Kinetics of Cl-dependent K Fluxes in Hyposmotically Swollen Low K Sheep Erythrocytes

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ABSTRACT A detailed kinetic study of K:Cl cotransport in hyposmotically swollen low K sheep red blood cells was carried out to characterize the nature of the outwardly poised carrier. The kinetic parameters were determined from the rate of K efflux and influx under zero-K-*trans* conditions in red cells with cellular K altered by the nystatin method and with different extracellular K or Rb concentrations. Although apparent affinities for efflux and influx were quite similar, the maximal velocity for K efflux was approximately two times greater than for influx. Furthermore, at thermodynamic equilibrium (i.e., when the ion product of K and Cl within the cell was equal to that outside) a temperature-dependent net K efflux was observed, approaching zero only when the external product reached approximately two times the internal product. The binding order of the ions to the transporter was asymmetric, being ordered outside (Cl binding first, followed by K) and random inside. K efflux but not influx was *trans*-inhibited by KCl. *Trans* inhibition of K efflux was used to verify the order of binding outside: *trans* inhibition by external Cl occurred in the absence of external K, but not vice versa. Thus K:Cl cotransport is kinetically asymmetric in hyposmotically swollen low K sheep red cells.

INTRODUCTION

Cell volume reduction of maturing mammalian erythrocytes in part involves Cl-dependent K fluxes (Lauf et al., 1984*a*; Lauf and Bauer, 1987), causing KCl and obligatory water losses mediated by a membrane carrier inhibited by the loop diuretic furosemide (Ellory et al., 1982; Lauf, 1984; Kaji, 1987). At least in red cells of man and sheep these fluxes are unaffected by the membrane potential, and thus are electrically silent (Brugnara et al., 1988). Although the stoichiometry has actually never been determined, transport of one Cl ion must accompany one K ion to obey electroneutrality. Therefore, the terms "Cl-dependent K fluxes" and "K:Cl cotransport" are used interchangeably (for review see Lauf, 1988*a*).

Different manipulations have been shown to significantly enhance K:Cl cotransport: cell swelling (Ellory and Dunham, 1980; Parker, 1983; Kaji, 1986; Lauf, 1988*c*),

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lowering intracellular pH (Brugnara et al., 1985; Zade-Oppen and Lauf, 1987), removal of cellular Mg (Lauf, 1985; Brugnara and Tosteson, 1987; Canessa et al., 1987; Fujise and Lauf, 1987), and the alkylation or oxidation of thiol groups (Lauf and Theg, 1980; Lauf et al., 1984*b*; Kaji and Kahn, 1985; Lauf, 1988*d*). However, the nature of the relationship between these interventions and the stimulation of transport activity remains to be established. A better understanding of the mechanisms by which transport occurs may shed some light on the various activating processes. Therefore, a full kinetic description of the interactions between K, Cl, and the transporter stimulated by different means is important.

In this work we present a detailed kinetic study of the KCl cotransport system in hyposmotically swollen low K (LK) sheep red blood cells. The sheep red blood cell constitutes a good model because, as part of the genetic low K/high K (LK/HK) polymorphism and at the exclusion of other K fluxes such as Ca-dependent K channels and Na:K:2Cl cotransport, K:Cl transport remains active in mature LK sheep red blood cells (Lauf, 1983*b*). Moreover, as previously reported, in hyposmotically swollen cells the Cl-independent K leak represents only a very small fraction of the total flux (Lauf, 1988*b*).

This study explores the order of ion addition to the endo- and exofacial domains of the transporter and determines the rate of both unidirectional K fluxes either under zero-K-*trans* conditions or simultaneously at equilibrium. Finally, we separately investigated the *trans* effects of both substrates, Cl and K. We conclude that K:Cl cotransport is functionally asymmetric.

Some of the results have been reported in abstract form (Delpire and Lauf, 1990*a*, *b*).

METHODS

Blood from genetically homozygous (LL) LK sheep was removed by jugular venipuncture into heparinized syringes just before the experiments. After determination of hematocrit (hct) and hemoglobin (hgb) content, the blood was centrifuged at 10,000 rpm for 1 min at 4°C to remove plasma and buffy coat. The red blood cells were then washed four times in an isosmotic (290 mosM) solution containing 150 mM NaCl and 5 mM PO_4, pH 7.4, at 4°C.

The volume of original cells was determined either by wet weight–dry weight analysis (Joiner and Lauf, 1978) and/or by the hgb/hct method (Lauf, 1983*a*), which is based on measuring the ratio between the optical density at 527 nm (OD^{527}) of an appropriate dilution (D) of hemolyzed blood and the hct: $OD^{527}_{pc} = OD^{527} \times D/\text{hct}$. The relative cell volume was derived from the ratio $OD^{527}_{pc}/OD^{527}_{sample}$.

Fluxes were performed at 37°C in hyposmotic media (240 mosM) containing (in mM): 120 (Na + K + Rb)Cl, 5 PO_4, 10 glucose, and 0.1 ouabain. At time zero packed cells were added to the temperature-equilibrated (37°C) solution to give a final hct of 5% (vol/vol).

The cellular K concentration, $[K]_i$, was modified with nystatin (mycostatin, 5,880 USP units/mg; Sigma Chemical Co., St. Louis, MO) using Na rather than choline or *N*-methyl-D-glucamine as a K substitute. Pilot experiments summarized in Table I showed a poor osmotic behavior of the cells when choline (or *N*-methyl-D-glucamine, data not shown) was used. Hence, cells were incubated at a hct of 2.5% for 90 min at 0°C in a solution containing (in mM): 150 (Na + K)Cl, 5 PO_4, 10 glucose, 60 sucrose (Joiner and Lauf, 1978), and 60 μg/ml nystatin (from a stock solution in dimethylsulfoxide; Sigma Chemical Co.). For complete K depletion cells were incubated in the above solution with nystatin for 1 h at 0°C, then centrifuged and

resuspended in the same solution without nystatin for another 30 min. After nystatin treatment the cells were washed seven times at 37°C in the same solution containing 50 mg/100 ml BSA (Sigma Chemical Co.) and volume-equilibrated at 4°C by four washings with the hyposmotic solution just before the flux measurement.

Influx

For zero-K-*trans* K influx in K-depleted cells, flux media were prepared with various $[K]_o$, and packed cells were added at $t = 0$ to yield a final hct of 5%. Aliquots of the cell suspension were removed at $t = 10$, 20, 30, 40, and 50 min and centrifuged at 10,000 rpm for 1 min at 4°C. The cell pellets were washed four times with a $MgCl_2$/Tris-Cl (290 mosM) solution at 0°C and lysed in a hemolysing solution containing 15 mM NH_4OH, 0.031% noncationic detergent (Acationox; Baxter Healthcare Corp., McGaw Park, IL), and 4 mM CsCl to reduce ionization

TABLE I

Relative Cell Volume after Choline or Na Substitution for K by the Nystatin Procedure

Nystatin	Concentration	Sucrose	osM	Time	RCV
μg/ml	*mM*	*mM*	*mosM*	*h*	
Choline					
a. 40	64	60	290	1	0.71
b. 40	94	60	350	1	0.77
40	155	30	420	1	0.95
c. 60	155	30	420	1	0.94
80	155	30	420	1	0.99
100	155	30	420	1	1.11*
d. 60	104	60	360	15	0.97–1.06‡
Sodium					
e. 60	150	60	360	1	1.09 ± 0.01 ($n = 18$)

Relative cell volumes of LK sheep red blood cells after treatment with nystatin, washing in presence of BSA (50 mg/ml), and equilibration in a 240-mosM solution. (*a*) With a nystatin treatment at normal osmolarity (290 mosM) the cells were still shrunken after the subsequent hyposmotic treatment. Increasing choline (*b*) or nystatin (*c*) concentrations did not yield volumes greater than 1. (*d*) Incubations overnight (15 h) gave volumes irreproducibly spread over a wide range and obviously depending on the quantity of choline entering the cells. (*e*). With Na_i replacement, volumes obtained were reproducible and reasonably close to those seen with untreated cells.

*Hemolysis. ‡RCV between 0.97 and 1.06.

interference. Cellular K was then measured using an atomic absorption spectrophotometer (model 5000; Perkin Elmer Corp., Norwalk, CT), while the optical density of hemoglobin was determined at 527 nm with a model 300-N spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). The ouabain-resistant (OR) K influx, $^iM_K^{OR}$, was expressed in millimoles/liter original cell per hour (mmol/LOC per h).

For the study of K *trans* effects, cells contained different $[K]_i$. Therefore, Rb was used as congener and its uptake was determined as reported earlier (Lauf, 1983*a*). Puratronic RbCl and Ultrapure Rb_2SO_4 were obtained from Alfa Products, Morton Thiokol Inc. (Danvers, MA).

Efflux

Zero-K-*trans* K efflux was measured in suspensions of cells containing different $[K]_i$ as adjusted by the nystatin technique. At $t = 0$ cells were added to the K-free flux medium. An aliquot was

directly removed and hemolyzed to determine $[K]_i$. Extracellular K and hgb were measured in the supernatants of samples of cell suspension removed at t = 10, 20, 30, 40, and 50 min and centrifuged at 10,000 rpm and 4°C for 1 min.

Ouabain-resistant K efflux ($^oM_K^{OR}$) was calculated from the first-order rate constant, $^ok_K^{OR}$, multiplied by $[K]_i$ (millimoles per liter original cell) as reported earlier (Lauf, 1983*a*).

To determine the presence of a *trans* effect, K effluxes were performed in flux media containing varying extracellular Rb concentrations, $[Rb]_o$, in either the presence or the absence of extracellular Cl (Cl_o).

Anion Replacement

For the *trans* effects of K or Cl on K efflux, external Cl was varied without affecting cellular Cl concentration by preincubating the cells for 45 min at 37°C with 10^{-6} M DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) in a solution containing (in mM): 120 NaCl, 5 PO_4, and 10 glucose. After centrifugation and washing the cells were resuspended in a Na_2SO_4 solution to avoid Cl movement via the conductive pathway. The solution contained (in mM): 92 Na_2SO_4, 5 PO_4, 10 glucose, and 0.1 ouabain (230–235 mosM; a lower osmolarity solution was needed to maintain cell swelling identical to that obtained with the 240 mosM NaCl solution). Under similar conditions at 290 mosM the cells lost ~10% of their Cl content over a period of 60 min as determined by chloridometry using a Buchler chloridometer: at t = 0, $[Cl]_i$ = 111 ± 3 mM; at t = 60, $[Cl]_i$ = 99 ± 3).

Statistics

K(Rb) influx was calculated by linear regression analysis based on five time points ($[K]_i$ as a function of time) with the slope (mmol/LOC per h) being the rate of influx. K efflux was calculated by multiplying $^ok_K^{OR}$ obtained by linear regression analysis of five time points $\{-\ln(1 - [K]^t/[K]^{t=\infty})\}$ with $[K]_i$ (mmol/LOC). $[K]^t$ represents the external K concentration at time = t and $[K]^{t=\infty}$ that at infinite time. The kinetic parameters K_m and V_{max} were calculated by linear regression analysis and verified by nonlinear fitting of the Michaelis-Menten equation as described by Cornish-Bowden (1976). The point of minimal distance for all intersecting lines in the case of a family of straight lines (double reciprocal plots) was calculated by the least-squares method, using a program reaching the minimal square distance by continuous approximation.

RESULTS

Na Independence

Using Na as a substitute for intracellular K, we first studied the Na dependence of the Cl-dependent K flux. In Fig. 1 K efflux is presented as a function of relative cell volume, at either low (5 mM) or high (115 mM) concentration of internal Na ($[Na]_i$). Note that the volume dependence of the K efflux was identical at both $[Na]_i$'s, and was linear within the volume range studied. Thus OR zero-K-*trans* K efflux is independent of $[Na]_i$, at least in the concentration range studied, consistent with our earlier report of independence of K influx on external Na (Lauf, 1983*a*).

Zero-K-trans K Fluxes

Fig. 2 shows the kinetics of OR K efflux under zero-K-*trans* conditions for LK red cells swollen in hyposmotic solution. The kinetic parameters calculated from a Lineweaver-Burke plot (Fig. 2, *insert*) were: K_m = 65 mM and V_{max} = 6.25 mmol/LOC

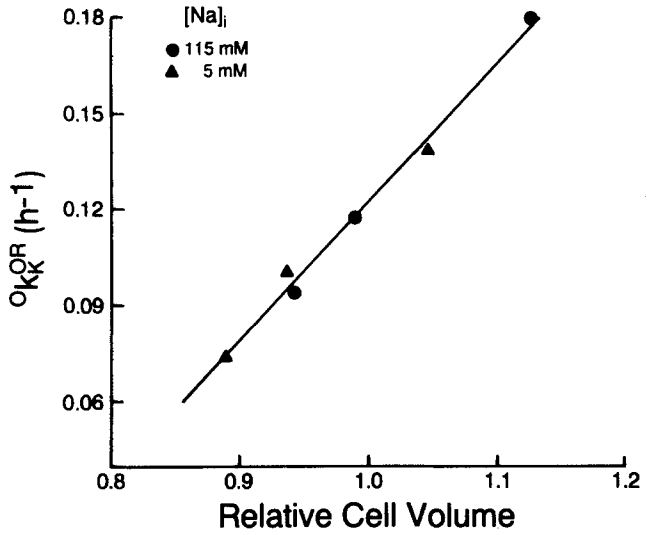

FIGURE 1. Rate constants of ouabain-insensitive K efflux as a function of relative cell volume. Measurements were performed either at high (115 mM) or low (5 mM) $[Na]_i$. Choline was substituted for Na by the nystatin method (incubation overnight). The three triangles show the lack of reproducibility and the dispersion of relative cell volume obtained with cells subjected to identical nystatin treatment using choline as Na substitute. Note, however, that the level of internal Na does not affect the volume-dependent rate of K efflux.

per h. Similar results were obtained in three different experiments: K_m = 67.5 ± 5.1 mM and V_{max} = 5.01 ± 0.96 mmol/LOC per h (n = 3 ± SD). Fig. 3 presents results of a typical K influx experiment in K-depleted cells equilibrated in hyposmotic medium. The kinetic constants determined were: K_m = 49 mM and V_{max} = 2.57 mmol/LOC per h (see insert). The means of three other experiments were: K_m = 54.4 ± 6.2 mM and V_{max} = 2.75 ± 0.47 mmol/LOC per h. The linearity of the two double reciprocal plots

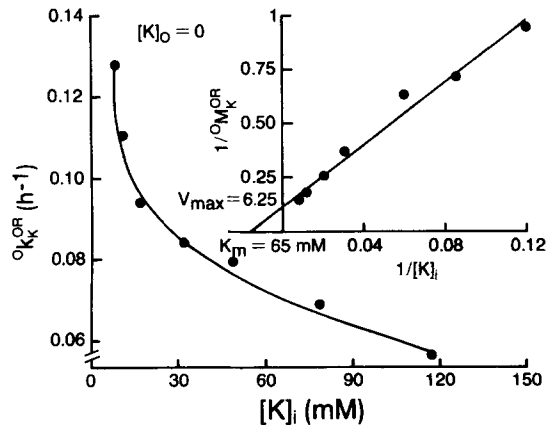

FIGURE 2. K efflux rate constants under zero-K-*trans* conditions as a function of various internal K concentrations. The cells were first preincubated at 0°C with different concentrations of K in the presence of nystatin (60 μg/ml). After several washings in the presence of BSA they were swollen in a 240-mosM, K-free NaCl solution and resuspended at 5% hct in the same solution prewarmed at 37°C. Each $^{o}k_{K}^{OR}$ value was calculated from five time points. *Inset,* Lineweaver-Burke double-reciprocal plot. K_m and V_{max} were 65 mM and 6.25 mmol/LOC per h, respectively.

indicated an absence of cooperativity for each unidirectional flux (Hill coefficient of unity).

Equilibrium Fluxes

We further characterized the transporter by determining unidirectional fluxes at equilibrium (where $[Cl]_o \times [K]_o = [Cl]_i \times [K]_i$) as previously reported for NEM-stimulated fluxes (Lauf, 1983*a*). To this end, Rb influx and K efflux were simultaneously measured in untreated, swollen cells, in which the chloride gradient was conserved while $[Rb]_o$ was varied. It has been previously shown that Rb and K are interchangeable, giving identical kinetic parameters (Lauf, 1983*a*). Thus, we were able to calculate the $[Rb]_o$ at which no net flux occurred. As shown in Fig. 4, Rb influx and K efflux were identical at 24 mM $[Rb]_o$, corresponding to 1.4 times the $[K]_i$. When the results were corrected for Cl concentration (i.e., the products of $[K]_i \times [Cl]_i$ and $[Rb]_o \times [Cl]_o$), the ratio of fluxes at equilibrium was ~2. This asymmetry between K efflux and Rb influx was highly temperature dependent, and disappeared when the temperature of the flux solutions was decreased to 20°C.

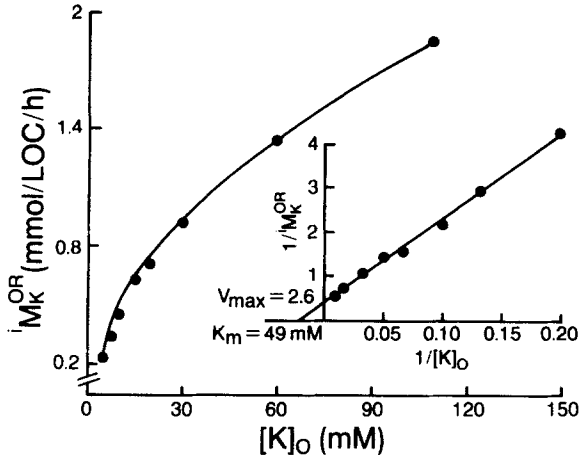

FIGURE 3. Ouabain-resistant K influx under zero-K-*trans* conditions, as a function of various $[K]_o$. The cells were previously depleted of K using nystatin (60 μg/ml). After seven washings in the presence of BSA the cells were swollen in a 240-mosM NaCl solution and resuspended at 5% hct in solutions containing different K concentrations. Each $^{i}M_K^{OR}$ value was obtained from five time points. *Inset*, Lineweaver-Burke plot determining K_m at 49 mM and V_{max} at 2.6 mmol/LOC per h.

Order of Ion Binding

The order of binding of K and Cl at both membrane sides of the cotransporter was examined. First we determined the rate of zero-K-*trans* K efflux from red blood cells loaded with different $[K]_i$ and at various equilibrium concentrations of chloride using NO_3 as replacement anion. The family of reciprocal plots for various $[K]_i$, shown in Fig. 5 *A*, intercepted the $1/[K]_i$ axis at a calculated value of −0.0243, which corresponds to 41 mM $[K]_i$. Note that the slope of the lines decreased as $[Cl]_i$ increased, and that even at high $[Cl]_i$ the slope remained positive. The alternate linear plots, with $[Cl]_i$ varied, are shown in Fig. 5 *B*. This family of lines also intercepted the horizontal axis at $1/[Cl]_i = -0.065$, corresponding to a $K_m$app value

of 15 mM. Furthermore, the slope remained positive at high $[K]_i$. A V_{max} of 4.3 mmol/LOC per h was obtained from a replot of $1/V_{max}$app versus $1/[Cl]_i$ (not shown). The observed mutual effects of $[K]_i$ and $[Cl]_i$ on K efflux are characteristic for random binding of these ions (see Appendix I).

Next we tested the effects of extracellular K and Cl on zero-K-*trans* K influx. The reciprocal plots in which K was varied gave a family of curves intercepting the vertical axis (Fig. 6 *A*). The negative intercepts obtained for $[Cl]_o$ = 10 and 20 mM reflect the larger experimental errors at small Cl concentrations. Note that the effective affinity

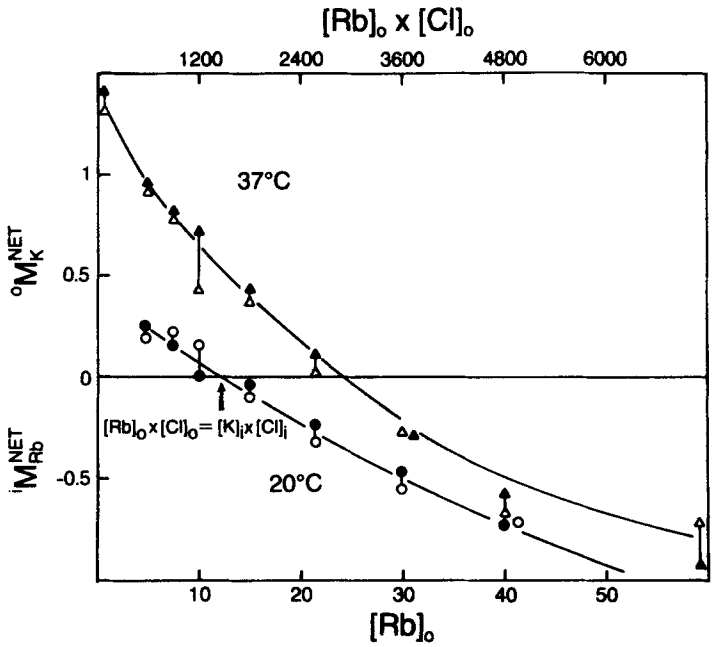

FIGURE 4. Difference between K efflux and Rb influx (net flux) in swollen cells not subjected to nystatin treatment, as a function of various $[Rb]_o$. At 37°C the point of zero net K(Rb) flux occurred at $[Rb]_o$ = 24 mM which was twice the concentration calculated from the point of zero net driving force ($[Rb]_o \times [Cl]_o = [K]_i \times [Cl]_i$). The open and filled triangles represent two different experiments. Each symbol represents the difference ${}^{o}M_K^{OR} - {}^{i}M_{Rb}^{OR}$, each flux being calculated from five time points. $[K]_i$ was measured at 18 mmol/liter intracellular cell water. At 20°C the point of zero net flux coincided with the point of thermodynamic equilibrium. The open and filled circles represent two different experiments.

for K increased with increasing $[Cl]_o$. The reciprocal plot of $1/{}^{i}M_K^{OR}$ vs. $1/[Cl]_o$ (Fig. 6 *B*) gave a series of straight lines intersecting in the second quadrant. The point of minimal distance for all intersecting lines (calculated by the least-squares method) gave a K_m value of 26 mM and a V_{max} of 0.9–1 mmol/LOC per h. Note that at high $[K]_o$ (30 mM) the slope of the plot approaches zero. This pattern is specific for an ordered mode of binding (see Appendix I).

Thus, results of our kinetic studies clearly demonstrate that the binding is ordered

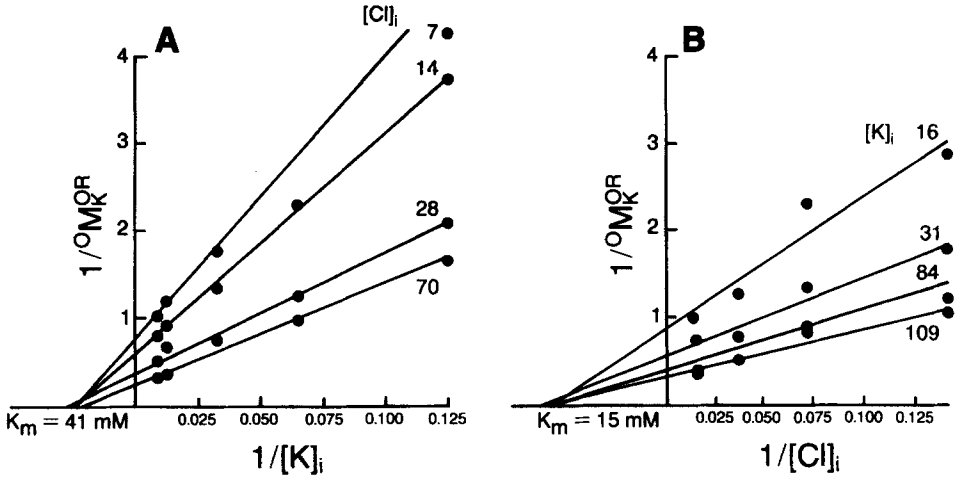

FIGURE 5. Ouabain-resistant K efflux of LK red blood cells loaded with different concentrations of K and Cl. Double reciprocal plots $1/{}^{o}M_K^{OR}$ vs. $1/[K]_i$ and $1/[Cl]_i$. The K concentration was varied using the nystatin technique. The cells were first incubated at 0°C in solutions containing different $[K]_o$ and nystatin (60 μg/ml). After the seven washings the cells were swollen by equilibration in a 240-mosM NaCl solution and then equilibrated with solutions containing different concentrations of Cl (NO_3 substitution). Each ${}^{o}M_K^{OR}$ value (mmol/LOC per h) was obtained from five time points. The common intersection of all the lines was calculated by the least-squares method. The apparent affinity for Cl was calculated to be 15 mM. The apparent affinity for K was 41 mM.

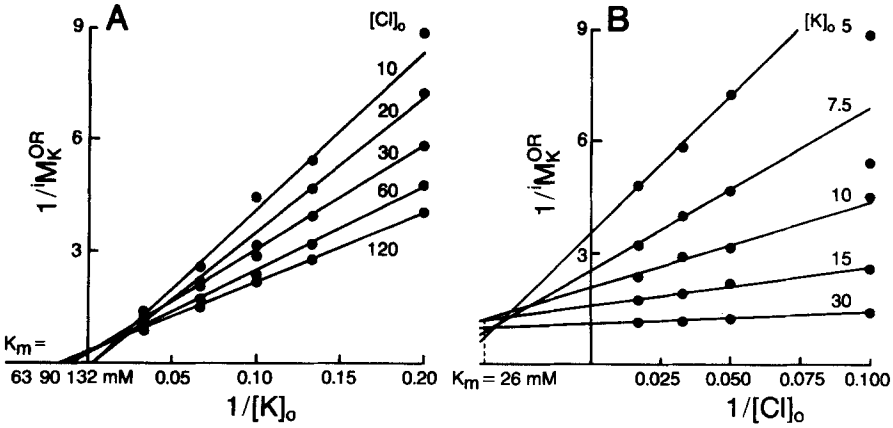

FIGURE 6. Ouabain-resistant K influx of K-depleted LK red blood cells incubated in solutions of various K and Cl concentrations. Double reciprocal plots $1/{}^{i}M_K^{OR}$ vs. $1/[K]_o$ and $1/[Cl]_o$. Each ${}^{i}M_K^{OR}$ value (mmol/LOC per h) was obtained from five time points. The points at $[Cl]_o$ = 10 and $[K]_o$ = 5 and 7.5 were omitted. The intersection of all lines (*B*) was calculated by the least-squares method. The apparent affinity for Cl was 26 mM (*B*). The effective affinity for K increased with $[Cl]_o$ (*A*).

externally (Cl binding first, followed by K) and random internally. The kinetic model of this system is depicted in Fig. 7.

Trans Effects Studies

To determine whether the presence of KCl at the *trans* side affects the unidirectional fluxes we performed the following experiments. First, K efflux was determined under different $[Rb]_o$ and at 120 mM $[Cl]_o$. In Fig. 8 OR K loss from cells loaded with 60

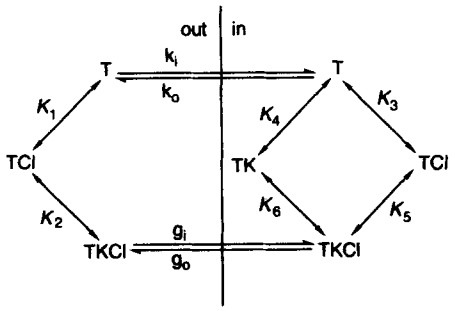

FIGURE 7. Model of the K:Cl cotransport considering an ordered binding outside and a random binding inside. The velocity equations derived according to Cha (1968) and Stein (1986*b*) are:

$$v_{in} = \frac{[Z_i\ Cl_o\ K_o] + Cl_i\ Cl_o\ K_i\ K_o}{Z_i\ Z_o\ R + Z_i\ R_i\ Cl_o\ K_o + Z_o\ R_o\ Cl_i\ K_i + R_e\ Cl_i\ Cl_o\ K_i\ K_o}$$

For v_{out}, $[Z_i\ Cl_o\ K_o]$ is replaced by $[Z_o\ Cl_i\ K_i]$

$$\begin{aligned} nR &= (1 + Cl_i/K_3)/k_o + (1 + Cl_o/K_1)/k_i \\ nR_i &= (1 + Cl_i/K_3)/k_o + 1/g_i \\ nR_o &= (1 + K_6/Cl_i)/g_o + (1 + Cl_o/K_1)/k_i \\ nR_e &= (1 + K_6/Cl_i)/g_o + 1/g_i \end{aligned} \quad \text{or} \quad \begin{aligned} nR &= (1 + K_i/K_4)/k_o + 1/k_i \\ nR_i &= (1 + K_i/K_4)/k_o + (1 + K_2/K_o)/g_i \\ nR_o &= (1 + K_5/K_i)/g_o + 1/k_i \\ nR_e &= (1 + K_5/K_i)/g_o + (1 + K_2/K_o)/g_i \end{aligned}$$

$$Z_i = K_3K_5k_o/g_o \quad \text{and} \quad Z_o = K_1K_2k_i/g_i$$

v_{in} and v_{out} represent the unidirectional inward and outward movement, respectively; n represents the number of transporters per unit area of membrane. The derivation of the kinetic scheme leads to two sets of R parameters, expressed as a function of Cl or K. These parameters are identical for the efflux (v_{out}) and the influx (v_{in}). The two sets are equivalent or interchangeable.

mM K was plotted as a function of time at different $[Rb]_o$. Note that K loss was identical at all *trans*-Rb concentrations chosen. However, with cells containing a low $[K]_i$ (15 mM) a *trans* inhibition was observed (Fig. 9). These results indicate that the level of the substrate at the *cis* side is critical for observing a *trans* effect. We then examined the K *trans* effect on K influx using external Rb as congener. As shown in Fig. 10, varying internal K between 0 and 100 mM had no significant effect on the influx of Rb. The kinetic parameters measured from a double reciprocal plot were: K_m = 53 mM and V_{max} = 2.4 mmol/LOC per h.

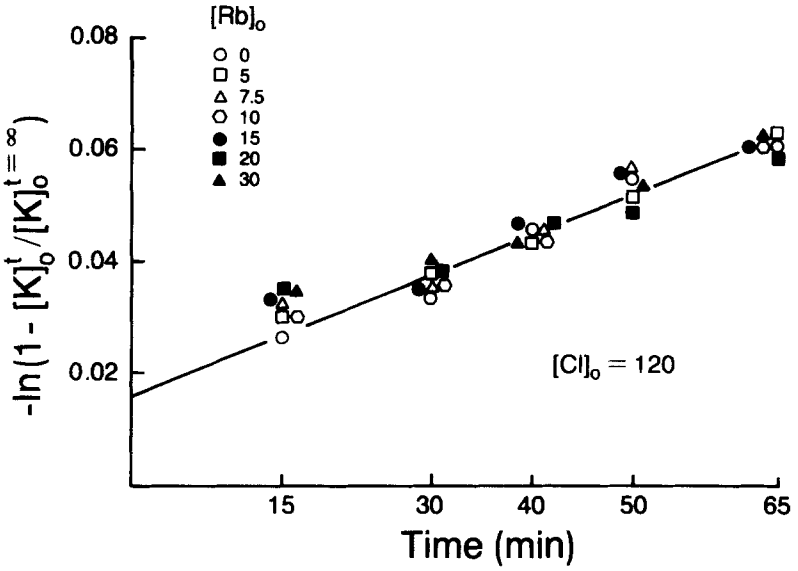

FIGURE 8. Lack of *trans* effect of external Rb on the ouabain-insensitive K efflux in swollen LK red blood cells with high $[K]_i$ (60 mM) using the nystatin technique. Fluxes were measured in 240-mosM media containing RbCl + NaCl (120 mM total). The direct plot shows the rate of K loss as a function of time.

Finally, we examined the *trans* effect of Cl in the absence of K, and inversely, the *trans* effect of K in the absence of Cl. First, using DIDS-treated cells containing 18 mM $[K]_i$, K efflux was measured at different $[Cl]_o$ (SO_4 replacement). With 10^{-6} M DIDS, the cells lost < 10% of their chloride content during a period of 60 min. At a higher concentration DIDS inhibited the Cl-dependent K fluxes (not shown). Fig. 11 shows the *trans* inhibition of K efflux by increasing $[Cl]_o$. Second, using DIDS-treated cells containing 18 mM $[K]_i$ incubated in Cl-free solution, K efflux was measured at

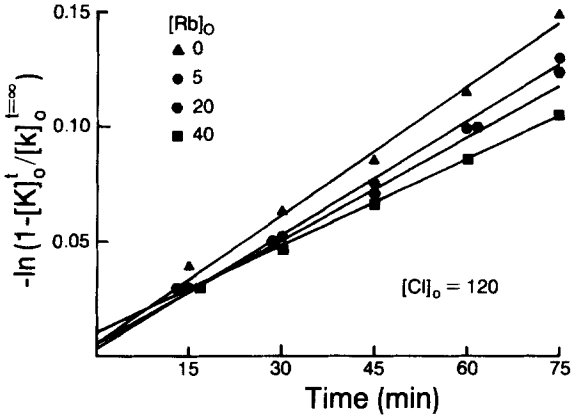

FIGURE 9. Evidence of *trans* effect of external Rb on ouabain-insensitive K efflux in normal swollen LK red blood cells with low $[K]_i$ (16 mM). Fluxes were measured in 240-mosM media containing RbCl + NaCl (120 mM total).

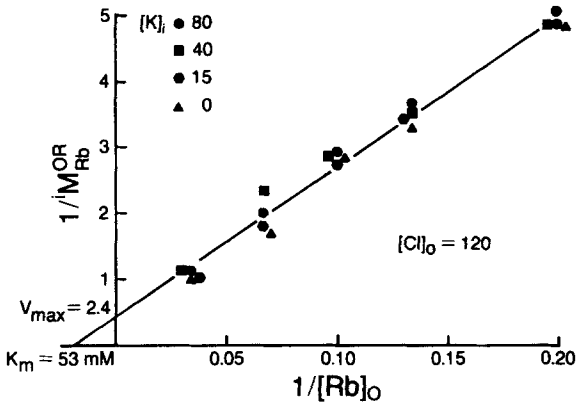

FIGURE 10. Effect of various intracellular K levels on the OR Rb influx in swollen red blood cells. The Lineweaver-Burke double reciprocal plot shows the absence of *trans* effect. K_m was measured to be 53 mM, and V_{max} 2.4 mmol/LOC per h.

different $[Rb]_o$'s. In contrast to the first condition, Rb_o failed to affect K efflux (Fig. 12). Thus Cl_o is required to express the K *trans* effects on K efflux.

DISCUSSION

One of the most informative findings of this study is the characterization of the order of K and Cl binding to the exo- and endofacial aspects of the sheep red blood cell cotransporter. Outside, the binding is *ordered* with Cl interacting first, following by K,

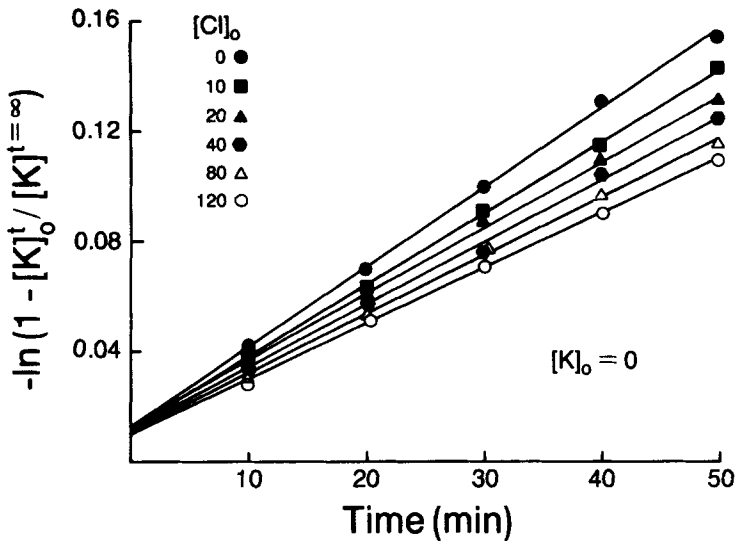

FIGURE 11. *Trans* inhibition of K efflux in normal swollen LK red blood cells by $[Cl]_o$ in the absence of $[K]_o$. The cells were preincubated with 10^{-6} M DIDS for 45 min at 37°C, centrifuged, and resuspended in Na_2SO_4 – NaCl (230–240 mosM) in the absence of DIDS. Therefore, they contained their normal Cl concentrations and were incubated with extracellular Cl concentrations ranging from 0 to 120 mM. Each rate constant value was obtained from five time points.

while inside the binding is *random*. The difference in the binding mode suggests a functional asymmetry of the cotransporter.

To determine the order of binding, we used the enzyme kinetic approach assuming rapid equilibrium (see Segel, 1975, and Appendix I). We measured the rate of K fluxes at different concentrations of K and Cl, substituted by Na and NO_3, respectively. The question of Cl substitution by other anions has been considered in the design of these experiments, because any Cl substituent affects the transporter rates (Lauf, 1988*c*). Due to its peculiar behavior in the sheep system (unpublished data), methane sulfonate used earlier (Lauf, 1988*c*) was eliminated. Although a non–Michaelis-Menten relationship between the flux and the Cl concentration (with NO_3

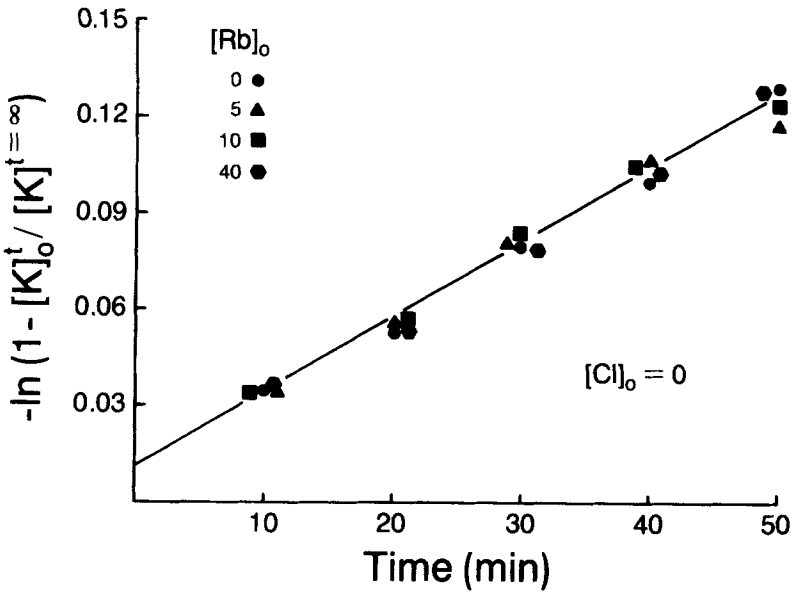

FIGURE 12. Insensitivity of ouabain-resistant K efflux to Rb_o in the absence of Cl_o in normal, swollen LK red blood cells. The cells were first incubated with 10^{-6} M DIDS for 45 min at 37°C, then centrifuged and resuspended in Cl-free Na_2SO_4 + Rb_2SO_4 solutions (230 mosM) in the absence of DIDS.

substitution) has been reported in NEM-stimulated cells, suggesting a possible competition for the transport site (Lauf, 1983*a*), a normal saturation behavior has also been demonstrated in diamide-treated cells (Lauf, 1988*a*). In the present study, using swollen cells, the linearity of the data suggests that NO_3 is indeed an acceptable substituent. Even if NO_3 binds to the transporter, it has no visible effect in the experimental approach chosen here and thus does not affect the conclusions derived from our substitution data. Nevertheless, the reactivity of foreign anions with the Cl-binding site warrants future consideration and is presently under investigation.

The random binding inside renders K flux more sensitive to changes of $[Cl]_i$ than the ordered binding to $[Cl]_o$. Indeed, when $[Cl]_o$ falls, only the apparent affinity for K

decreases; therefore, increasing $[K]_o$ recovers the flux to its original value. In contrast, a decrease in $[Cl]_i$ diminishes the maximum velocity of the flux, and high $[K]_i$ may not compensate for this effect. Ordered outside binding makes the K flux at low $[Cl]_o$ more sensitive to a variation in $[K]_o$.

Our results also show that the intracellular binding of one ion does not change the affinity of the transporter for the second ion (as demonstrated by intercepts on the horizontal axis (Fig. 5, *A* and *B*)). Hence, the interaction of cytoplasmic K and Cl with the transporter can be compared to a noncompetitive activation.

The ordered binding outside was also confirmed by the *trans* effects of one ion in the absence of its cotransported species. As represented Fig. 13, only Cl_o *trans*-inhibited K efflux in the absence of Rb_o, while Rb_o failed to *trans*-inhibit in the absence of Cl_o. These results are consistent with a model in which the free transporter interacts with Cl_o in the absence of K_o (Rb_o); i.e., Cl binds first, followed by K, but not vice versa (see Appendix III). It has previously been reported that the binding of furosemide is dependent on the extracellular concentration of Rb (Lauf, 1984). The

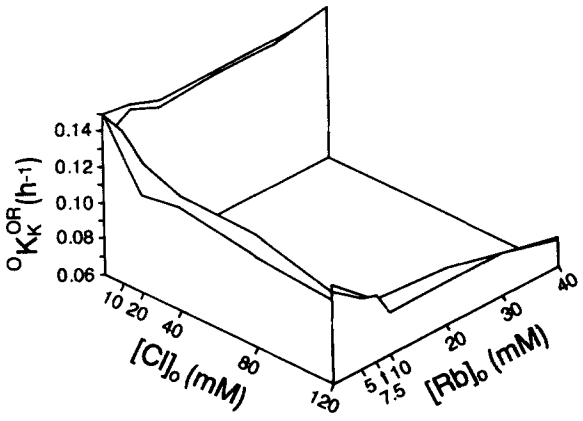

FIGURE 13. Summary of $[Cl]_o$ and $[Rb]_o$ effects on pseudo–first order rate constants of K efflux $^{o}k_K^{OR}$. The K efflux was maximal in the absence of external Cl and Rb. As $[Cl]_o$ increased, K efflux decreased. In the presence of 120 mM $[Cl]_o$, increasing $[Rb]_o$ further decreased the rate of the flux. However, in the absence of $[Cl]_o$, $[Rb]_o$ failed to affect the K efflux. The two lines represent two different experiments.

order of binding presented here suggests, therefore, that the affinity of furosemide is higher for the tertiary complex than for the free carrier.

Our results constitute the first demonstration of such an asymmetry in the order of binding for the binary K:Cl cotransporter. In the case of Na:K:2Cl cotransport in duck red cells, ordered binding was demonstrated at both sides of the cotransporter (McManus, 1987). However, for various other cotransport systems, different modalities of substrate binding have been reported in the literature (Table II). Unfortunately, few studies have investigated the binding mode at both faces of the membrane. For the Na:glucose cotransporter in rabbit intestine, ordered binding was suggested at both sides of the membrane but with a different order of addition: Na first at one side, glucose first at the other (Hopfer and Groseclose, 1980). Although these results are indeed consistent with a first on–first off model, it cannot yet be determined at which side Na bound first.

On the other hand, in the presence of Cl_o, Rb_o *trans*-inhibited K efflux (Figs. 9 and 13). One may consider $RbCl_o$ as a substrate for the cotransporter outside. By analogy

to a simple carrier (Lieb, 1982), *trans* acceleration, *trans* inhibition, and no *trans* effect may be observed depending on the respective values of the R parameters (see definition in the legend of Fig. 7). However, in the case of the KCl cotransporter the presence of Cl_o and K_o "by themselves" greatly complicates the issue of the *trans* effect. We challenged our model by manipulating (with the help of a computer) the

TABLE II

Order of Binding in Different Cotransport Systems

Cotransport	System and reference	Order
$H:H_2PO_4$	*Saccharomyces cerevisiae* (Roomans and Brost-Pauwels, 1979)	H first
H:lactose	*Escherichia coli* (Garcia et al., 1983)	H first
H:L-Lactate	Human erythrocyte (De Bruijne et al., 1985)	H first
H:sorbose	*Saccharomyces fragilis* Van den Broek and Van Steveninck, 1980)	Random
$H:Ca:SO_4$	*Penicillium notatum* (Cuppoletti and Segel, 1975)	H, Ca random, then SO_4
$Na:H_2PO_4$	*Saccharomyces cerevisiae* (Roomans and Brost-Pauwels, 1979)	Random
Na:cycloleucine	Guinea pig intestine (Alvarado and Mahmood, 1974)	Random
Na:Cl:glycine	Pigeon erythrocyte (Imler and Vidaver, 1972)	Cl first
Na:*p*-aminohippurate	Newt kidney (Kikuta and Hoshi, 1979)	Na first
Na:succinate	Rabbit kidney (Kippen et al., 1982)	Na first
Na:glucose	Renal brush border (Hopfer and Groseclose, 1980)	Na first
Na:glucose	Bovine intestine (Kaunitz and Wright, 1984)	Na first
Na:glucose	Rabbit intestine (Hopfer and Groseclose, 1980)	Na first at one side, glucose first at other
Na:K	3T3 (Altan et al., 1984)	Random
Na:K:2Cl	Hela cells (Miyamoto et al., 1986)	Na-Cl-K-Cl
Na:K:2Cl	Duck red blood cells (McManus, 1987)	Na-Cl-K-Cl
K:Cl	LK sheep red blood cells (Delpire and Lauf, 1990*a*, *b*, and this study)	Random inside, ordered outside with Cl before K

*Modified from Stein, 1986*a*.

numerous parameters. We achieved a very good fitting of all our *trans*-effect data (when it was not exactly of the same magnitude, it was always in the right direction). The fact that Rb_o further *trans*-inhibited K efflux rather than relieving the inhibition by Cl_o places the rate-limiting step of K:Cl cotransport on the translocation of the loaded carrier.

As demonstrated in this study, the *trans* effect was also dependent on the *cis* concentration level. Indeed, with cells containing high $[K]_i$, no *trans* effect was measurable, while in cells with low $[K]_i$, K efflux was *trans*-inhibited by Rb_o. These results suggest that the magnitude of the *trans* effect is inversely related to the concentration of the substrate at the *cis* side. After NEM stimulation *trans* inhibition of external Rb on K efflux was previously reported (Lauf, 1983*a*). The response of OR K fluxes in LK sheep red blood cells then is in contradiction to that in human red blood cells, where a *trans* stimulation was observed (Kaji, 1989).

The K_m values calculated for K were 67 mM for the efflux and 55 mM for the influx. These values are significantly higher than those reported for the Na:K:2Cl cotransporter in red cells: 5.5 mM in human red cells (Chipperfield, 1981), 3.5 mM in rat erythrocytes (Duhm and Göbel, 1984), and 10.2 mM in ferret red cells (Hall and Ellory, 1985). Furthermore, the K_m values for K are significantly different from those reported for K:Cl cotransport in human red blood cells: 140 mM for the influx and 33 mM for the efflux (Kaji, 1989).

Another important finding is the evidence of a significant difference between the efflux and influx levels. The comparison between K_m/V_{max} ratios for both unidirectional fluxes is generally considered as a good test for characterizing a cotransport system and distinguishing it from a simple carrier model (Lieb, 1982). For a simple carrier the ratios must be identical, while for a cotransport carrier they must be a function of the transmembrane gradient of the accompanying substrate, and they sometimes depend on the membrane potential (if the transporter carries a net charge). In our case the K_m/V_{max} ratios were 13 for the efflux and 20 for the influx. Therefore, our ratios ($r_i/r_o = 1.5$) are inconsistent with the transmembrane gradient of chloride (~ 0.7, see Appendix II).

Could the existence of another pathway influence the parameters causing such ratio differences? Considering that the membrane potential of the red blood cell is mainly due to the chloride permeability, a diffusional K outward leak would be most affected. However, such a passive pathway would simultaneously increase V_{max} and reduce the affinity. On the other hand, the results of Fig. 5*A* show a significant reduction of V_{max} as $[Cl]_i$ decreased. This finding confirms the high Cl dependence of the efflux and excludes the possibility of high K movement through a path not mediated by the cotransporter as previously reported (Lauf, 1988*b*).

Our results suggest that the fluxes are not equivalent at equilibrium (i.e., when $[K]_i \times [Cl]_i = [K]_o \times [Cl]_o$). This was also confirmed by simultaneously measuring both unidirectional fluxes in swollen cells in the absence of nystatin treatment and varying $[Rb]_o$. At equilibrium a significant net efflux still occurred. This asymmetry cannot be due to the fact that Rb is used in one case and K in the other. Indeed, as shown earlier (Lauf, 1983*a*), comparing Figs. 3 and 10, we observe identical K_m values for K and Rb (49 and 53 mM), as well as similar V_{max} values (2.6 and 2.4 mmol/LOC per h). Considering the model in terms of rate constants, the product of all rate constants for the efflux ($k_i g_o K_1 K_2$, see Fig. 7) is higher than the product for the influx ($k_o g_i K_3 K_5$). Recall that for the intestinal glucose transport (Stein, 1986*b*) the equivalence was not obtained unless the membrane potential was included: $k_i g_o K_1 K_2 = e^{(zT - zTGN)\mu} k_o g_i K_3 K_5$, where zT and $zTGN$ are the charges of the free and loaded transporter and μ is the membrane potential (the glucose carrier carried a positive

charge due to the Na ion). However, KCl cotransport in sheep as well as human red blood cells has been shown to be independent on the membrane potential (Brugnara et al., 1988).

Therefore, our results suggest a missing component in explaining the deviation from equilibrium and strongly argue for the nondiffusional nature of the K:Cl cotransport system in LK sheep red blood cells. This finding is in contrast to that previously reported for alkylation- or oxidation-stimulated K fluxes (Lauf, 1983*a*, 1988*b*), where zero net K efflux was much closer to the point of unity of the KCl product ratios. It remains to be seen whether the observation of a cAMP dependence of K:Cl cotransport in pig red blood cells (Kim et al., 1989) and the participation of a phosphorylation/dephosphorylation mechanism in the activation of the K:Cl cotransport in rabbit red blood cells (Jennings and Al-Rohil, 1989) or other factors can account for such asymmetry in fluxes.

APPENDIX I

To determine the order of ion binding to the cotransporter, we used the "rapid equilibrium" approach (Segel, 1975). To derive the kinetic scheme presented in Fig. 7, both steady-state and rapid equilibrium assumptions were considered. Formally, the velocity equations of a particular mode of binding (random or ordered) are identical for both approaches.

Random Binding

At rapid equilibrium (Segel, 1975),

$$\begin{array}{ccc} & & \overset{K_1}{\rightleftharpoons} \\ \mathrm{T} + \mathrm{K} & & \mathrm{TK} \\ + & & + \\ \mathrm{Cl} & & \mathrm{Cl} \\ K_2 \uparrow\downarrow & & \downarrow\uparrow \alpha K_2 \\ \mathrm{TCl} + \mathrm{K} & \underset{\alpha K_1}{\rightleftharpoons} & \mathrm{TKCl} \xrightarrow[k_p]{} \mathrm{T} + \mathrm{K} + \mathrm{Cl} \end{array}$$

$$\frac{1}{v} = \frac{\alpha K_1}{V_{max}}\left[1 + \frac{K_2}{[\mathrm{Cl}]}\right]\frac{1}{[\mathrm{K}]} + \frac{1}{V_{max}}\left[1 + \frac{\alpha K_2}{[\mathrm{Cl}]}\right] \tag{A1}$$

where v is the velocity, K_1 and K_2 are the dissociation constants for K and Cl, and α is the factor by which the dissociation constant of one ion is modified by the binding of the other ion to the transporter. V_{max} is defined as the product of the rate constant, k_p, and the sum of all states of the transport molecule.

The velocity equation of K efflux, v_{out}, described in Fig. 7 can be also written as follows:

$$\frac{1}{v_{out}} = F_2\left[1 + \frac{F_1/F_2}{[\mathrm{Cl}]_i}\right]\frac{1}{[\mathrm{K}]_i} + F_4\left[1 + \frac{F_3/F_4}{[\mathrm{Cl}]_i}\right] \tag{A2}$$

with $F_1 = K_1K_2k_i/g_i[(1 + \mathrm{Cl}_o/K_1)/k_i + 1/k_o]$

$F_2 = K_1K_2K_3k_ik_o/g_i$

$F_3 = K_3/g_o$

$F_4 = 1/g_o + (1 + \mathrm{Cl}_o/K_1)/k_i$

(see the definition of parameters in legend to Fig. 7).

Eqs. A1 and A2 have the same form. Their characteristics are: (*a*) a positive slope at high concentrations of [Cl], (*b*) an intercept on the horizontal axis if $\alpha = 1$, and (*c*) the intercept on the vertical axis depending on [Cl].

Ordered Binding

The rapid equilibrium approach yields:

$$\begin{array}{l} T + Cl \overset{K_2}{\rightleftharpoons} TCl \\ \qquad\qquad\quad + \\ \qquad\qquad\quad K \\ \qquad\qquad\quad \downarrow\uparrow K_1 \\ \qquad\qquad\quad TKCl \xrightarrow[k_p]{} T + K + Cl \end{array}$$

$$\frac{1}{v} = \frac{K_1}{V_{max}}\frac{K_2}{[Cl]}\frac{1}{[K]} + \frac{1}{V_{max}}\left[1 + \frac{K_2}{[Cl]}\right] \tag{A3}$$

if K binds first and

$$\frac{1}{v} = \frac{K_1}{V_{max}}\left[1 + \frac{K_2}{[Cl]}\right]\frac{1}{[K]} + \frac{1}{V_{max}} \tag{A4}$$

if Cl binds first.

The velocity equation of K influx, v_{in}, described in Fig. 7 can be transformed as follows:

$$\frac{1}{v_{in}} = F_2\left[1 + \frac{F_1/F_2}{[Cl]_o}\right]\frac{1}{[K]_o} + F_3 \tag{A5}$$

with $F_1 = K_3K_4k_o/g_o[(1 + Cl_i/K_3)/k_o + 1/k_i]$

$F_2 = K_3K_4k_o/(g_ok_iK_1)$

$F_3 = 1/g_i + (1 + Cl_i/K_3)/k_o$

Eqs. A4 and A5 are similar in their form. If we transform $1/v_{in}$ as a function of $1/[Cl]_o$, the equation will be similar to Eq. A3. The characteristics of the ordered binding are: (*a*) a positive slope and an intercept on the vertical axis of the double reciprocal plot $1/v$ vs. 1/[ion] (binding second); (*b*) a slope equal to zero at infinite concentrations of the cotransported ion; and (*c*) an intersection of the lines in the second quadrant for the reciprocal plot $1/v$ vs. 1/[ion] (binding first).

In conclusion, although the equations derived from the "rapid equilibrium" assumption do not sufficiently account for the cotransport model, these simple equations are valid in determining the order of binding.

APPENDIX II

The velocity equations of unidirectional fluxes (Fig. 7) simplify under zero-K-*trans* conditions to:

$$v_{in} = \frac{Cl_oK_o}{Z_oR + R_iCl_oK_o} \tag{A6}$$

$$v_{out} = \frac{Cl_iK_i}{Z_iR + R_oCl_iK_i} \tag{A7}$$

From the reciprocal transformation, the kinetic parameters can be determined as:

$$K_m = \frac{Z_oR}{R_iCl_o} \quad \text{and} \quad V_{max} = 1/R_i, \tag{A8}$$

for influx, and

$$K_m = \frac{Z_i R}{R_o Cl_i} \quad \text{and} \quad V_{max} = 1/R_o, \tag{A9}$$

for efflux.

The ratios K_m/V_{max} are defined as

$$r_{in} = Z_o R/Cl_o$$

$$r_{out} = Z_i R/Cl_i$$

Therefore, for a cotransport system the ratios K_m/V_{max} for influx and efflux are related to the chloride gradient:

$$\frac{r_{in}}{r_{out}} = \frac{Z_o}{Z_i}\frac{Cl_i}{Cl_o} \tag{A10}$$

Because the affinities for K influx and efflux represent external and internal concentrations of K ($[K]_o$ and $[K]_i$), respectively, Eq. A10 is similar to $[K]_i \times [Cl]_i = [K]_o \times [Cl]_o$ (driving forces relation). For a cotransport that strictly responds to K and Cl driving forces, the multiplication of the constants $K_1K_2k_o/g_o$ and $K_3K_5k_i/g_i$ are equivalent; that is, $Z_o = Z_i$.

APPENDIX III

Under zero-K-*trans* or zero-Cl-*trans* conditions, the velocity equations for K efflux and influx are Eqs. A6 and A7. As described Fig. 7, the R parameters contain Cl or K terms. However, the R parameters appearing in the efflux equation (R and R_o) contain Cl_o but not K_o. This comes from the ordered binding realized in the model. If the binding was random, both Cl_o and K_o would appear in the R or R_o parameters. This can be shown for the influx where the binding at the *trans* side is random. In this case, we can see from Fig. 7 that the R and R_i parameters contain both Cl_i and K_i.

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