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Kinetics of K-Cl Cotransport in Frog Erythrocyte Membrane: Effect of External Sodium

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Abstract. In frog red blood cells, K-Cl cotransport (i.e., the difference between ouabain-resistant K fluxes in Cl and NO_3) has been shown to mediate a large fraction of the total K^+ transport. In the present study, Cl^--dependent and Cl^--independent K^+ fluxes via frog erythrocyte membranes were investigated as a function of external and internal K^+ ($[K^+]_e$ and $[K^+]_i$) concentration. The dependence of ouabain-resistant Cl^--dependent K^+ (^{86}Rb) influx on $[K^+]_e$ over the range 0–20 mM fitted the Michaelis-Menten equation, with an apparent affinity (K_m) of 8.2 ± 1.3 mM and maximal velocity (V_{max}) of 10.4 ± 1.6 mmol/l cells/hr under isotonic conditions. Hypotonic stimulation of the Cl^--dependent K^+ influx increased both K_m (12.8 ± 1.7 mM, $P < 0.05$) and V_{max} (20.2 ± 2.9 mmol/l/hr, $P < 0.001$). Raising $[K^+]_e$ above 20 mM in isotonic media significantly reduced the Cl^--dependent K^+ influx due to a reciprocal decrease of the external Na^+ ($[Na^+]_e$) concentration below 50 mM. Replacing $[Na^+]_e$ by $NMDG^+$ markedly decreased V_{max} (3.2 ± 0.7 mmol/l/hr, $P < 0.001$) and increased K_m (15.7 ± 2.1 mM, $P < 0.03$) of Cl^--dependent K^+ influx. Moreover, $NMDG^+$ Cl substitution for NaCl in isotonic and hypotonic media containing 10 mM RbCl significantly reduced both Rb^+ uptake and K^+ loss from red cells. Cell swelling did not affect the Na^+-dependent changes in Rb^+ uptake and K^+ loss. In a nominally $K^+(Rb^+)$-free medium, net K^+ loss was reduced after lowering $[Na^+]_e$ below 50 mM. These results indicate that over 50 mM $[Na^+]_e$ is required for complete activation of the K-Cl cotransporter. In nystatin-pretreated cells with various intracellular K^+, Cl^--dependent K^+ loss in K^+-free media was a linear function of $[K^+]_i$, with a rate constant of 0.11 ± 0.01 and 0.18 ± 0.008 hr^{-1} ($P < 0.001$) in isotonic and hypotonic media, respectively. Thus K-Cl cotransport in frog erythrocytes exhibits a strong asymmetry with respect to transported K^+ ions. The residual, ouabain-resistant K^+ fluxes in NO_3 were only 5–10% of the total and were well fitted to linear regressions. The rate constants for the residual influxes were not different from those for K^+ effluxes in isotonic (~0.014 hr^{-1}) and hypotonic (~0.022 hr^{-1}) media, but cell swelling resulted in a significant increase in the rate constants.

Key words: Erythrocytes — Potassium-chloride cotransport — Potassium affinity — Cell volume

Introduction

The K-Cl cotransporter has been identified in a wide variety of animal cells. The properties of this cotransporter have been intensively investigated mainly in red blood cells of different species (for review *see* Lauf et al., 1992). There is significant variability between cells among different species in the magnitude of ion fluxes via the K-Cl cotransporter and its sensitivity to transport and protein phosphatase/kinase inhibitors. One of the intrinsic properties of this transporter is the activation by cell swelling and involvement in cell volume regulation. A great deal of research over the past ten years has been focused on the study of regulation of K-Cl cotransport in erythrocytes of some mammals (Kim et al., 1989; Kelley & Dunham, 1996; Bize et al., 1998). Although the functional characteristics of K-Cl cotransport have been extensively investigated, the data concerning kinetic properties of the transporter are limited to studies on human (Kaji & Kahn, 1985; Brugnara, 1989; Kaji, 1989) and sheep erythrocytes (Bergh, Kelley & Dunham, 1990; Delpire & Lauf, 1991*a*,*b*). These studies have demonstrated that the Cl^--dependent K^+ fluxes have a relatively low affinity for external and internal K^+ and provide evidence for functional asymmetry of K-Cl cotransport in these cells. Some uncertainty appears to exist regard-

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ing the effect of cell swelling on the kinetic constants for Cl^--dependent K^+ fluxes.

Our previous studies (Gusev, Agalakova & Lapin, 1995, 1997) have shown that Cl^--dependent K^+ transport mediates more than 50% of the total K^+ fluxes across frog erythrocyte membranes. This Cl^--dependent K^+ flux is stimulated by cell swelling and most likely represents K-Cl cotransport activity. The purpose of this study was to investigate the kinetic parameters of the K-Cl cotransporter in frog red blood cells. Our data indicate that the apparent affinity for external potassium of the K-Cl cotransporter is relatively high for K^+ influx but is extremely low for K^+ efflux. Analysis of the kinetics of Cl^--dependent K^+ transport shows that swelling increases the V_{max} and decreases the apparent affinity. A striking finding in the present study was the observation that reducing external Na^+ below 50 mM markedly inhibited both Cl^--dependent K^+ influx and efflux in these cells.

Materials and Methods

Cell Preparation

The experiments were performed on erythrocytes of frogs (*Rana temporaria*) in the period from November to March. The animals were kept in aquaria at a temperature of 2–4°C. Red blood cells were obtained as reported before (Gusev, Agalakova & Lapin, 1995; Agalakova et al., 1997). Occasionally, blood from two or three frogs was pooled. Red cells were gently washed by centrifugation three times in standard chloride or nitrate saline, removing the buffy coat. Washed cells were resuspended at a hematocrit of 30–40% and incubated in the standard saline for 60 min at 20°C.

Solutions

The standard chloride saline comprised (in mM): 102 NaCl, 3 KCl, 1 $MgCl_2$, 10 Tris-HCl, 10 glucose (pH 7.6 at 20°C), the osmolality of this solution was 230 mosm/kg water. In K^+-free incubation medium, 3 mM KCl was replaced with 3 mM NaCl. To vary the K^+ concentration in the incubation media, the K^+-free medium was mixed with a solution containing 105 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.6 at 20°C). Hypotonic K^+-free incubation medium (~160 mosm/kg water) contained (in mM): 65 NaCl, 1 $MgCl_2$, 10 Tris-HCl (pH 7.6 at 20°C). The corresponding isotonic medium had the same composition with the addition of 80 mM sucrose. In the experiments in NO_3 both external and internal Cl^- were replaced by NO_3^-. N-methyl-D-glucamine ($NMDG^+$) was used to replace Na^+ in the incubation media. The Cl and NO_3 salts of $NMDG^+$ were prepared from the base by titration with hydrochloric or nitric acid, respectively. In experiments in which cellular K^+ (Na^+) was manipulated, erythrocytes were treated with the nystatin techniques as described elsewhere (Kaji, 1986; Brugnara, 1989). The nystatin-loading solutions contained varying K^+ and Na^+ concentrations (Na + K = 105 mM) and 40 mM sucrose. Aliquots of nystatin-treated cells were washed with an ice-cold solution containing 70 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.6 at 4°C) to determine the intracellular K^+ and Na^+ content. Ion concentrations were expressed as mmol/l cell water assuming that the cell water content in frog erythrocytes is 63% under isotonic conditions (Gusev et al., 1997).

K^+ Flux Measurements

Ouabain-insensitive K^+ influx was measured by conventional radioactive tracer technique, using ^{86}Rb as a tracer for K^+. Aliquots of the cell suspension of washed cells were diluted into the uptake incubation media to a final hematocrit of 2–3%. Then ^{86}Rb (0.02–0.05 MBq/ml final) was added to the cell suspensions and uptake of ^{86}Rb was measured over a 30–60 min, during which time was linear. After incubation, cells were washed rapidly three times with an ice-cold saline and were extracted with 10% trichloracetic acid. Aliquots of the uptake media and supernatants were removed for determination of radioactivity. The radioactivity of the samples was measured as Cerenkov radiation directly in the aqueous medium using a RACBETA (LKB Wallac) liquid-scintillation spectrometer.

In experiments in which Rb^+ substituted for K^+ in the incubation media, Rb^+ influx and K^+ efflux were measured simultaneously. The experiments were performed with the same techniques as the influx assay above. After incubation, aliquots of flux media were removed to determine the K^+ concentrations. Cells were washed three times and lysed with distilled water for determination of Rb^+ content. To measure net K^+ loss, frog erythrocytes were incubated in nominally K^+-free media for 30–60 min as described previously (Gusev et al., 1997). During 30–60 min, the K^+ loss did not exceed 10% of the total cellular K^+ content, was approximately linear, and *corresponded to* the rate of K^+ efflux from the cells. The net K^+ loss was calculated from the change in potassium concentration in the flux media.

Sodium and potassium concentrations in cell lysates were measured by flame photometry (Flapho model 40). Potassium concentrations in flux media and rubidium in cell lysates were determined by emission flame spectroscopy (Perkin-Elmer model 370 Atomic absorption spectrophotometer).

All flux experiments were performed at 20°C. Fluxes are expressed in mmol K^+ per liter original packed cell volume per hour (mmol/(l × hr).

Materials

Ouabain and nystatin were obtained from Sigma Chemical. ^{86}Rb was purchased from ISOTOP (Russia). Ouabain was prepared as a 10 mM stock solution in distilled water and used at a final concentration of 0.1 mM. Stock solutions of nystatin (4 mg/ml) in dimethylsulfoxide were made fresh daily and protected from light. All other chemicals were analytical grade.

Statistics

Results were expressed as means ± SE. Comparison between means was performed by paired or unpaired *t* test with $P < 0.05$ being taken as the level of significance. A curve-fit computer program was used to perform a regression analysis (SigmaPlot, version 3.0, Jandel Scientific, Corte Madera, CA). Best-fit curves relating K^+ influx to external K^+ concentration were calculated using a hyperbolic relationship derived from the Michaelis-Menten equation: $V = V_{max} \cdot S/(K_m + S)$, where V_{max} is the maximal velocity of K^+ influx, S is the K^+ concentration and K_m is the Michaelis constant. Regression lines were calculated individually from each experiment.

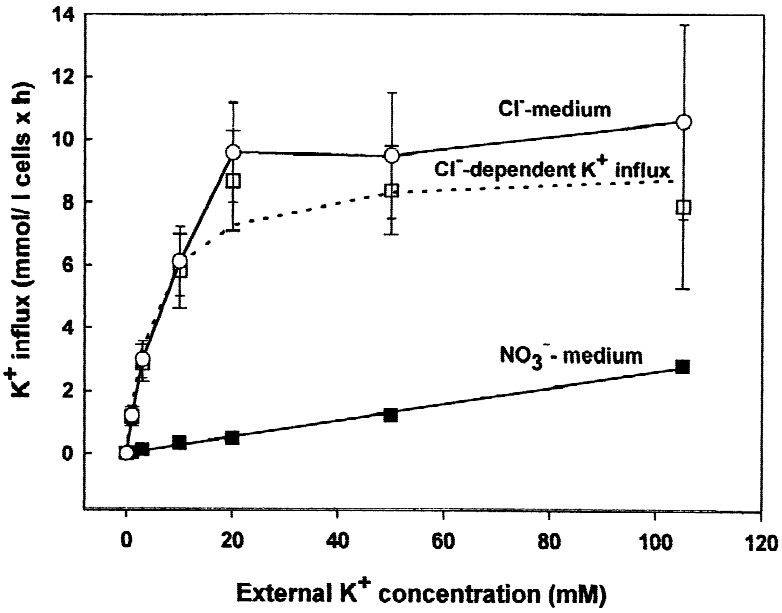

Fig. 1. Cl^--dependent and Cl^--independent K^+ influx plotted as a function of external K^+ concentration $[K^+]_e$. Erythrocytes were washed and preincubated in Cl or NO_3. Medium Na^+ and K^+ concentrations were varied reciprocally (Na^+ plus K^+ = 105 mM). Ouabain (0.1 mM) was present in all media. K^+ influx was measured as ^{86}Rb uptake for 60 min. The Cl^--dependent K^+ influxes were calculated as the difference in K^+ influxes in the presence (open circles) and absence (filled squares) of Cl. Results from these 4 experiments were averaged and shown as means ± SE. Error bars were omitted when smaller than the symbols. Cl^--dependent K^+ influx (broken line) was a Michaelis function of $[K^+]_e$, such that $V = 9.2 \times [K^+]_e/(5.2 + [K^+]_e)$ ($R = 0.54$). In NO_3 K^+ influx was a linear function of $[K^+]_e$, such that $V = 0.02 \times [K^+]_e$, ($R = 0.997$). Data points were fitted with a computer program (SigmaPlot).

Results

K^+(^{86}Rb) Influx as a Function of External K^+ Concentration

To determine the kinetics parameters of K-Cl cotransport, frog erythrocytes were preincubated in Cl and NO_3 containing 1 to 105 mM K^+ and 0.1 mM ouabain for 30 min. ^{86}Rb uptake was measured for 60 min. As can be seen in Fig. 1, K^+ influx in frog red cells incubated in Cl increased considerably with $[K^+]_e$ increasing up to 20 mM. The K^+ influx in frog erythrocytes in NO_3 was substantially smaller than that observed in Cl. At 20 mM $[K^+]_e$, K^+ influx in the presence of NO_3 was only about 5% of that in Cl and was a linear function of $[K^+]_e$ up to 105 mM as described by a linear regression equation: $V = (0.026 \pm 0.001) \times [K^+]_e$ ($R = 0.997$, $n = 4$). In these paired experiments, the Cl^--dependent component of K^+ influx was calculated as the difference between K^+ influx in Cl and NO_3 (Fig. 1). The Cl^--dependent K^+ influx was a saturable function of $[K^+]_e$ described by the Michaelis-Menten equation with a Michaelis constant (K_m) of 5.3 ± 1.7 mM and a maximal velocity (V_{max}) of 9.2 ± 0.7 mmol/(l cells × hr) ($R = 0.54$). It is evident from Fig. 1 that the *total* and Cl^--dependent K^+ influx was unchanged with increasing $[K^+]_e$ above 20 mM. The Cl^--dependent K^+ influxes were 8.7 ± 2.0, 8.3 ± 1.9 and 7.9 ± 3.0 mmol/(l × hr), respectively. As will be shown below, the kinetics of this transport were more accurately described using $[K^+]_e$ in the range from 0 to 20 mM. The analysis gave an apparent K_m value of 12.3 ± 2.7 mM and a V_{max} of 14.2 ± 1.4 mmol/(l × hr), showing a much higher regression coefficient ($R = 0.94$).

Effect of Cell Swelling on K^+(^{86}Rb) Influx as a Function of External K^+ Concentration

Similar to erythrocytes of other species, the K-Cl cotransporter in frog red cells is significantly activated by cell swelling (Gusev et al., 1995, 1997). Therefore, it is important to elucidate the changes in the kinetic properties of K-Cl cotransport following its activation in swollen cells. We compared K^+ influxes in frog erythrocytes incubated in isotonic (Fig. 2*A*) and hypotonic media (Fig. 2*B*) with the same Na^+ and K^+ concentrations plus/minus sucrose, respectively. Unexpectedly, K^+ influx in isotonic Cl was decreased when $[K^+]_e$ was increased above 20 mM. The magnitude of K^+ influx at 50 mM $[K^+]_e$ was considerably lower than that at 20 mM $[K^+]_e$ ($P < 0.001$). In contrast, K^+ influx in frog erythrocytes incubated in hypotonic Cl increased as a function of $[K^+]_e$ ranging from 1 to 50 mM. In NO_3 a linear relationship was observed between K^+ influx and $[K^+]_e$. K^+ influxes as a function of $[K^+]_e$ were fitted by simple linear regression equations: $V = (0.012 \pm 0.001) \times [K^+]_e$ ($R = 0.95$) and $V = (0.019 \pm 0.002) \times [K^+]_e$ ($R = 0.95$) for isotonic and hypotonic NO_3, respectively. The slopes of the linear regressions represent rate constants (rate coefficients) for K^+ influxes. The rate constant for K^+ influx in red blood cells incubated in hypotonic NO_3 was significantly higher than that in isotonic NO_3 ($P = 0.0066$, *t* test).

From the data presented in Fig. 2 the Cl^--dependent K^+ influx in frog erythrocytes as a function of $[K^+]_e$ was calculated as the difference between the K^+ fluxes in chloride and nitrate media (Fig. 3). The Cl^--dependent K^+ influx in isotonic medium reached a maximal magnitude at 20 mM $[K^+]_e$ and then decreased at higher $[K^+]_e$. In isotonic medium, the dependence of this flux on $[K^+]_e$

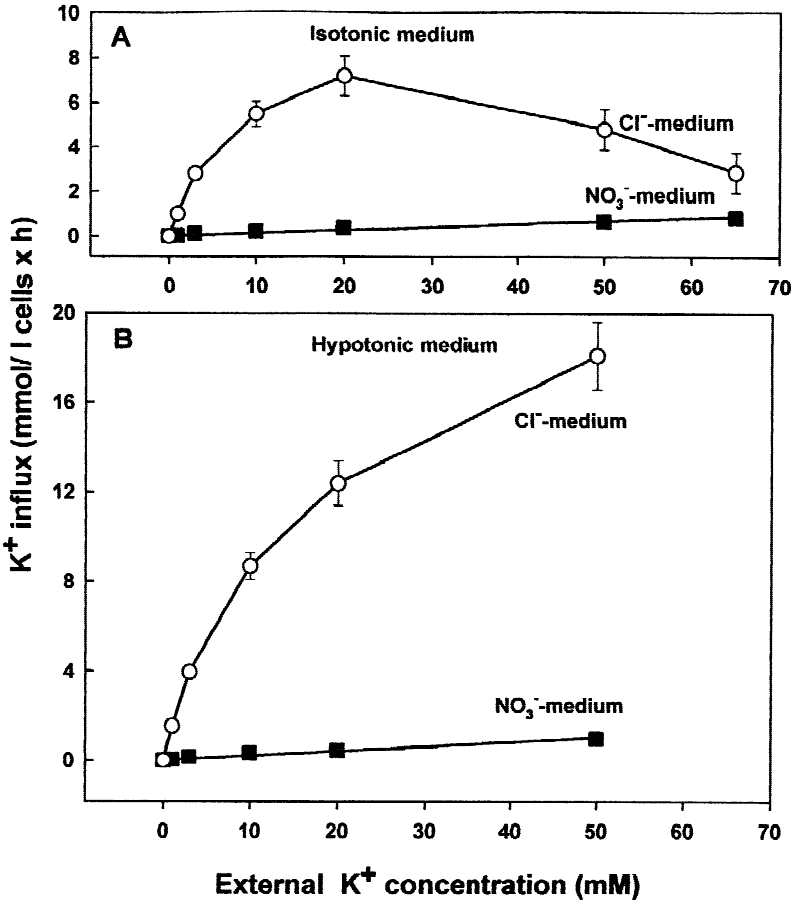

Fig. 2. K^+ influx as a function of external K^+ concentration in isotonic (*A*) and hypotonic media (*B*). K^+ and Na^+ concentrations in the media were varied reciprocally ($Na^+ + K^+ = 65$ mM) and isotonic media contained 80 mM sucrose. Ouabain (0.1 mM) was present in all media. K^+ influxes were determined from ^{86}Rb uptake for 30 min in Cl and for 60 min in NO_3. Results from 6–7 separate experiments were averaged and shown as means ± SE. Error bars were omitted when smaller than the symbols. In NO_3, lines were drawn using a linear regression analysis: $V = 0.012 \times [K^+]_e$ ($R = 0.95$) and $V = 0.019 \times [K^+]_e$ ($R = 0.95$) for isotonic and hypotonic media, respectively. Regression lines were fitted using a computer program (SigmaPlot).

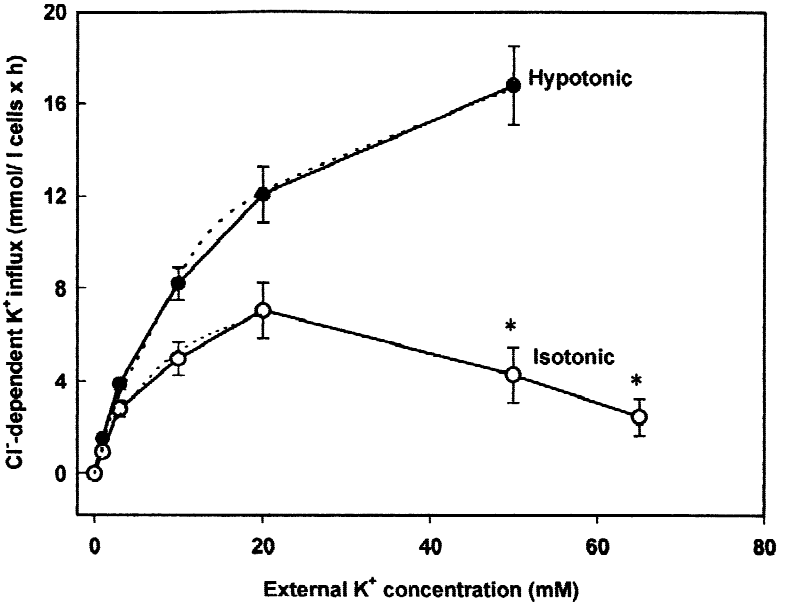

Fig. 3. Effect of cell swelling on Cl^--dependent K^+ influx. All data points were calculated from data given in Fig. 2. Cl^--dependent K^+ influxes (*V*) were a Michaelis function of external $[K^+]_e$ (0–20 mM for isotonic, 0–50 mM for hypotonic media), such that $V = 9.9 \times [K^+]_e/(8.8 + [K^+]_e)$ and $V = 22.0 \times [K^+]_e/(16.1 + [K^+]_e)$ with $R = 0.89$ and $R = 0.94$ for isotonic and hypotonic media, respectively. The hyperbolic functions are shown as dotted curves. Asterisks indicate a significant difference ($P < 0.01$) as compared with 20 mM $[K^+]_e$.

in the concentration range of 0 to 20 mM was well described by the Michaelis-Menten equation with a V_{max} of 9.8 ± 2.2 mmol/l/hr and an apparent K_m of 8.4 ± 1.8 mM ($R = 0.89$). These values agree well with those obtained in the previous series of experiments (Fig. 1). In swollen cells, Cl^--dependent K^+ influx was a saturable function

of $[K^+]_e$ in the concentration range from 0 to 50 mM, with a V_{max} of 22.0 ± 0.7 mmol/l/hr and an apparent K_m of 16.1 ± 1.2 mM. These kinetic parameters were similar to those calculated from the part of the curve at 0–20 mM $[K^+]_e$: V_{max} = 20.2 ± 2.9 mmol/l/hr and K_m = 12.8 ± 1.7 mM (R = 0.94). Thus activation of K-Cl cotransport in swollen frog erythrocytes was associated with both an increase in maximal velocity (P = 0.009) and a small decrease of its affinity for external K^+ (P = 0.03).

Effect of External Na^+ Concentration on K^+ Influx

In the experiments described above, a rather surprising observation was the decline in the Cl^--dependent K^+ influx after increasing $[K^+]_e$ above 20 mM (Fig. 3). Since the changes in $[K^+]_e$ were achieved by replacing external Na^+ $[Na^+]_e$, one simple explanation for this phenomenon is that removal of $[Na^+]_e$ can inhibit K^+ transport in frog red blood cells. Therefore the following experiments were designed to clarify this possibility. Frog erythrocytes were incubated in isotonic media with substitution of K^+ for Na^+ or N-methyl-D-glucamine ($NMDG^+$) so that the total cation concentration was kept constant (65 mM). In NO_3 K^+ influxes in frog erythrocytes were relatively low and were independent of the presence of $[Na^+]_e$ (*data not shown*). The Cl^--dependent K^+ influxes calculated from these experiments are shown in Fig. 4. Again, as $[K^+]_e$ was increased from 20 to 50 mM and $[Na^+]_e$ was reduced from 45 to 15 mM, the influx of K^+ (^{86}Rb) decreased considerably (7.52 ± 1.15 *vs.* 5.78 ± 1.08 mmol/l cells × hr, $P < 0.001$). The Cl^--dependent K^+ influx in red blood cells incubated in Na^+ media was a saturable function of $[K^+]_e$ within 1–20 mM with an apparent K_m of 7.9 ± 1.9 mM and a V_{max} of 11.2 ± 2.3 mmol/l cells/hr (R = 0.86).

In the absence of $[Na^+]_e$, the K^+ influx in red cells at all $[K^+]_e$ was much lower than that in the presence of $[Na^+]_e$ (Fig. 4). In Na^+-free media, there was a hyperbolic dependency between K^+ influx and $[K^+]_e$ from 0 to 50 mM which could be described by the Michaelis-Menten equation. The kinetic constants for the Cl^--dependent K^+ influx in Na^+-free media were 15.7 ± 2.1 mM and 3.2 ± 0.7 mmol/l × hr for an apparent K_m value and a V_{max} value, respectively (R = 0.88). These data indicate that the removal of $[Na^+]_e$ produced a significant reduction ($P < 0.001$) in both maximal velocity for Cl^--dependent K^+ influx and its affinity for $[K^+]_e$ ($P < 0.03$).

Effect of $[Na^+]_e$ on Rb^+ Uptake and K^+ Loss

To characterize further the dependence of K^+ transport on the Na^+ concentration in the medium $[Na^+]_e$, experiments were performed on frog erythrocytes incubated in media with different concentrations of RbCl substituting for NaCl so that the sum of both salts was kept constant

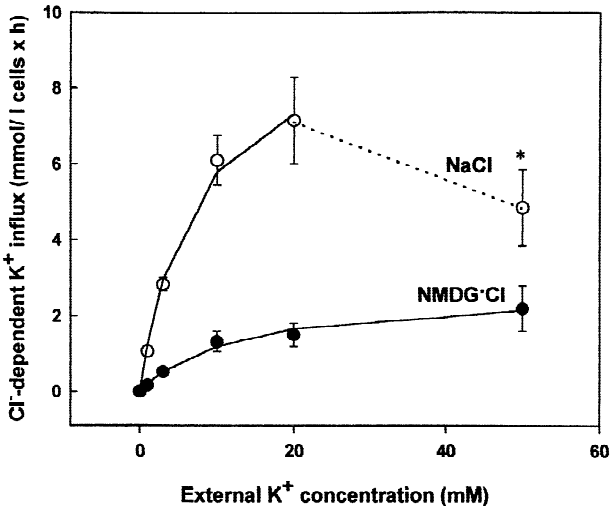

Fig. 4. Effect of removal of external Na^+ on Cl-dependent K^+ influx plotted as a function of $[K^+]_e$. Erythrocytes were preincubated in Cl and NO_3. Then Cl^-- and NO_3^- cells were washed with corresponding Na^+-containing or Na^+-free media ($NMDG^+$). $[K^+]_e$ concentrations were varied reciprocally (K^+ plus Na^+ or K^+ plus $NMDG^+$ were 65 mM). All media also contained (in mM): 80 sucrose, 1 $MgCl_2$, 10 Tris-HCl, 0.1 ouabain (pH 7.6 at 20°C). Results from these 5 experiments were averaged and shown as means ± SE. Cl^--dependent K^+ influxes were a Michaelis function of $[K^+]_e$ both in the presence of Na^+ (0–20 mM $[K^+]_e$) and in the absence of Na^+ (0–50 mM $[K^+]_e$). In Na^+-medium: $V = 11.2 \times [K^+]_e/(7.9 + [K^+]_e)$, R = 0.87. In medium without Na^+: $V = 2.7 \times [K^+]_e/(12.2 + [K^+]_e)$, R = 0.88. The curves were drawn to a standard Michaelis-Menten equation using a computer program (SigmaPlot).

(65 mM). It is evident from Fig. 5*A* that the Rb^+ influx was increased when external Rb^+ $[Rb^+]_e$ was raised from 10 to 20 mM. However, further increase in $[Rb^+]_e$ up to 50 mM in isotonic media led to a significant decrease in Rb^+ influx. As can be seen in Fig. 5*B*, K^+ efflux was not significantly changed as the $[Rb^+]_e$ varied from 0 to 20 mM in isotonic media (8.2 ± 0.7, 8.8 ± 0.9 and 11.0 ± 0.9 mmol/(l × hr) at 0, 10 and 20 mM $[Rb^+]_e$, respectively). Again, there was a significant reduction in K^+ loss from the cells (Fig. 5*B*) in isotonic media following increase of the $[Rb^+]_e$ from 20 to 50 mM and reducing $[Na^+]_e$ from 45 to 15 mM. In hypotonic media, the K^+ loss was diminished gradually as a result of increasing $[Rb^+]_e$ from 0 to 50 mM. These data support results of our previous series of experiments and suggest that the removal of $[Na^+]_e$ exerts a transinhibitory effect on K^+ efflux from frog erythrocytes as well as a *cis*-inhibitory effect on influx.

In the next series of experiments we compared Rb^+ uptake and K^+ loss in frog red blood cells incubated in NaCl and Na^+-free ($NMDG \cdot Cl$) media at the same $[Rb^+]_e$ (10 mM). These experiments clearly demonstrated that both Rb^+ uptake (Fig. 6*A*) and K^+ loss (Fig. 6*B*) were substantially reduced in isotonic and hypotonic Na^+-free media as compared with standard Na^+ media.

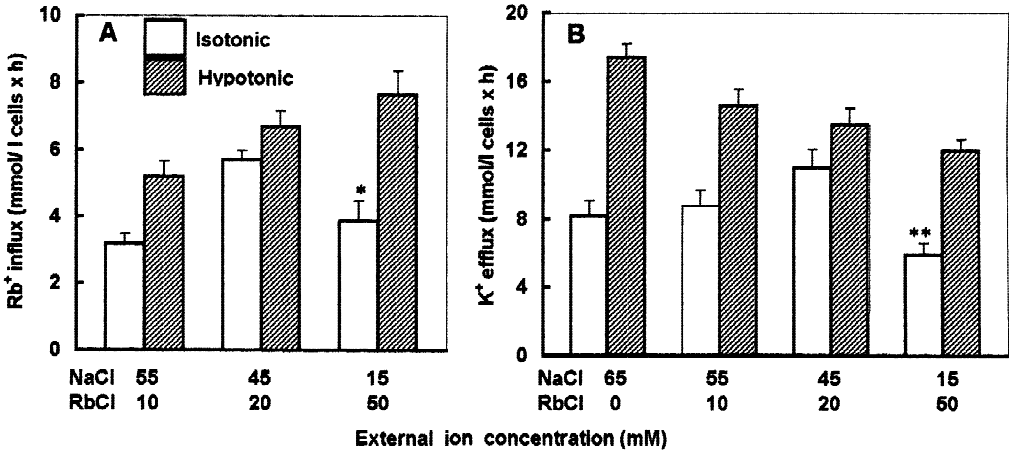

Fig. 5. Dependence of Rb^+ uptake (*A*) and K^+ efflux (*B*) from frog erythrocytes on extracellular Rb^+ (Na^+) concentration. Red cells were incubated in Cl containing various Rb^+ concentrations. Rb^+ uptake and K^+ loss were measured simultaneously for 60 min in isotonic (white columns) and 30 min in hypotonic (hatched columns) media. Data are expressed as means ± SE from 5 separate experiments. $*P < 0.02$, $**P < 0.005$ *vs.* isotonic medium with 45 mM Na^+ (paired *t* test).

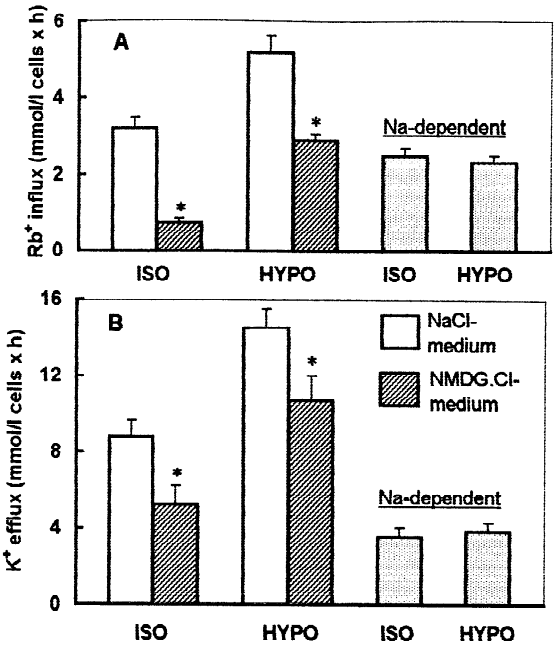

Fig. 6. Inhibition of Rb^+ uptake (*A*) and K^+ efflux (*B*) in frog erythrocytes by removal of external Na^+. Red cells were incubated for 60 min in isotonic (ISO) and 30 min in hypotonic (HYPO) media. All media contained 10 mM RbCl and 55 mM NaCl (white columns) or 55 mM NMDG · Cl (hatched columns); isotonic media contained 80 mM sucrose. The Na^+-dependent components of Rb^+ influx and K^+ efflux (black columns) were calculated from the data in each experiment. Data are means ± SE from 5 separate experiments. $*P < 0.003$ (paired *t* test) as compared with Na^+-containing media.

All fluxes of Rb^+ and K^+ in swollen cells were significantly higher than those in isotonic media. However, the calculated Na^+-dependent components of Rb^+ uptake and K^+ loss were identical in isotonic and hypotonic media. Thus the volume-sensitive components of Rb^+ uptake obtained by subtracting the flux in isotonic from that in hypotonic media were indistinguishable in red blood cells incubated in NaCl-medium (1.97 ± 0.35 mmol/(l × hr) and NMDG · Cl-medium (2.15 ± 0.19 mmol/(l × hr). The K^+ losses induced by cell swelling were also similar in both media (5.65 ± 0.76 and 5.48 ± 0.44 mmol/(l × hr) in NaCl and Na^+-free medium, respectively).

Effect of $[Na^+]_e$ on K^+ Loss in K^+-Free Media

In the following experiments frog erythrocytes were incubated in nominally K^+-free isotonic media with varying concentrations of Na^+ ($NMDG^+$ substitution) in the presence of Cl^- or NO_3^- as the principal anion. All media contained the same total salt concentrations, 105 mM. The results of these experiments are presented in Fig. 7. The K^+ loss was similar in both nitrate media in the absence and in the presence of Na^+, whereas removal of Na^+ from chloride medium caused a twofold inhibition of K^+ loss from frog erythrocytes. Equimolar replacement of 55 mM Na^+ with $NMDG^+$ in the incubation medium had no significant effect on K^+ loss. However, decreasing $[Na^+]_e$ below 50 mM resulted in a marked fall in the K^+ loss. Thus it was evident that the concentration of Na^+ in the incubation media plays an important role in transactivation of Cl^--dependent K^+ transport from frog red blood cells.

Dependence of K^+ Loss in K^+-Free Media on Intracellular K^+ Concentration

In these experiments red blood cells were prepared by the nystatin technique with varying intracellular K^+ con-

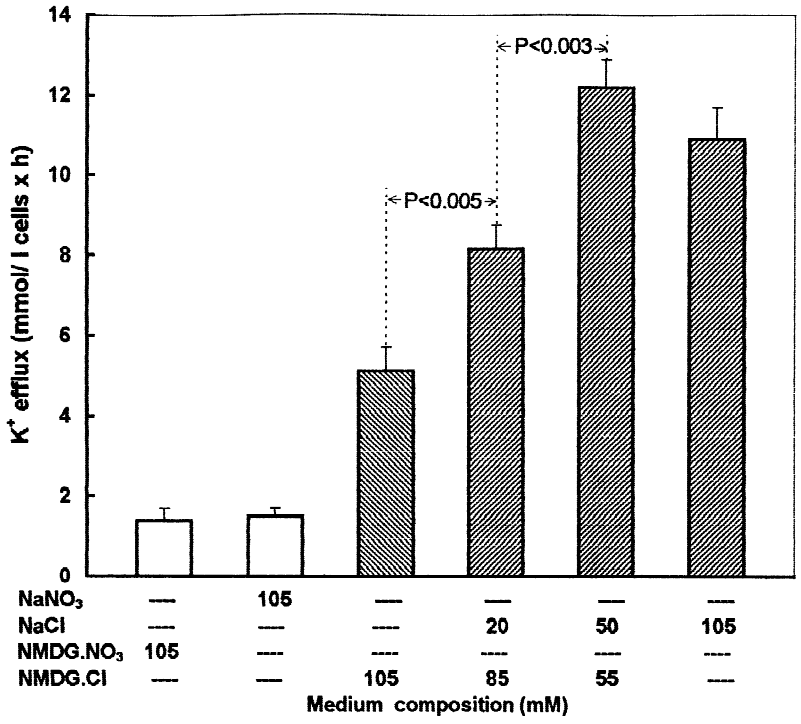

Fig. 7. Effects of ion replacement on net K^+ loss in isotonic nominally K^+-free media. Red blood cells were washed and incubated in corresponding media for 60 min, then K^+ concentrations in flux media were measured. Data are presented as means ± SE for 4 separate experiments.

centrations in Cl and NO_3. The red blood cells were incubated in isotonic and hypotonic nominally K^+-free media for 30–60 min and K^+ release from the cells was measured. As expected, the rate of K^+ loss from erythrocytes incubated in NO_3 was smaller than that observed in Cl (Fig. 8). When red cells were incubated in isotonic and hypotonic NO_3, the relationship between K^+ loss and intracellular K^+ concentration was linear in each experiment with a correlation coefficient (R) within 0.76–0.99. The slopes of the linear regressions represent rate constants for K^+ transport from frog erythrocytes. The average values of rate constants calculated from separate experiments in NO_3 were 0.015 ± 0.002 and 0.028 ± 0.008 hr^{-1} for isovolemic ($N = 7$) and swollen erythrocytes ($N = 5$), respectively. The rate of K^+ loss was stimulated by 87% in hypotonic NO_3 and the stimulation was statistically significant ($P = 0.032$, paired t test, $N = 5$). When data were pooled from all separate experiments, a plot of K^+ loss *vs.* internal K^+ concentration yielded straight lines with slopes of 0.016 and 0.026 hr^{-1} for control and swollen cells, respectively (Fig. 8).

In these paired experiments, Cl^--dependent components were calculated as the differences in K^+ loss in Cl and NO_3 (Fig. 8). Unexpectedly, the dependence of the Cl^--dependent K^+ loss on intracellular K^+ concentration in frog erythrocytes was also best fitted by a linear regression. In seven separate experiments in isotonic media, the magnitudes of linear regression correlation coefficients (R) varied from 0.85 to 0.99. A highly linear correlation between K^+ loss and intracellular K^+ concentration was observed in hypotonic media ($R = 0.95$–0.97, $N = 5$). The slopes of the regressions represent rate constants for Cl^--dependent K^+ transport from red cells. The mean values of the rate constants calculated from separate experiments were 0.108 ± 0.009 and 0.215 ± 0.008 hr^{-1} in isotonic and hypotonic media, respectively. Figure 8 shows the linear relationship between K^+ loss and intracellular K^+ concentration for pooled data of all experiments with a slope of 0.12 and 0.21 hr^{-1} for normal and swollen red cells, respectively. The stimulation of Cl^--dependent K^+ transport from frog erythrocytes in hypotonic media was significant ($P < 0.001$, paired t test).

Discussion

Data from the present study demonstrated that the K-Cl cotransporter in frog erythrocytes displays kinetic properties different from these of other red blood cells. The most interesting finding here is the effect of extracellular Na^+ on K^+ transport mediated by K-Cl cotransport via the frog erythrocyte membrane. As shown in three series of experiments (Figs. 2–5), total and Cl^--dependent K^+ influx was appreciably inhibited when external Na^+ concentration in isotonic chloride media was below 50 mM. Replacement of all external Na^+ with $NMDG^+$ led to a marked reduction in influx and efflux of K^+ in frog red blood cells in isotonic and hypotonic media (Figs. 6–7). Our results of the first series (Fig. 1) conflict somewhat with the data of all the following experiments. The Cl^--dependent K^+ influx was significantly reduced in a nomi-

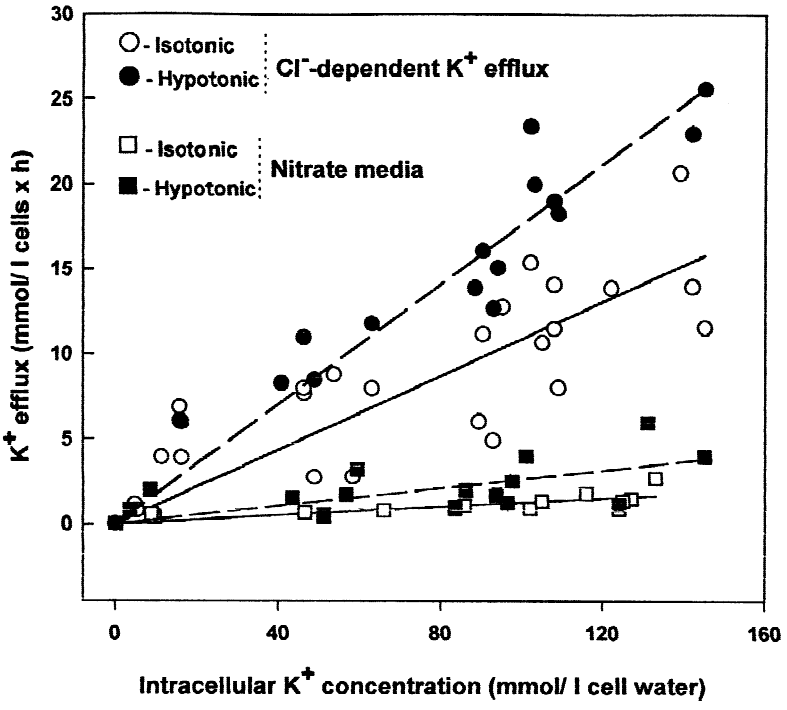

Fig. 8. Relationship between K^+ loss and intracellular K^+ concentration ($[K^+]_i$). Nystatin-pretreated cells with various $[K^+]_i$ were incubated in nominally K^+-free media for 60 min (isotonic) and 30 min (hypotonic) media and net K^+ loss were measured. All media contained 65 mM Na^+, isotonic media additionally contained 80 mM sucrose. Results from 7 (isotonic) and 5 (hypotonic) separate experiments were pooled, data points represent individual values. $[K^+]_i$ is the arithmetic mean intracellular concentration which was calculated as the difference between $[K^+]_i$ before cell incubation and half of K^+ loss during cell incubation. Dependence of Cl^--independent (squares) and Cl^--dependent (circles) K^+ loss on $[K^+]_i$ were described by linear regressions. In isotonic media (solid lines): $y = 0.015 \times [K^+]_i$ ($R = 0.82$) for NO_3 and $y = 0.12 \times [K^+]_i$ ($R = 0.87$) for Cl^--dependent component. In hypotonic media (dashed lines): $y = 0.028 \times [K^+]_i$ ($R = 0.79$) for NO_3 and $y = 0.21 \times [K^+]_i$ ($R = 0.94$) for Cl^--dependent component. The lines are drawn according to linear regressions of all data points (SigmaPlot).

nally Na^+-free isotonic medium with 65 mM $[K^+]_e$ (Fig. 3) but not in a Na^+-free medium with 105 mM $[K^+]_e$ (Fig. 1). Although a small decrease in K^+ influx, but statistically insignificant, was observed when $[K^+]_e$ was raised from 20 to 105 mM (8.7 ± 2.0 and 7.9 ± 3.0 mmol/(l × hr) at 20 and 105 mM $[K^+]_e$, respectively). A possible explanation for this contradiction would be an increase in the cell volume at too high $[K^+]_e$ which, in turn, could result in an activation of K^+ influx. In this case, two opposite effects on K^+ influx occur in medium with 105 mM KCl: inhibition and stimulation due to the removal of $[Na^+]_e$ and the increase in cell volume, respectively. When all internal and external Cl^- ions were replaced with NO_3^-, K^+ influxes were very small and independent of external Na^+. It was evident that only Cl^--dependent K^+ fluxes were affected by decreasing external Na^+ below 50 mM.

It is unlikely that the K^+ influx changes associated with decreasing external Na^+ concentration could be related to coupled Na^+ and K^+ transport. We showed earlier in frog red blood cells that bumetanide, a selective inhibitor of Na-K-2Cl cotransport, had no effect on K^+ and Na^+ transport. In addition the Na^+ influx is independent of the external K^+ concentration and of replacement of Cl with NO_3 in the incubation media (Gusev et al., 1995; Agalakova et al., 1996, 1997). Moreover, our present experiments provide evidence for similar effects of external Na^+ concentration on K^+ loss from frog erythrocytes. Our results are in contrast to studies on human (Kaji & Kahn, 1985; Kaji, 1986) and low potassium (LK) sheep erythrocytes (Lauf, 1983) which showed that K-Cl cotransport activity does not change following removal of external Na^+. Little is known concerning the properties of K-Cl cotransport system in nucleated erythrocytes of birds, amphibia and fish. Currently, there is one report demonstrating existence of a Na^+-dependent K-Cl cotransport in red blood cells of hagfish (Ellory & Wolowyk, 1991). Similar Na^+-dependent K-Cl cotransport has been recently proposed for vascular smooth muscle cells (Orlov, Tremblay & Hamet, 1996).

The effect of external Na^+ on K-Cl cotransport in frog erythrocytes should be taken into consideration for determination of the kinetic constants of the transporter. The results of our experiments indicate that decreasing the Na^+ concentration in the incubation media up to 45 mM did not affect the Cl^--dependent K^+ influx. Therefore, the kinetic parameters of this cotransport can be correctly measured varying $[K^+]_e$ within 0–20 mM. The Table summarizes the mean values of the kinetic parameters for external K^+ dependence of K-Cl cotransport from all series of experiments. There were no differences in these parameters between two series of experiments in isotonic media when the Na^+ concentration was decreased to 85 mM (Table, column 1) and to 45 mM (Table, column 2). Similar values of K_m and V_{max} were obtained in two series of experiments in isotonic media (columns 2 and 4 of Table). Pooled values for an apparent K_m and a V_{max} of Cl^--dependent K^+ influx in isotonic media were 8.2 ± 1.3 mM and 10.4 ± 1.6 mmol/l × hr ($N = 13$). Cell swelling caused a twofold increase in V_{max} and a 50% decrease in the affinity of Cl^--dependent K^+ influx in frog red blood cells. These K_m values for ex-

Table. Apparent affinity (K_m) for external K^+ and maximal velocity (V_{max}) of Cl^--dependent K^+ influx in frog erythrocytes

Kinetic parameters (n)	Isotonic media		Hypotonic media	Isotonic media	
	($Na^+ + K^+$) 105 mM (4)	($Na^+ + K^+$) 65 mM (7)	($Na^+ + K^+$) 65 mM (5)	($Na^+ + K^+$) 65 mM (6)	($NMDG^+ + K^+$) 65 mM (5)
K_m (mM)	12.3 ± 2.7	8.4 ± 1.8	12.8 ± 1.7†	7.9 ± 1.9	15.7 ± 2.1*
V_{max} (mmol/l × hr)	14.2 ± 1.4	9.8 ± 2.2	20.2 ± 2.9††	11.2 ± 2.3	3.2 ± 0.7**

The kinetic constants were calculated from the relationship between Cl^--dependent K^+ influx as a function of $[K^+]_e$ in the range from 0 to 20 mM whereas a 0–50 mM $[K^+]_e$ interval was used in Na^+-free experiments ($NMDG^+$). The K_m and V_{max} values are means ± SEM calculated from experiments shown in Figs. 1–4. Numbers in parentheses represent the number of separate experiments. Frog erythrocytes were incubated in media containing different salts at a constant total concentration (65 or 105 mM), isotonic media with 65 mM concentration contained additionally 80 mM sucrose. *P < 0.03, **P < 0.001 as compared with the corresponding Na^+-containing medium; †P < 0.05, ††P < 0.001 as compared with the corresponding isotonic medium.

ternal K^+ in frog erythrocytes were appreciably lower and the V_{max} values higher than those found for erythrocytes of some mammalian species. In isotonic media, the apparent K_m values for $[K^+]_e$ of Cl^--dependent K^+ influxes were ~17 mM and ~50–80 mM in human (Kaji & Kahn, 1985) and LK sheep erythrocytes (Bergh et al., 1990; Delpire & Lauf, 1991*b*), respectively. The external K^+ affinity of the Cl^--dependent volume-sensitive K^+ fluxes in human and in LK sheep red cells were quite low (K_m ~50–120 mM). In contrast to human and sheep erythrocytes, the removal of external Na^+ caused a significant reduction in Cl^--dependent K^+ influx in frog erythrocytes and decreased the maximal velocity and affinity of the K-Cl cotransporter for K_e^+ (Fig. 4 and Table, column 5).

Under the conditions of the present study, we failed to provide evidence for saturation kinetics for the Cl^--dependent K^+ efflux from frog erythrocytes as a function of internal K^+ concentration. In fact, K^+ transport from cells incubated in both isotonic and hypotonic media declined proportionally with the internal K^+ concentration (Fig. 8). Thus, the Cl^--dependent K^+ efflux may be characterized by rate coefficients of 0.11 and 0.21 hr^{-1} for isovolumic and swollen cells, respectively. The Cl^--dependent K^+ efflux from frog erythrocytes was significantly higher than that observed in LK sheep red blood cells (Bergh et al., 1990; Delpire & Lauf, 1991*b*), whereas this K^+ transport was not found in human erythrocytes under isotonic conditions (Kaji, 1989). In sheep erythrocytes, Cl^--dependent K^+ efflux exhibited a low affinity for internal K^+ in isotonic (K_m ~84 mM) and in hypotonic media (K_m ~58–68 mM). Volume-sensitive Cl^--dependent K^+ efflux from human erythrocytes (Kaji, 1989) had also a low affinity for $[K^+]_i$ (K_m ~47 mM). However, K^+ efflux from CC cells (Brugnara, 1989) increased monotonically with the $[K^+]_i$ concentration similar to frog erythrocytes. Varying $[K^+]_e$ had a small or no effect on K^+ efflux in human and LK sheep red cells. In the present study, K^+ efflux from frog erythrocytes showed also small *trans*-effects by 10–20 mM external Rb^+ (Fig. 5). In contrast to $[K]_e$, decreasing external Na^+ concentration below 50 mM was associated with a significant *trans*-inhibitory effect on K^+ efflux from frog erythrocytes (Figs. 5–6). A role of internal Na^+ concentration in K^+ transport in frog erythrocytes remains unclear. In our experiments varying $[K^+]_i$ was associated with reciprocal changes in cellular Na^+ content. Therefore, one possible explanation for the linear relationship between Cl^--dependent K^+ efflux and $[K^+]_i$ may be attributed to the changes in intracellular Na^+ concentration.

Taken together, the results of the present study clearly demonstrated that both influx and efflux of K^+ mediated by K-Cl cotransport in the frog erythrocyte membrane are inhibited by lowering external Na^+ below 45–50 mM. In a nominally Na^+-free medium, the activity of K-Cl cotransport was reduced to 30–50% of that observed in normal saline. The mechanism by which the cotransporter's function is influenced by the external Na^+ concentration remains obscure and may be indirect. The decrease in Na^+ concentration in the medium could inhibit Na^+/Mg^{2+} exchange resulting in an increase in erythrocyte Mg content and in inhibition of K-Cl cotransport as was found in erythrocytes of some species (Lauf et al., 1992).

Of great interest is the fact that the stimulation of K^+ transport by cell swelling did not require the presence of Na^+ in the medium. In the media with varying K^+ (Na^+) concentrations (Fig. 3), the volume-sensitive component calculated as the difference between mean values of the Cl^--dependent K^+ influxes in isotonic and hypotonic media was linear related to $[K^+]_e$ ($V = 0.25 \cdot [K^+]_e$, $R = 0.997$). Thus the decrease in external Na^+ concentration did not affect the volume-sensitive component of Cl^--dependent K^+ influx in frog erythrocytes. Similarly, in

media with 10 mM RbCl (Fig. 6), the calculated volume-sensitive components of Rb^+ influx and K^+ efflux were also independent of the presence of external Na^+. Based on the results of this study, we cannot rule out the possibility that the Cl^--dependent K^+ transport in frog erythrocytes occurs via two different K-Cl cotransport pathways. One of them is Na^+-dependent, volume-independent with a relatively high affinity for external K^+ and another is volume-sensitive, Na^+-independent with a very low affinity for external K^+. This suggestion is speculative enough at present and further experiments will be necessary to elucidate this hypothesis. Such studies would require evidence for effects of K-Cl cotransport inhibitors, regulation of this cotransport activity by intracellular messengers as well as identification of transporters at the molecular level.

The residual Cl^--independent K^+ fluxes in frog red blood cells were only small fractions of the total K^+ transport. Removal of external Na^+ did not affect the Cl^--independent K^+ fluxes. The kinetic analysis of Cl^--independent K^+ fluxes with respect to $[K^+]_e$ and $[K^+]_i$ in normal and swollen cells revealed linear regressions, showing the absence of other pathways with saturable kinetics. Mean values of the rate coefficients for both K^+ influx and K^+ efflux in isotonic media were 0.012 ± 0.009 and 0.013 ± 0.01 hr^{-1}; this difference was not significant. In hypotonic media, there was also no statistical difference in the rate coefficients between influxes (0.019 ± 0.002 hr^{-1}) and effluxes of K^+ (0.023 ± 0.005 hr^{-1}). However, the rate coefficients in swollen cells were significantly higher than those found in isosmotic media. Thus, the residual K^+ transport presents a nonsaturable pathway having the characteristics of a channel or a carrier-mediated transporter. This pathway for K^+ in frog erythrocyte membrane has the same permeability in both directions and is activated by cell swelling similar to volume-sensitive Cl^--independent K^+ transport that is well documented in fish erythrocytes (Guizouarn et al., 1993; Berenbrink et al., 1997). The contribution of this Cl^--independent pathway in total K^+ transport in frog red blood cells is relatively small. It is evident that at physiological conditions K^+ efflux via the Cl^--independent pathway considerably exceeds K^+ influx, presumably largely because $[K^+]_i$ exceeds $[K^+]_e$.

In summary, the results of our studies indicate that the K-Cl cotransporter of frog erythrocytes with respect to its kinetic properties is different from the systems described in human and LK sheep red blood cells. K^+ influx via K-Cl cotransport in frog red blood cells is characterized by a relatively high affinity for $[K^+]_e$ and large maximal capacity. By contrast, Cl^--dependent K^+ efflux from frog erythrocytes displays an extremely low affinity for $[K^+]_i$ showing nonsaturable kinetics over the $[K^+]_i$ range tested (5–140 mM). Unlike human and LK sheep erythrocytes, the activity of the K-Cl cotransport in frog red blood cells is strongly dependent on $[Na^+]_e$ ranging from 0 to 50 mM. It is noteworthy that, similar to our findings, a number of studies on epithelial tissues have demonstrated that Na^+ ions are able to stimulate another transporting protein, Cl^--HCO_3^- exchanger, whereas the Na^+ ions are not translocated by the carrier (Orsenigo, Tosco & Faelli, 1994). Like other ion transporters, it would be expected that various isoforms of K-Cl cotransport could be revealed in different cell types. Recently, two distinct isoforms of the K-Cl cotransporter (KCC1 and KCC2) with different affinities for $[K^+]_e$ were described (Gillen et al., 1996; Payne, Stevenson & Donaldson, 1996; Payne, 1997).

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