference between the electrical potential inside, \( \Phi_i \), or outside, \( \Phi_o \), the cell, minus the standard potential.

Thus, for [K], and [Cl], and for [K], and [Cl], respectively, Eq. 1 becomes:

\[ \bar{\mu}_{KCI} = \bar{\mu}_{KCI} + \bar{\mu}_{ClCI} = RT \left\{ \ln \left( \frac{[K]}{[Cl]} \right) \right\} + F \theta (z_K + z_Cl). \]  

and

\[ \mu_{KCI} = \bar{\mu}_{KCI} + F \Phi \]  

Subtracting Eqs. 2b from 2a yields:

\[ \Delta \mu_{KCI} = \Delta \mu_K + \Delta \mu_{Cl} = RT \ln \left\{ \frac{([K]/[Cl])}{([K]/[Cl])} \right\} \]  

When \( \Delta \mu_{KCI} = 0, \frac{[K]}{[Cl]} = \frac{[Cl]}{[Cl]} \) net flux through the K-Cl cotransporter must be zero, i.e., the flux reverses from outward to inward or vice versa (flux reversal point or FRP), at a [K]ₙ (or [Rb]ₙ used as K congener) as per Eq. 4:

\[ \text{FRP} = \frac{[Rb]}{[K]} = \frac{[K]}{[Cl]} \cdot \frac{[Cl]}{[Cl]} = \frac{[K]}{[Cl]}. \]  

The FRPs were calculated from the relationship between net flux rates and the logarithm of the \([Rb]_i/[K]_i\) ratios (Lauf et al., 1994).

RESULTS

Water and Ions in DIDS-treated LK Red Cells after pH Equilibration

Fig. 1 shows the effect of titration with CO₂ to pH₉, 6.5 or with NaCO₃ to pH₉, 9 on cell water and ion concentrations of Cl⁻ and NO₃⁻-equilibrated LK red cells subsequently treated with 0.1 mM DIDS and 10 μM diamox. Cell water and hence volume were maintained near 1.84 Kg/Kg dcs (Fig. 1, A, legend), i.e., ~3% below that found at pH 7.4 for normal red cells (see MATERIAL AND METHODS). Like human hemoglobin A (Freedman and Hoffman, 1978), intracellular oxygenated sheep hemoglobin, with its isoelectric point of ~7 (Blunt and Huisman, 1975), provides the fixed and titratable charges. Acidification or alkalinization of the cell suspension caused the expected pH shifts in both Cl and NO₃ (Fig. 1, B). As expected from the Donnan distribution of permeable cations and anions, Cl and NO₃ either entered or left the red cell compartment through the conductive pathway (Gunn et al., 1989), as measured for CI in Fig. 1, C. Subsequent treatment with DIDS and diamox (to further reduce proton generation by the carbonic anhydrase catalyzed hydration of CO₂) of volume-clamped and pH-titrated (but not pH-clamped) cells did not change this result. Although diamox did not affect the parameters depicted in A–C, it was kept throughout the pretreatment step with DIDS.

Water and Ions in DIDS pH₉-clamped Control and Low Mg, LK Red Cells versus pH₉

Table I displays the cell water content of control and low Mg, LK red cells with pH₉ clamped at different values by DIDS in CI or NO₃ media. The average mean water contents for each pH₉ were close to 1.87 and 1.81 in Cl and 1.83 and 1.82 in NO₃, in control and low Mg, cells, respectively, and there was no significant difference between the three pH₉ values. Hence, the techniques developed here provided cells with similar volumes at all pH₉ permutations chosen.

Fig. 2 shows the mean pH₉ values of control (interrupted lines) and low Mg (solid lines) LK red cells each at different pH₉ (6.5, 7.4/7.5, and 9) imposed on the cells after DIDS treatment. Comparing these data with those in Table I suggests that DIDS effectively clamped pH₉ at any pH₉. The fact that low Mg, cells always displayed
TABLE I

<table>
<thead>
<tr>
<th>pH</th>
<th>Cl_</th>
<th>NO_3</th>
<th>pH</th>
<th>Cl_</th>
<th>NO_3</th>
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<td>6.5</td>
<td>1.781 (0.016)</td>
<td>1.781 (0.016)</td>
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<td>1.842 (0.052)</td>
<td>1.862 (0.051)</td>
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<tr>
<td>8.3</td>
<td>1.840 (0.019)</td>
<td>1.823 (0.081)</td>
<td>8.5</td>
<td>1.812 (0.024)</td>
<td></td>
</tr>
</tbody>
</table>

*Kg/Kg dry cell solids (n = 3, ±SD) for each pH at pH = 6.5, 7.4/5, and 9.

slightly higher pHi values is not readily explained; it is, however, not important for interpretation of the data shown below. Fig. 3 shows [Cl]_i in cells with pHi clamped at different pHi values: [Cl]_i increased from ~52 to 166, and from 44 to 143 meq/Kg cell water in controls (A) and low Mg (B) cells, as pHi was lowered from 8.3 to 6.5 and from 8.5 to 6.8, respectively (see also Table II). This Cl shift was not affected by the subsequently imposed pHi, as the slopes of all three lines were not significantly different from zero. In contrast to pHi and [Cl]_i, [K]_i remained at 22–25 meq/Kg cell water and did not change with pHi, (Table II).

An inverse change of pHi and [Cl]_i indicates effective DIDS-clamping which sharply reduced the dissipation of the imposed pH and Cl gradients while pHi was altered in the subsequent flux experiments.

Ouabain-resistant K and Rb Fluxes in CI and NO3 Before and After DIDS Treatment

Although DIDS reversibly inhibits K-Cl cotransport at pH 7.4 (Delpire and Lauf, 1992), potential DIDS effects on K-Cl cotransport at pH values different from pH 7.4 are unknown, particularly at pH 9 where DIDS covalently attaches via the second cyanate group to band 3 protein in human red cells (Jennings and Passow, 1979; Okubo et al., 1994). DIDS also affects passive K fluxes in low ionic strength media suggesting involvement of band 3 protein (Jones and Knauf, 1995), and Maldonado and Cala (1994) labeled a swelling-stimulated K/H exchanger with DIDS in Amphiuma red blood cells.

Fig. 4 shows that in low Mg, cells, both K effluxes and Rb influxes measured in Cl at 20 mM [Rb]_o were higher the higher the pH of equilibration (A). Most notably, at pH 9 the Rb influxes in Cl controls exceeded the K effluxes, and DIDS stimulated both. Consistent with Delpire and Lauf (1992), no such effects were dis-
concerned at lower pH values, particularly at pH 7.4. Fig. 4 B shows that both K and Rb fluxes in NO3 increased with the pH of equilibration. However, a substantial increase was seen for both fluxes in the presence of DIDS at pH 9 suggesting activation of diffusional K fluxes. C contains the calculated K-Cl cotransport activities (dCl). Although the stimulatory effect of DIDS on Rb influxes in Cl was canceled, the alkaline pH activation remained unabated. The Cl-dependent K effluxes were slightly higher in the presence of DIDS, an effect of probably borderline significance. These data are consistent with alkaline stimulation of K-Cl cotransport, in particular of Rb-Cl influx in the absence of DIDS reported by us earlier for Mg-depleted LK red cells (Lauf et al., 1994).

### Determination of Flux Reversal Points (FRPs)

The assessment of the FRPs requires measurements of both Cl-dependent Rb influx and K efflux at varying [Rb]o. 18 experiments were carried out to determine the FRPs: 9 in controls and 9 in low Mg LK red cells. To illustrate the flux behavior and the assessment of the FRPs, Fig. 5 presents 3 experiments with controls and 6 with low Mg cells. Shown are Cl-dependent Rb influx (Fig. 5 A and D) and K efflux (B and E), and net K-Cl cotransport, calculated as the difference between the two former (C and T) as a function of [Rb]o in volume- and pHi 6.6-clamped controls (A–C) and in pHi 6.8- (open symbols) and 8.6-clamped (closed symbols) low Mg (D–F) LK red cells, respectively.

**Rb-Cl influx.** As expected for carrier mediated K-Cl cotransport (Delpire and Lauf, 1991a, b) Cl-dependent Rb influx (Rb-Cl influx) in both control (Fig. 5 A) and low Mg (Fig. 5 D) red cells saturated with increasing [Rb]o. Fig. 5, A–C displays the experiments with control cells clamped at pHi of 6.6 and subsequently exposed to pHi 6.5, 7.5, and 9. Varying pHi between 6.5 and 9 had no effect. The average kinetic parameters (n = 3, mean ± SD) calculated from Wolf-Hanes plots from Fig. 5 A, were 0.9 ± 0.23 mmol/(locxh) for the Vmax values and 35.1 ± 8.8 mM [Rb]o for the Km values. Hence pHi, but not pHo, determined Vmax but not Km of Rb-Cl influx. These data provide the new information that acid pH activation of K-Cl cotransport in cells with normal Mg and unity volume reported by us earlier (Lauf et al., 1994) is due to internal proton effects.

Fig. 5 D shows kinetically indistinguishable saturating

### Table II

<table>
<thead>
<tr>
<th>pHi</th>
<th>Controls</th>
<th>Low Mg</th>
<th>pHi</th>
<th>Controls</th>
<th>Low Mg</th>
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<tr>
<td></td>
<td>C</td>
<td>K</td>
<td></td>
<td>C</td>
<td>K</td>
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<td>6.6</td>
<td>166.4(5.8)</td>
<td>24.4(1.0)</td>
<td>6.8</td>
<td>143.4(7.2)</td>
<td>25.1(0.4)</td>
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<td>7.4</td>
<td>98.9(2.1)</td>
<td>25.3(0.5)</td>
<td>7.6</td>
<td>81.4(6.4)</td>
<td>22.8(0.5)</td>
</tr>
<tr>
<td>8.3</td>
<td>52.0(2)</td>
<td>24.1(0.7)</td>
<td>8.6</td>
<td>44.2(4.3)</td>
<td>21.8(0.3)</td>
</tr>
</tbody>
</table>

*meq/Kg cell water for (n = 3, ±SD) for pHi = 6.5, 7.4/5, and 9.
Rb-Cl influxes when $pH_0$ was varied from 6.5 to 9 in low Mg, pH 6.8 cells, albeit Rb-Cl influx was more than twice that of controls (A). The average values for $V_{max}$ were $2.46 \pm 1.06 \text{ mmol/(locxh)}$ and for $K_m$ $31.3 \pm 6.6 \text{ mM } [\text{Rb}]_i$. Compared to the latter as well as to the controls (Fig. 5 A), saturation of Rb-Cl influx at $>20 \text{ mM } [\text{Rb}]_i$ in pH 8.6 low Mg, cells increased by greater than twofold as $pH_0$ was raised from 6.5 to 9 (bold numbers), an effect primarily due to changes in $V_{max}$ (calculated values were 3.6 and 8.5 mmol/(locxh), respectively) but not in $K_m$ (24.1 and 35.9 mM $[\text{Rb}]_i$, respectively). At the intermediate $pH_0$ of 7.5, the values for $V_{max}$ were 4.9 mmol/(locxh) and for $K_m$ 31.3 mM$[\text{Rb}]_i$. These findings are commensurate with our earlier report that alkaline $pH_0$, even in the absence of a pH clamp, increases $V_{max}$ but not $K_m$ of Rb-Cl influx in Mg depleted LK red cells (Lauf et al., 1994) and add the new information that in alkaline pH-clamped cells, $V_{max}$ is sensitive to $pH_0$.

$K\text{-Cl efflux.}$ In low Mg cells (Fig. 5 E) K-Cl efflux was about twofold larger than in controls (Fig. 5 E) exhibiting a small trans-inhibition with increasing $[\text{Rb}]_i$. The twofold stimulation in low Mg, cells over controls is commensurate with the effect of Mg removal on Rb-Cl influx shown above. Trans-inhibition has been attributed to the level of $[K]$ at the cis-side of the transporter as well as to the fact that the slow step of the transporter is the translocation of the loaded form (Delpire and Lauf, 1991a, b). Furthermore, at 25 mM $[\text{Rb}]_i$, which is about equivalent to $[K]$ (see Table II), lowering Mg stimulated K-Cl efflux less than Rb-Cl influx (compare panels E vs. B and D vs. A), in particular at
high pH$_i$ (D and E, filled symbols), and no pH$_o$ effects were evident on K-Cl efflux. The apparent insensitivity of K-Cl efflux to pH$_i$ can be in part explained by the high $K_m$ values of the K-Cl cotransporter reported earlier by us (Delpire and Lauf, 1991a, b), i.e., the pH$_i$ effect should be observed at [K]$_i > 25$ mM as shown in D for Rb influx as function of [Rb]$_o$.

Net K-Cl flux and FRP. The FRPs resulting from the x-axis intercept of the net fluxes, i.e., the differences between the K-Cl efflux (panels B and E) and Rb-Cl influx (panels A and D) are indicated in Fig. 5 C for controls and Fig. 5 F for low Mg$_i$ cells. The FRPs (i.e., the [Rb]$_o$ at which net K-Cl cotransport is zero) were ~40 mM in both low pH$_i$ controls and low Mg$_i$ cells, and ~12 mM in pH$_i$ 8.5 low Mg$_i$ cells. Based on our earlier work on LK sheep red cells in the absence of DIDS, differences between the FRPs signal changes in the driving forces associated with pH titration (Lauf et al., 1994): The shift in the FRPs was commensurate with a greater than threefold decrease of [Cl]$_i$ as pH$_i$ increased from 6.8 to 8.5 (Fig. 3).

FRPs in Relation to Ion Gradients after DIDS Treatment

The experimentally determined flux reversal points from 15 experiments on control (A) and low Mg$_i$ (B) LK red cells are shown in Fig. 6. In panel A only 6 of the 9 control experiments are displayed since at alkaline pH no significant K-Cl cotransport was measured. The FRPs in controls with pH$_i$ of 7.4 and 6.5 (A), and in low Mg$_i$ cells with pH$_i$ 8.5, 7.6, and 6.8 (B) increased, independently of pH$_o$, from near 30 to above 40 mM [Rb]$_o$, and from near 10 to near 40 mM [Rb]$_o$, respectively. Thus, the FRP values approximately followed the shifts of [Cl]$_i$ in Fig. 3.

Eq. 4 states that the FRPs should be a linear function of the Cl ratio, or more precisely, of [Cl]$_i$ since [Cl]$_o$ was constant, with a slope equal to [K]$_i$. Fig. 7 shows plots of the FRPs from Fig. 6, calculated according to Eq. 4 from the measured [K]$_i$, [Cl]$_i$, and known [Cl]$_o$ as a function of [Cl]/[Cl]$_o$ for controls (A) and low Mg$_i$ (B) cells. The mean slopes for the data obtained from

![Figure 6](image-url)  
**Figure 6.** Flux reversal points for control (A) and low Mg$_i$ LK red cells (B) as a function of pH$_i$, for three different pH$_i$ per condition (numbers). Regression lines were computer fitted. Mean FRPs (mM) [Rb]$_o$ for n = 3 (±SD) were 41.4 (3.9) and 27.4 (2.8) for pH 6.6 and 7.4 controls, and 40.2 (4.3), 20.6 (1.8), and 11.4 (1.2) for pH 6.8, 7.6, and 8.6 low Mg$_i$ cells.

![Figure 7](image-url)  
**Figure 7.** FRP as a function of the chloride ratios in controls (A) and low Mg$_i$ (B) LK red cells. The slopes of the regression lines were: (A) 22.6 (r = 0.821, filled circles) and 24.3 (r = 0.995, open circles) mM [K]$_i$, and (B) 28.7 (r = 0.973) and 26.5 (r = 0.997) mM [K]$_i$ for values measured (filled circles) and calculated (open circles) from [K]$_i$ · [Cl]/[Cl]$_o$ for controls (A) and low Mg$_i$ (B) cells, respectively.
the fluxes (closed symbols) and those calculated according to Eq. 4 (open symbols) were 22.6 and 24.3 mM for controls, and 28.7 and 26.5 mM for low Mg cells, respectively, and hence approximated [K], (24.6 and 23.3 meq/Kg cell water, see Table II). Thus the product of the chemical gradients was the thermodynamic driving force for both basal and low Mg-activated K-Cl cotransport. Consequently, a plot of the Donnan ratio of cations versus anions transported by the carrier should reveal a slope of unity if K-Cl cotransport is electroneutral. In Fig. 8 the slopes of the experimental data relating [Rb], (determined from the FRP)/[K], to [Cl],/[Cl]o, were 1.11 (r = 0.912) for controls (filled symbols) and 1.08 (r = 0.976) for low Mg cells (open symbols).

Discussion

The principal aim of this study was to fully describe the response of the basal and activated erythrocyte K-Cl cotransporter to its thermodynamic driving forces in volume- and pH-clamped LK sheep red cells. Although uphill transport of K driven by the Cl gradient and independence of K-Cl cotransport from the membrane potential were shown in earlier studies, these were not done at constant (original) cell volume (Brugnara et al., 1989; Kaji, 1993). In our study, DIDS was used to vary simultaneously pH and hence [Cl], while keeping cell volume and [K], constant, and to vary [K],, using Rb, as congener, while maintaining [Cl],, constant (Fig. 3, Table II). Our principal conclusion is that K-Cl cotransport is electroneutral in control and in low Mg LK red cells because the experimentally determined FRP (in mM [Rb],) provided with [K], a ratio that equaled [Cl],/[Cl],, i.e., [K(Rb)],/[K], = [Cl]/[Cl],. The measured FRPs (i.e., [Rb],) of the net K-Cl cotransport shifted linearly and, as thermodynamically predicted, with [Cl],/[Cl], (Figs. 5-7), commensurate with the Donnan ratio of the transported ions (Fig. 8). A transport system with such properties is able to use the chemical potential of one ion (K or Cl) to move the counter ion (Cl or K) against its chemical potential. Furthermore, the thermodynamically derived stoichiometry of the transported K and Cl ions was unity (Fig. 8). Our data do not permit to assess whether unity coupling occurs between one or two K and Cl ions, respectively. In swollen cells Dunham and Ellory (1981) showed sigmoidal kinetics of both K and Cl fluxes in LK sheep red cells with Cl varied on both sides of the membrane. However, our present results (see also the saturation kinetics of Figs. 5) are consistent with earlier kinetic data (Hill coefficient of unity for K, Lauf, 1984).

Our findings are also in agreement with our earlier work showing that K-Cl cotransport is independent of Na (Delpire and Lauf, 1991a) and exclude the contribution of proton gradients, particularly at acid pHo, to the driving force.

Although the data clearly show that K-Cl cotransport is electroneutral, we cannot prove beyond doubt that changes in Φm over the pH range studied could have altered unidirectional flux rates. At alkaline pH, DIDS-treated cells are expected to be hyperpolarized which would cause Rb influx > K efflux in NO3 as shown in Fig. 4 B but not electroneutral Rb-Cl flux > K-Cl efflux. If indeed in DIDS-treated cells, the experimentally achieved [Cl],/[Cl], ratios determine Φm, then the permeability (P) to Cl should be much larger than that to K, i.e., PCl >> PK. A plot of the FRPs versus the calculated OCl, ranging from +12 to −23 mV between pH 6.5 and 9, respectively, should be linear. However, such an approach may be irrelevant for two reasons. First, DIDS inhibits PCl in human red cells (Kaplan et al., 1983; Knauf et al., 1983). To test whether DIDS alters PCl as function of pH we measured PK in valinomycin-treated control LK cells according to Knauf et al. (1977). The valinomycin-induced changes in PK were highest at low pH as compared to alkaline pH (data not shown) suggesting that PCl is affected by DIDS differently at pH 6.5 than at pH 9. Second, at alkaline pH, DIDS augmented both K efflux and Rb influx in NO3, i.e., the (Cl-independent) PK, whereas it did not affect K-Cl cotransport (Fig. 4). This indirect experimental evidence suggests that Φm indeed affected unidirectional fluxes without having a major effect on K-Cl cotransport, a conclusion agreeing with Brugnara et al. (1989) and Kaji (1993).

The data presented here contrast with two conclusions of our earlier work. First, Zade-Oppen and Lauf (1991) showed that DIDS, present during the flux experiments, stimulates K and Rb fluxes in Cl preferen-

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**Figure 8.** Relationship between the ratios of the concentrations of the transported cations [Rb],,/ [K], with [Rb], determined from the FRP and the measured ratio of [Cl],/[Cl]o in control and low Mg LK red cells. The slope for controls (closed circles) is 1.12 (r = 0.912, n = 8 experiments) and for low Mg cells 1.08 (r = 0.976, n = 10 experiments).
tionally at acid pH, whereas no experiments were done in NO₃⁻. In Fig. 4 we again find that DIDS stimulated Rb influx in Cl, however, at alkaline pH, but affected much less K efflux. Later, Delpire and Lauf (1992) showed later that DIDS inhibits K-Cl cotransport reversibly. Besides DIDS, alkaline pH also enhanced Rb influx in NO₃⁻ (Fig. 4 and see also above), i.e., the basal K permeability. This is consistent with recent findings by Ortiz-Carranza et al. (1996b). The present study on Cl-dependent K and Rb fluxes excludes "other" effects of DIDS on K fluxes such as those shown by Jones and Knauf (1985) in human red cells. Delpire and Lauf (1991a) reported a twofold difference between the calculated and measured FRPs, in swollen LK cells and in Cl only, the Cl-dependent component being unknown. Based on the present work this difference may be due to different pHᵢ and hence [Cl]ᵢ. Lauf et al. (1994) found a similar small difference for net K-Cl cotransport in osmotically "clamped" cells, as opposed to the present study were both osmotic and pHᵢ were clamped. This discrepancy is more difficult to explain. It is possible that DIDS eliminated K flux components which may have contributed to the small discrepancies in the FRPs of the preceding studies on swollen and volume-clamped cells.

The design of the experiments to demonstrate electroneutrality of K-Cl cotransport provided also new information on the kinetic behavior of the system which needs to be addressed in future work. Whereas the bulk of the kinetic data were consistent with our previous work in volume- but not pH-clamped LK red cells (Lauf et al., 1994), the data in Fig. 5 clearly indicate separate effects of pHᵢ and pHᵢ, on Rb-Cl influx: in both control and low Mg, low pHᵢ cells, internal protons determined Rb-Cl influx, whereas in low Mg, alkaline pHᵢ cells both internal and external protons were modulatory. The reason for this discrepancy cannot be explained based on the data presented in this study. We have recently shown elsewhere that alkaline pH per se stimulates K-Cl cotransport, in particular when combined with extraction of Mg by A23187 and EDTA at pH 9 (Ortiz-Carranza et al., 1996a). Consistent with this report, in the present study K-Cl efflux was higher in alkaline pHᵢ cells (Fig. 5 E, filled symbols) than in controls (Fig. 5 B).

However, Rb-Cl influx in alkaline pHᵢ cells with low Mgᵢ was more sensitive to pHᵢ₀ suggesting complex kinetic effects of protons on K-Cl cotransport. Since by design of the experiments the FRPs respond solely to [Cl]ᵢ and the apparent affinities of the transporter for Rbo were about equal in 15 experiments (mean values ± SD of 32.6 ± 7.2 and of 37.2 ± 7.1 mM for controls and low Mgᵢ cells, respectively), the kinetic effects of pHᵢ₀ on Rb-Cl influx at high [Rbo]₀ seen in Fig. 5 D are of no consequence regarding the thermodynamic conclusions.

Both basal and low Mg-activated K-Cl cotransport behaved thermodynamically identical. Together with the findings of Brugnara et al. (1989) for the NEM-activated K-fluxes in LK sheep red cells and those of Kaji (1993) for the swelling-induced human K-Cl cotransporter, we may conclude that all K-Cl cotransport modes displayed through these activation mechanisms are electroneutral and must obey the Donnan relationship of the transported cations and anions as shown here (Fig. 8). Since DIDS has been recently shown to reduce the Cl permeability of human red blood cells close to that of bilayers (Gasbjerg et al., 1994), yet does not inhibit K-Cl cotransport in human red blood cells (Kaji, 1986), it should be feasible to test this conclusion in red blood cells from other species, including human.

Our demonstration that basal and stimulated K-Cl cotransport modes in LK sheep red cells is electroneutral follows similar approaches to another cotransporter: Geck et al. (1980) established the thermodynamically electroneutral nature of Na-K-2Cl cotransport in Ehrlich tumor cells by demonstrating that the slopes for Cl fluxes were twice those for Na and K fluxes, and Duhm and Göbel (1984) showed that the electrochemical gradient ratios of the transported ions were the determinants also in human red blood cells.

Although the detailed mechanism by which K and Cl are transported across the membrane awaits further clarification, two papers published most recently at the time of completion of the final revision of this study report cloning and functional expression of two isoforms of the K-Cl cotransporter from rabbits, rat and human (Gillen et al., 1996; Payne et al., 1996).

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